RECEPTOR-MEDIATED CATECHOLAMINE RELEASE FROM CHROMAFFIN CELLS: THE ROLE OF PROTEIN KINASE C

Thesis submitted for the degree of

Doctor of Philosophy

At the University of Leicester

By

Nouf AL-Rasheed BSc. (Hons).

Department of Cell Physiology and Pharmacology,

University of Leicester

April 2006

UMI Number: U489430

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI U489430
Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

RECEPTOR-MEDIATED CATECHOLAMINE RELEASE FROM CHROMAFFIN CELLS: THE ROLE OF PROTEIN KINASE C

Nouf AL-Rasheed

Modulation of neurotransmitter release by ionotropic nicotinic receptors and $G\alpha_{q/11}$-coupled receptors may be essential for events such as regulation of cardiovascular function and the stress response. Previous studies indicated that the exocytotic mechanism is enhanced by protein kinase C (PKC). However, the circumstances in which PKC plays a role, the extent of regulation and the mechanisms underlying both PKC activation and its regulation of exocytosis are unclear. In this study, bovine chromaffin cells were used to explore the role of PKC, particularly the various PKC isoforms, in nicotinic receptor- and $G\alpha_{q/11}$-coupled receptor-mediated catecholamine release.

This study reveals that bovine chromaffin cells express PKC-α, -β, -ε and -ι and that they were differentially activated by nicotinic receptors and a range of $G\alpha_{q/11}$-coupled GPCRs. Thus, nicotinic receptor stimulation recruited PKC-α, -β and -ε from the cytoplasm to the plasma membrane, indicative of activation. In contrast, activation of $G\alpha_{q/11}$-coupled receptors with histamine activated all the expressed PKC isoforms and angiotensin II only activated PKC-α and -ε.

Inhibition of PKC using general or isoform-selective inhibitors potentiated catecholamine release in response to activation of $G\alpha_{q/11}$-coupled receptors, most likely as consequence of potentiated phospholipase C-mediated signalling. However, inhibition of PKC, particularly PKCα, markedly inhibited nicotinic receptor-mediated catecholamine release. PKCα is a classical isoform of PKC, activated by Ca^{2+} and diacylglycerol (DAG).

The current study suggests that Ca^{2+} influx across the plasma membrane in response to nicotinic receptor activation is largely through the nicotinic receptors themselves. Furthermore, this Ca^{2+} entry activates PLC, generating both $Ins(1,4,5)P_3$ and DAG. This activation of PLC contributes significantly to the activation of PKC. The mechanism through which PKC facilitates the release of catecholamines requires the PKC-dependent phosphorylation of myristoylated alanine-rich C protein kinase substrate (MARCKS), the subsequent disassembly of the cortical F-actin cytoskeleton and probably therefore, increased access of a reserve exocytotic vesicle pool to release sites at the plasma membrane. Moreover, this study also suggests that PLC-dependent generation of DAG recruits Munc13-1, a known vesicle priming agent. Thus, activation of Munc13-1 may also contribute to nicotinic receptor-mediated catecholamine release in a manner dependent on the activation of PLC.

ACKNOWLEDGEMENTS

Initially, I would like to express my thanks to the College of Pharmacy and Department of Pharmacology, King Saud University for allowing me to pursue my postgraduate studies to fulfil my PHD. My gratitude also goes to Royal Embassy of Saudi Arabia Cultural Bureau who generously funds this research.

I would like to express my sincere gratitude and full appreciation to Dr. Gary Willars for his close day-to-day supervision, constructive comments, support, encouragement, extraordinary patience and excellent supervion over the last 3 years. Moreover, I feel deeply indebted to Dr. Elizabeth Seward for constant attention and advice. I would like also to thank Dr. Claudia Bauer for her kindness and regular supplement of chromaffin cells. I must also extend my gratitude to several people in University of Leicester and University of Sheffield who have willingly helped in various ways over the last 3 years.

Special thanks must go to my parents, sisters and relatives for their support throughout my life in educations. I would like to express my sincere gratitude for my husband, Thamer AL-Rasheed for unlimited support and encouragement throughout my PHD. Finally, special mention must go to my lovely children, Noura and Abdulelah who have been the inspiration for me to accomplish writing this thesis with full of enthusiasm.

Dedicated to my husband and children.

CONTENTS Page Number

1: INTRODUCTION

1: INTRODUCTION

Exocytosis from neuroendocrine cells can be triggered by different receptor types such as ionotropic nicotinic receptors and $G\alpha_{q/11}$-coupled receptors. These different receptors may differentially regulate exocytosis by using different mechanisms: importantly, one possibility is through PKC. PKC is well documented to regulate exocytosis, although there is debate about the contribution of various PKC isoforms to the release process and the mechanisms underlying PKC activation. Work described within this thesis focuses on examining the role of PKC in catecholamine secretion in response to ionotropic nicotinic receptors and $G\alpha_{q/11}$-coupled receptors.

1.1 NEUROENDOCRINE SECRETORY MODELS USED FOR STUDYING EXOCYTOSIS

Secretory granules and their regulated exocytosis have been most extensively studied in a number of cell types. These have served either as model systems that have certain experimental advantages or have been of particular interest due to their crucial physiological roles and alterations in disease states. A well-used cell type is the adrenal chromaffin cell and its tumour counterpart, PC12 cells, both of which have allowed biochemical and electrophysiological investigations (Gillis and Chow, 1997; Morgan and Burgoyne, 1997; Burgoyne and Morgan, 1998a). Among the wide range of secretory cells such as neurons (Bean *et al.*, 1994; Zucker, 1996), pancreatic β-cells (Easom, 2000), mast cells (Lindau and Gomparts, 1991), platelets (Lemons *et al.*, 1997) and neutrophils (Nusse and Lindau, 1988), PC12 cells and chromaffin cells were selected in this study for a variety of reasons that will be discussed later.

1.1.1 PC12 cell line

Pheochromocytomas are neuroendocrine tumours of adrenal chromaffin cells, characterized by over production of catecholamines (Parmer and Zinder, 2002). They are rare in all species except rats but occur with increased frequency in several human familial syndromes. Pheochromocytomas are inducible in rats by a variety of non-genotoxic substances that may act indirectly by stimulating chromaffin cell proliferation (Tischler, 2002).

Inially the work of this project was conducted using a PC12 cell line, which was originally isolated by Greene and Tischler (1976) from a rat pheochromocytoma. It has been extensively employed in neurobiological studies and for almost 30 years has served as a research tool. Under conventional condition these cells resemble chromaffin cells. Nevertheless, when treated with nerve growth factor (NGF) PC12 cells stop growing, enlarge and acquire a neuronal-like phenotype. For example, they develop flattened, non-spherical cell bodies and extend long, branching neurites (Greene and Tischler, 1976). Their secretory vesicles are redistributed from the somata to the neurites, and they secrete both catecholamines and acetylcholine in a Ca^{2+}-dependent manner (Harkins and Fox, 1998). Because of these properties, PC12 cells are popular model of both neurosecretory and neuronal-type cells (Janigro *et al.*, 1989). PC12 cells express a variety of $G\alpha_{q/11}$-coupled receptors and ligand gated ion channels as well as T-, L-, N-, and P/Q-type Ca^{2+} channels (Shafer and Atchison, 1991). Moreover, PC12 cells synthesize and store catecholamines in secretory vesicles that have many characteristics in common with neuronal large dense-core vesicles, and release their contents by Ca^{2+}-dependent exocytosis (Shoji-Kasai *et al.*, 2001). In addition, the ability to produce homogeneous populations of cells

from pheochromocytomas expands the usefulness of these tumours as models for neurotoxicology and for developmental and degenerative neural disorders (Tischler, 2002). An additional advantage in using PC12 cells is the possibility of selecting mutant subclones useful for the analysis of various cellular functions. In spite of these advantages, there are also disadvantages including, an apparently less stable phenotype. Thus, it is liable to change its morphology when cultured for long periods and it becomes heterogeneous after a large number of cell divisions due to spontaneously generated mutations (Shoji-Kasai *et al.*, 2001).

PC12 cells have been widely used in studies investigating the molecular mechanisms involved in neurotransmitter release (Ritchie, 1979; Stallcup, 1979; Williams and McGee, 1982; Pozzan *et al.*, 1986; Appell and Barefoot, 1989; Weiss and Atlas, 1991; Rhoads *et al.*, 1993; Suh and Kim, 1994). They have also been used extensively to study signalling, particularly by GPCRs (Fasolato *et al.*, 1988; Rhee *et al.*, 1989; Berridge, 1993; Nardone *et al.*, 1994; Suh *et al.*, 1995; Koizumi *et al.*, 2002; Kudlacek *et al.*, 2003; Ohta *et al.*, 2004). Despite such reports, experiments conducted on PC12 cells in this study failed to show the expression of $G\alpha_{q/11}$-coupled receptors in these cells (see Chapter 3) and attention was therefore directed to its physiological counterpart, the neuroendocrine chromaffin cells. These are relatively easy to prepare and maintain in culture, have a stable phenotype (Schwarz, 1994; Powell *et al.*, 2000), can be stimulated to elicit robust catecholamine secretion (Burgoyne, 1991), and express a range of $G\alpha_{q/11}$-coupled receptors (O'Sullivan and Burgoyne, 1989; Plevin and Boarder, 1988; Artalejo *et al.*, 1990; Dahmer and Perlman, 1988; Wilson, 1988; Marley, 2003; Burgoyne, 1991) and ion channels (Kilpatric *et al.*, 1982; Bormann and Clapham, 1985; Lara *et al.*, 1998; Teschemacher and Seward, 2000).

1.1.2 Bovine chromaffin cells

The adrenal glands, located at the superior poles of the two kidneys, are composed of two distinct layers, the adrenal cortex and adrenal medulla. The outer adrenal cortex, which develops from the abdominal mesothelium surrounding the medulla during embryogenesis, synthesizes and secretes the adrenocortical hormones (i.e., mineralocorticoids and glucocorticoids). The adrenal medulla, which comprises the central 20% of the gland, originates from the neural crest. The adrenal medulla is a modified sympathetic ganglion, which secretes the catecholamines adrenaline and noradrenaline in response to sympathetic neural stimulation from the splanchnic nerves (Morgan and Burgoyne, 1997; Perlman and Chalfie, 1977; Eaton and Duplan, 2004).

Chromaffin cells of the adrenal medulla store their secretory products in specialized membrane-bound organelles, the chromaffin vesicles. In response to stimuli, these vesicles fuse with the plasma membrane and release their soluble contents (catecholamines, ATP, peptides) to the cell exterior by exocytosis (Rosē *et al.*, 2002). In addition, chromaffin granules are the counterparts of LDCVs (large dense core vesicles) found in at least two compartments, the release-ready granule pool and the reserve pool comprising the vast majority of granules (Trifaró *et al.*, 1985). Thus, these cells serve as an excellent model for studying the biosynthesis and functional role of the different peptides and proteins contained within LDVs. There is large body of evidence indicating that the co-stored neuropeptides and proteins are also co-released together with the catecholamines from cultured bovine chromaffin cells (Livett *et al.*, 1981; Laslop and Mahata, 2002).

A number of factors have contributed to the success of the bovine chromaffin cell model. Of great importance is convenience. Large numbers of chromaffin cells can be obtained in relatively pure populations from the adrenal medulla. Other normal neuroendocrine cells have until recently not been as easily accessible, and chromaffin cells has therefore had broad appeal (Burgoyne, 1991; Tischler, 2002). Chromaffin cells of bovine source have been intensively studied by those interested in endocrine mechanisms, neuronal function and the basic cell biology of the secretory process (Burgoyne, 1984a; Knight *et al.*, 1989). Bovine chromaffin cells provide an ideal model system for studying regulated exocytosis and in the last few decades they have been used extensively as a model system to study Ca^{2+}-triggered exocytosis. Secretion of catecholamines can be evoked by a wide variety of stimuli and readily quantified using a variety of approaches including electrophysiological, and biochemical analyses (Burgoyne, 1991; Holz *et al.*, 1992; Lawrence *et al.*, 2002) In addition, they offer the advantage of allowing the use of techniques for studying the kinetics of exocytosis in single cells with high temporal resolution. Thus, measurement of membrane capacitance and electrochemical detection of catecholamine release allows the study of exocytosis in the millisecond range (Ashery *et al.*, 2000). For those interested in neuronal function, chromaffin cells possess the advantage that they are derived during embryogenesis from the same precursors as sympathetic neurons and possess some properties in common with neurons such as the expression of voltage-dependent Na^{+} and Ca^{2+} channels and a variety of 'neuronal-specific' proteins (Burgoyne, 1991). Furthermore, it has recently become possible to over-express recombinant proteins in chromaffin cells including those located at synapses, making them an ideal system to study their role in regulated exocytosis

(Ashery *et al.*, 1999; Duncan *et al.*, 1999). Despite these advantages there are some important points to consider when using chromaffin cells. Foremost is the existence of species differences. Furthermore, populations of chromaffin cells are heterogenous and may have varied responsiveness to neurotrophic or mitogenic factors *in vitro*, and utilization of varied signal transducers in physiological processes (Suzuki and Kachi, 1996). To overcome these problems chromaffin cells should be used after only short culture periods and where possible the results confirmed with freshly isolated cells. In addition, differential plating may reduce the percentage of contaminating cells in chromaffin cell culture (Waymire *et al.*, 1983).

1.2 NEUROTRANSMITTER RELEASE

Exocytosis is the process by which a membrane-bound vesicle fuses with the plasma membrane of a cell, resulting in the release of vesicle contents (Craig *et al.*, 2003). Exocytosis takes place along two pathways. Firstly, constitutive exocytosis which occurs in all eukaryotic cells. In this pathway the vesicles fuse with the plasma membrane in the absence of external signals. In contrast, in regulated exocytosis, vesicles only undergo exocytosis in response to particular stimuli; for example an increase in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$). Regulated exocytosis plays diverse roles including the release of neurotransmitters and hormones (Lin and Scheller, 2000). In neurons, synaptic vesicles that are considered the key organelles in this process, undergo a complex cycle of fusion and fission events that regulate the release process at the synapse (Betz *et al.*, 1998). In 1950, Katz and his colleagues suggested the idea of synaptic vesicles and since then, synaptic vesicle exocytosis has been explored using a wide variety of techniques, which has led to the identification of many of the proteins that

participate not only in synaptic vesicle fusion but also in other steps of synaptic vesicle trafficking such as targeting, docking and priming. Modifications to any of these steps may modulate synaptic transmission and this may play a role in synaptic plasticity underlying events such as learning and memory (Lin and Scheller, 2000).

1.2.1 Biosynthesis of catecholamines

Chromaffin cells in the adrenal medulla are specialized for the synthesis, storage and secretion of catecholamines. These cells are innervated by preganglionic sympathetic neurons in the splanchic nerves and activation by these nerves appears to be the most important determinant of adrenomedullary function (Perlman and Chalfie, 1977).

Chromaffin cells store catecholamines, nucleotides, opioid peptides, dopamine β-hydroxylase (DBH) and chromogranin A (CgA) in membrane-bound organelles: the chromaffin granules (Trifaró, 1977). Upon stimulation, soluble contents of the vesicles are released to the cell exterior by exocytosis. Therefore, chromaffin vesicles are considered true organelles, which in addition to their soluble contents contain enzymes involved in catecholamine synthesis (Trifaró, 2002). At least three types of chromaffin cells are present in the adrenal medulla: adrenaline (85%), noradrenaline (14-15%), and dopamine (1%) containing cells. Adrenaline, noradrenaline, and dopamine are synthesized in the chromaffin cell from tyrosine which is a common precursor (Udenfriend, 1953). Tyrosine is converted initially to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH). This enzyme is found only in catecholamine–containing cells. It is probably free in the cytosol and is considered the rate-limiting enzyme in catecholamine synthesis (Levitt *et al.*, 1965; Ikeda *et al.*, 1966). TH is inhibited by the end-

product of the biosynthetic pathway or by DOPA and as this provides the mechanism for regulation of the rate of synthesis (Weiner and Rabadjija, 1968). Following the production of DOPA, this is converted to dopamine by DOPA decarboxylase, a cytosolic enzyme confined to catecholamine-synthesising cells. Dopamine can be converted to noradrenaline by DBH, a membrane bound mixed-function oxidase present in chromaffin vesicles (Winkler and Westhead, 1980; Brooks and Treml, 1983; Pender and Burgoyne, 1992; Trifaró, 2002). DBH is not, however subject to rapid degradation or uptake and its concentration in the plasma can be used as index of overall sympathetic nerve activity. The subsequent formation of adrenaline from noradrenaline is catalysed by phenylethanolamine N-methyltransferase (PNMT) (Axelrod, 1962; Trifaró, 2002). This enzyme is present in the population of adrenaline-releasing chromaffin cells of the adrenal medulla but not in the smaller proportion of noradrenaline releasing cells. The adrenaline-releasing chromaffin cells, which appear only after birth, lie adjacent to the adrenal cortex and there is evidence that the production of PNMT is triggered specifically by glucocorticoid hormones produced by the cortex (Kalcheim *et al.*, 2002).

Following synthesis of catecholamines and other neurotransmitter amines in the cytoplasm, they are taken up into storage vesicles by vesicular monoamine transporters (VMATs) to be ready for subsequent exocytotic events (Henry *et al.*, 1994; Parmer and Zinder, 2002; Flatmark *et al.*, 2002). VMATs act as an electrogenic antiporters (exchangers) of protons and monoamines using a proton electrochemical gradient (Johnson, 1988; Kanner and Schuldiner, 1987). Two homologous but distinct VMAT genes have been cloned from rat, bovine and human adrenal glands (Erickson and Eiden, 1993). In rat, $VMAT_1$ is expressed in

the adrenal gland whereas $VMAT_2$ is expressed in the brain. In contrast, bovine adrenal glands express both $VMAT_1$ and $VMAT_2$ (Krejci *et al.*, 1993).

1.2.2 The life cycle of granules and synaptic vesicles

Neurons possess two classes of secretory vesicles, namely LDCVs and synaptic vesicles. LDCVs store neuropeptides and some small molecules such as ATP and catecholamines. Classical neurotransmitters such as acetylcholine, GABA (gamma aminobutyric acid), glutamate and glycine are stored in small synaptic vesicles (Kelly, 1993). Neuroendocrine cells such as chromaffin cells have only LDCVs and these are commonly referred to as chromaffin granules. They are known to store catecholamines, neuropeptides (neuropeptide Y and enkaphalin), nucleotides and proteins (e.g. chromogranin A and B, secretogranin II) (Huttner and Natori, 1995). In contrast, PC12 cells possess both LDCVs, which contain monoamines, and small synaptic like-microvesicles (SLMVs) thought to store acetylcholine (Weihe *et al.*, 1996; Ninomiya *et al.*, 1997). In addition, SLMVs are also present in other endocrine cells such as pancreatic β-cells, which store and secrete molecules such as GABA, the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) and the degradative enzyme GABA-transaminase (Reetz *et al.*, 1991; Braun *et al.*, 2004). Furthermore, in pancreatic β-cells, GABA and GAD are excluded from insulin-containing LDCVs (Garry *et al.*, 1988). Although both classes of vesicles share the ability to undergo Ca^{2+}-triggered exocytosis, they differ in their biogenesis. Immature granules bud directly from the *trans*-Golgi network (TGN). In contrast, synaptic vesicles derive from the endosome and acquire specific proteins via constitutive secretion and endocytosis from the plasma membrane (Régnier-Vigouroux *et al.*, 1991).

In neurons, synaptic vesicles are not directly bound to the actin cytoskeleton and a small proportion are tightly docked to neurotransmitter sites at the presynaptic plasma membrane ('the active zone'). The vast majority of synaptic vesicles are cross-linked by the vesicle protein synapsin I, which itself is associated with the actin cytoskeleton (Trifaró and Vitale, 1993; Burgoyne and Morgan, 1995; Calakos and Scheller, 1996)

The life cycles of synaptic vesicles and chromaffin granules are shown in Figure 1.2.2. Neuronal exocytosis occurs through a pathway consisting of multiple functionally definable stages including secretory vesicle recruitment, priming, and docking, followed by Ca^{2+}-triggered membrane fusion (Burgoyne and Morgan, 1993a; Südhof, 1995). In neurons, following the action potential, a small proportion of synaptic vesicles at the active zone (neurotransmitter release site on the presynaptic plasma membrane) undergo exocytosis within 200μs in response to high Ca^{2+} concentrations at the mouths of presynaptic Ca^{2+} channels where synaptic vesicles are tethered (Burgoyne and Morgan, 1995; Calakos and Scheller, 1996). Influx of Ca^{2+} through voltage-operated Ca^{2+} channels (VOCCs) is the classical mechanism of initiating transmitter release. There are multiple types of Ca^{2+} channels (L-, T-, N-, Q-, P-, and R-types). However, it is now widely accepted that N- and P/Q-type Ca^{2+} channels are the predominant species in presynaptic nerve terminals and these channels couple to proteins that form the release machinery for synaptic vesicles (Burgoyne, 1991; Artalejo *et al.*, 1994). Mobilization of synaptic vesicles from a reserve pool of synapsin I-linked vesicles allows replenishment of those lost from the active zone as a result of rapid exocytosis. Ca^{2+} influx leads to activation of calmodulin-dependent protein kinase II (present in synaptic vesicles) which phosphorylates synapsin I leading to the

liberation of synaptic vesicles from the cytoskeletal constraint allowing them to move to and dock at the active zone (Ryan, 1999). In contrast, in chromaffin cells, the initial stage is recruitment of granules to the subplasmalemmal area, which follows disassembly of a cortical actin barrier (Cheek and Burgoyne, 1986). This is an ATP- and Ca^{2+}-dependent step, although it can also be activated by alternative Ca^{2+}-independent pathways (Burgoyne and Morgan, 1998a). The protein(s) which Ca^{2+} acts on to trigger cytoskeletal disassembly are unclear but there is evidence that scinderin, a Ca^{2+}-dependent actin-severing protein is involved in this process in chromaffin cells (Trifaró and Vitale, 1993; Roth and Burgoyne, 1995). Thus, it is proposed that Ca^{2+} acts at least two distinct stages in exocytosis: an early stage of actin rearrangement and a late stage of membrane fusion (Morgan and Burgoyne, 1997). This mobilisation step is followed by vesicle docking, which consists of the formation of protein complexes linking the presynaptic plasma membrane with the vesicle membrane. This complex set of interactions is thought to complete the docking of synaptic vesicles and primes them within the active zone (Lin and Scheller, 2000). Fusion is then triggered by influx of Ca^{2+} through presynaptic voltage-gated Ca^{2+} channels following membrane depolarization. Finally, the synaptic vesicle membrane and protein components are retrieved via endocytosis and are recycled for additional rounds of release. Clathrin-coated budding is implicated in the endocytotic process (Heuser, 1989; Palfrey and Artalejo, 1998). The binding of clathrin to the membrane is enhanced by certain adaptor proteins. Synaptotagmin serves as a receptor for the clathrin adaptor protein AP-2 (Zhang *et al.*, 1994). The clathrin coat forms a regular lattice around the pit, which finally pinches off as a small coated vesicle. In addition, the pinching off of the vesicle depends on a

cytoplasmic GTPase, dynamin, which forms a constricting helical ring around the neck of the vesicle during endocytosis. Endocytosed vesicles may then proceed through recycling endosomes or may shed their coat of clathrin and directly re-enter the reserve or releasable pool of vesicles (Murthy and Stevens, 1998).

Exocytosis in neurons and neuroendocrine cells has now been shown to occur through two distinct modes (Burgoyne *et al.*, 2001; Artalejo *et al.*, 1998). In addition to the classical model of exocytosis that involves full incorporation of vesicles into the plasma membrane ('full fusion'), followed by clathrin-mediated endocytosis, exocytosis can occur by a mechanism in which the vesicle rapidly pinches off without full integration into the plasma membrane. Retrieved vesicles are rapidly refilled with cargo or those that have not dispensed their full cargo are immediately available for further exocytosis (Valtorta *et al.*, 2001). This so called 'kiss-and-run' mode of exocytosis is generally favoured by strong stimulation and high Ca^{2+} conditions and provides a mechanism for very fast vesicle recycling in response to intense signalling (Ales *et al.*, 1999; Stevens and Williams, 2000). In the kiss-and-run mode of exocytosis, complete integration of vesicle and plasma membranes rarely occur (Sim *et al.*, 2003). For example, in PC12 cells, secretory granules are recycled virtually intact after exocytosis (Taraska *et al.*, 2003). In chromaffin cells, high Ca^{2+} levels are required to switch the mode to kiss-and-run (Ales *et al.*, 1999). Recently, three forms of kiss-and-run have been described: 'pure', which releases only very small molecules, 'mixed' where the fusion pore size is greater to allow exocytosis of larger molecules, and 'full', where the release of all vesicle cargo is permitted (Tsuboi and Rutter, 2003; Sim *et al.*, 2003).

Some of the most striking advances in neurobiology over the past decade have been the identification of proteins responsible for neurotransmitter release. Therefore, the next part of this section will discuss briefly these proteins that participate in vesicle targeting and docking.

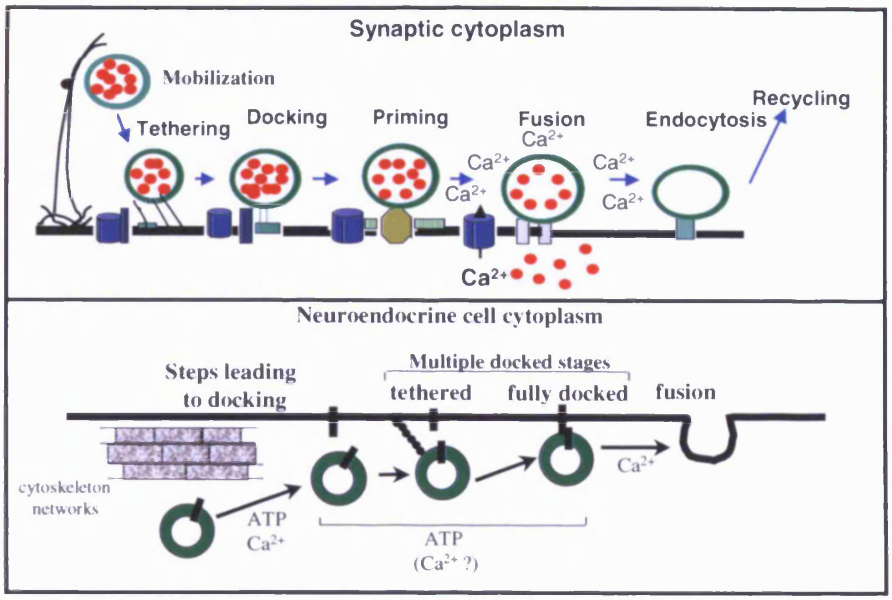


Figure 1.2.2. The stages of exocytosis in neurons and neuroendocrine cells. Secretory granules or synaptic vesicles are excluded from the cell periphery by an actin cytoskeleton network. Disassembly of the actin barrier allows granules or vesicles to approach the plasma membrane and at one or more stages of exocytosis (before, during, or after), the exocytotic machinery undergoes ATP-dependent priming. In this process, granules or vesicles become attached to the plasma membrane and may be initially tethered before becoming tightly docked via the exocytotic machinery. Finally, membrane fusion is triggered by Ca^{2+} in an ATP-independent stage. Adapted from Burgoyne and Morgan, 1998a and Schiavo and Stenbeck, 1997.

1.2.3 Proteins that are involved in exocytosis

The exocytotic process is regulated by a number of proteins such as those in SNARE complex. The most well known among the proteins involved in exocytosis are the SNARE (soluble n-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) proteins (Figure 1.2.3). There are two distinct categories of SNARE proteins that have been identified. SNAREs present on the vesicle (or donor) compartment are known as v-SNAREs (synaptic vesicle associated SNARE) while those on the target (or acceptor) compartment are known as t-SNAREs (target-membrane associated SNARE). The SNARE hypothesis was proposed in 1993, before most of the current knowledge became available, as the first working model to explain vesicle docking and fusion in molecular terms (Söllner *et al.*, 1993). It was postulated that association of the v-SNARE, VAMP (vesicle-associated membrane protein, also known as synaptobrevin) with the t-SNAREs, syntaxin and SNAP-25 (synaptosomal–associated protein 25kDa) leads to the formation of a core complex needed for membrane fusion and the release of the vesicle's contents. This 'core complex' is very stable which is reflected in its resistance to SDS denaturation, protease digestion, clostridial neurotoxin cleavage and temperature up to ~90°C (Leeders and Sheng, 2005). *In vivo* its disassembly is carried out by two proteins, the ATPase, NSF (n-ethylmaleimide-sensitive fusion protein) and an adaptor protein, α-SNAP (soluble NSF attachment protein), which act as molecular chaperones to prepare the SNARE proteins for subsequent events in docking or fusion or both as they allow recycling of SNARE components (Bock and Scheller, 1997; Chen and Scheller, 2001; Leenders and Sheng, 2005). There is no doubt that the proteins underlying the SNARE hypothesis are key players in steps leading to vesicle

docking and fusion. In addition, an essential point of the SNARE hypothesis was the role of v- and t-SNARE pairing in determining the specificity of targeting (Burgoyne and Morgan, 1998a). Synaptotagmin serves importantly as a Ca^{2+} sensor through Ca^{2+}-dependent interactions with both SNAP-25 and syntaxin. Synaptotagmin possesses two C2 domains that are homologous to the Ca^{2+}-binding domain of protein kinase C (PKC). The first C2 domain (C2A) binds syntaxin in a Ca^{2+}-dependent fashion. Furthermore, synaptotagmin has been reported to have a Ca^{2+}-dependent interaction with SNAP-25 (Schiavo *et al.*, 1997). The role of synaptotagmin in synaptic vesicle docking and fusion is most likely based on such interactions. An influx of Ca^{2+} into the cytosol drives these interactions, which subsequently cause conformational changes and allows both core complex formation and membrane fusion to progress (Jan and Stevens, 2000). Although there are 13 synaptotagmin isoforms, not all of them are able to bind Ca^{2+} (Burgoyne and Morgan, 2003). Only some (synaptotagmin I, II, III, V, VII) bind Ca^{2+} (Li *et al.*, 1995). Currently there is little information regarding the role of synaptotagmin in chromaffin cell exocytosis, where the only isoform definitely reported is synaptotagmin I whose Ca^{2+} affinity is poorly matched to the concentrations of Ca^{2+} likely to mediate granule exocytosis (Morgan and Burgoyne, 1997).

Over the past decade, significant progress has been made in identifying other proteins involved in the modulation and regulation of vesicular release and the nature of the protein-protein interactions that underlie these events (Chen and Scheller, 2001; Li and Chin, 2003; Murthy and De Camilli, 2003). For instance, rabphilin3A, DOC2 (double C2 domain), Munc13 (mammalian uncoordinated mutant 13), and RIM (rab3 interacting molecule), all of which may also serve as

Ca^{2+} sensors, like synaptotagmin contain PKC-like C2 domains and bind both Ca^{2+} and phospholipids. Moreover, they have been identified as important factors in synaptic vesicle priming (Augustin *et al.*, 1999b; Verhage *et al.*, 2000; Schoch *et al.*, 2002). DOC2 is a soluble protein and its over-expression in PC12 cells enhances Ca^{2+}-dependent granule exocytosis (Morgan and Burgoyne, 1997). The mechanism underlying this enhancement is unknown, although DOC2 has been shown to interact with both Munc18 (also known as nSec1) and another C2 domain containing protein, Munc13. Rabphilin3A is soluble protein containing two tandem C2 domains that mediate binding to phospholipids (especially phosphatidylinositol 4, 5-bisphosphate). Evidence for a role of this protein in granule exocytosis comes from work on chromaffin cells based on its binding in a GTP-dependent manner to Rab3a (a low molecular GTPase) (Yamaguchi *et al.*, 1993; Chung *et al.*, 1995). Munc13 (four isoforms: 1, 2, 3 and 4) is the mammalian homolog of *C. elegans* UNC-13 and contains domains homologous to both the C1 and C2 domains of PKC (Brose *et al.*, 1995; Duncan *et al.*, 1999). These proteins are phorbol ester/diacylglycerol binding proteins (Betz *et al.*, 1998). The first evidence for its role in exocytosis comes from a study in neurons (Augustin *et al.*, 1999a). In addition, over-expression of Munc13-1 in chromaffin cells increases the magnitude of both the exocytotic burst and the subsequent slower phase of release, which is consistent with a role for this protein in vesicle priming (Ashery *et al.*, 2000; Rhee *et al.*, 2002; Junge *et al.*, 2004).

Other Ca^{2+}-binding proteins that may have a role in granule exocytosis include calmodulin, annexin II, and CAPS (Ca^{2+}-dependent activator protein for secretion). Calmodulin also plays a late role in certain forms of vesicle trafficking (Okabe *et al.*, 1992). Annexins are a family of EF hand (Ca^{2+} binding domain)-

containing proteins are able to bind phospholipids, which may promote lipid fusion (Ali *et al.*, 1989). CAPS is another candidate that has been proposed to mediate a late post-docking stage in the exocytotic pathway specific for dense-core vesicles (Walent *et al.*, 1992; Richmond and Broadie, 2002).

Other components of the exocytotic machinery which are not known to associate with the SNARE complex include Munc18, cysteine string (Csp), and Rab3a. Munc18 identified initially in chromaffin cells as nSec1 (Hodel *et al.*, 1994), is well known as a presynaptic syntaxin-binding protein that is involved in neurotransmitter release by binding tightly to syntaxin and holding it in a closed conformation, thereby preventing its assembly into a SNARE complex (Dulubova *et al.*, 1999; Verhage *et al.*, 2000). By an unknown mechanism, Munc18 releases syntaxin in a state that is primed for its interaction with other SNARE proteins, thus permitting the formation of SNARE complexes (Leenders and Sheng, 2005). Csp is a secretory vesicle membrane protein, which interacts with several presynaptic proteins including syntaxin and synaptotagmin (Nie *et al.*, 1999; Evans and Morgan, 2002). Csp is essential for a Ca^{2+}-dependent step of synaptic vesicle exocytosis, downstream of Ca^{2+} entry (Ranjan *et al.*, 1998; Dawson-Scully *et al.*, 2000). More recently, Csp has been shown to be expressed in chromaffin cells (Chamberlain and Burgoyne, 1996) where it is associated with chromaffin granules (Chamberlain *et al.*, 1996) suggesting a role in exocytosis. However, it is also possible that Csp is involved in endocytotic recycling after exocytosis (Südhof, 1995) since it interacts with hsc70, a protein that is required for uncoating clathrin-coated vesicles. Rab3 (four isoforms, A-D) is localized to both synaptic vesicles and chromaffin granules. Functional studies have indicated the involvement of rab3A in exocytosis of both vesicle classes (Holz *et al.*, 1994;

Geppert *et al.*, 1994), although its precise molecular function remains unknown (Morgan and Burgoyne, 1997).

In conclusion, the SNARE hypothesis, which proposes that vesicle targeting is based on specific interactions between vesicular (v-SNARE) and target membrane proteins (t-SNAREs) (Kretzschmar *et al.*, 1996), facilitates an understanding of the general mechanisms of the fusion of either synaptic vesicles or secretory granules. By combining electrophysiological and imaging techniques, it has recently been possible to study in detail the events involved in vesicle fusion, mainly on the basis of studying transmitter release from neuroendocrine cells, where transmitter is packaged in LDCVs that contain an electron-dense material (Lindau and Almers, 1995).

The identities of many of the proteins that participate in membrane fusion during exocytosis and that have key roles in the regulation of the fusion machinery are well established (Lin and Scheller, 2000) and summarized above. The exocytotic process can also be positively and negatively regulated by presynaptic receptors; either G protein-coupled receptors or ligand gated ion channels (Majewski and Iannazzo, 1998). Receptor activation in chromaffin cells results either in depolarisation and opening of voltage-dependent channels or direct activation of phospholipase C with subsequent generation of the Ca^{2+}-mobilising signal inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) which is in some cases accompanied by opening of store-operated Ca^{2+} channels (SOCs). The details of regulation of exocytosis by G protein-coupled receptors and ligand gated ion channels will be considered later (Section 1.3.3 and 1.4.4, respectively).

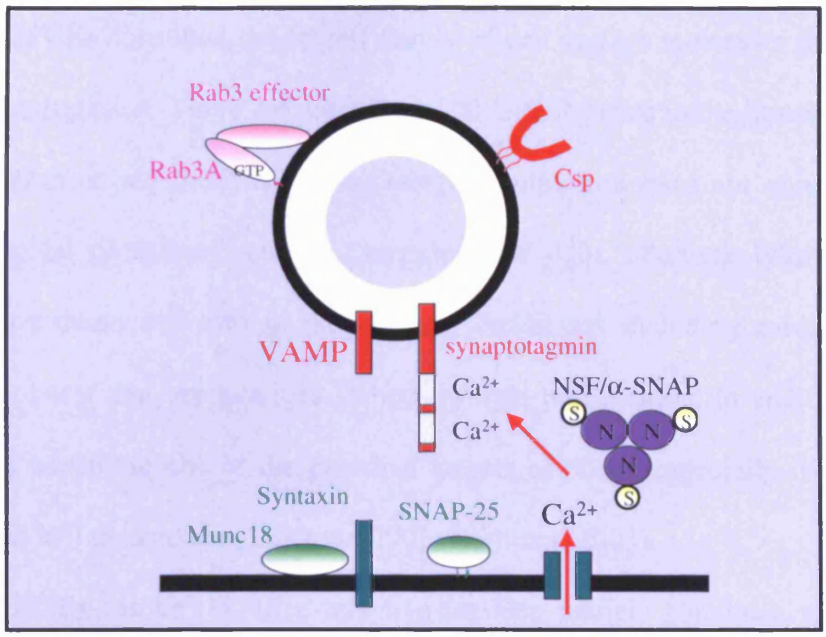


Figure 1.2.3. Major proteins expressed in chromaffin cells that are involved in regulating secretory granule exocytosis. The SNARE complex comprises three proteins: VAMP/synaptobrevin, syntaxin and SNAP-25 and interaction between these proteins leads to formation of the SNARE complex, which is essential for membrane fusion. NSF and α-SNAP act as molecular chaperones to help disassemble SNARE complexes to release SNARE proteins to engage in further membrane fusion events. Munc18 binds Syntaxin; phosphorylation of Munc18 by PKC would allow it to dissociate from syntaxin so that syntaxin could take part in SNARE complex assembly. Rab effectors are involved in the initial tethering of vesicles to target membranes. Synaptotagmin is likely to function as a component of the SNARE complex and acts as a Ca^{2+}-sensitive clamp. Voltage-gated Ca^{2+} channels (VOCCs) are also associated with syntaxin and upon cell depolarisation that triggers Ca^{2+} influx via VOCCs, the dissociation of synaptotagmin occurs allowing membrane fusion to proceed in a SNARE-dependent fashion. Csp acts to stabilise VAMP, allowing it to interact with plasma membrane-associated syntaxin and SNAP-25 to form an active SNARE complex for membrane fusion. This picture is modified from Burgoyne and Morgan (2003).

1.3 G PROTEIN-COUPLED RECEPTORS (GPCRs)

1.3.1 The superfamily of GPCRs

GPCRs constitute the largest family of cell surface molecules involved in signal transmission. There are more than 800 GPCR genes in the human genome (Fredriksson *et al.*, 2003) and these receptors play key roles not only in many physiological processes, acting as receptors for light, odorants, hormones and neurotransmitters but also in pathological conditions including cardiovascular disease, cancer and irregularities in body weight homeostasis. In addition, these receptors constitute one of the principal targets of drugs, especially in the CNS (reviewed in Tsao and Von Zastrow, 2001; Hermans, 2003).

GPCRs can be classified into five families, namely glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin forming the GRAFS classification system, which is based on the initials of the family names. Several classification system have been used, some systems group the receptors by how their ligand binds and other are based on structural features. The most commonly used systems uses clans (or classes) A, B, C, D, E, and F. This A-F system is designed to include all GPCRs in both vertebrates and invertebrates and clans D and E do not exist in human. Class A, B and C correspond to the rhodopsin, secretin and glutamate families respectively. The rhodopsin family constitutes the largest family and forms four main groups (α, β, γ and δ) with 13 sub-branches. The α-group of rhodopsin has five branches such as the prostaglandin receptors, amine receptors, opsin receptors, melatonin receptors, and MECA receptors (this includes melanocortin receptors, endothelial differentiation G-protein coupled receptors, cannabinoid receptors and adenosin binding receptors). The β-group includes 36 receptors such as tachykinin receptors, cholecystokinin receptors, neuropeptide Y

receptors, endothelin-related receptors, neuromedin B receptor, vasopressin receptors, gonadotrophin-releasing hormone receptors. The γ-group of rhodopsin receptors constitutes three main branches including SOG receptors (suboesophageal ganglion receptors), MCH receptors (melanin-concentrating hormone) and the chemochine receptors. The δ-group of the rhodopsin family has four main branches: the MAS-related receptors (a large family of GqPCRs called MAS-related genes (Mrgs) also known as sensory neuron-specific receptors or dorsal root receptors); glycoprotein receptors; purine receptors; and olfactory receptors. The secretin/glucagon receptor family contains the receptors for distinct hormones and peptides (e.g., calcitonin, glucagons, parathyroid hormone, secretin, vasoactive intestinal polypeptide, growth hormone-releasing hormone (GHRH), pituitary adenylate cyclase-activating polypeptide (PACAP) and diuretic hormone. The glutamate receptor family includes receptors for glutamate receptors and the calcium-sensing receptor.

1.3.2 Structural features of GPCRs

GPCRs are referred to as seven-transmembrane domain (7TM) receptors based on their highly conserved backbone structure (Muller, 2000). Thus, all members of this family are composed of a single peptide containing seven hydrophobic regions of similar length separated by hydrophilic loops of variable sizes. In other words, these receptors are characterised by the presence of seven α-helices crossing the plasma membrane separated by intracellular and extracellular loops. The NH_2 terminus is exposed to the extracellular environment and the COOH terminus is intracellular. The transmembrane domains of these receptors show most homology but there is much less homology between the N- and C-termini of different receptors. This diversity plays a role in allowing activation by

different ligands, coupling to different G-proteins and regulation by different intracellular proteins. For example, rhodopsin family receptors display short amino-terminal tails and have highly conserved amino acid residues within each transmembrane helix. The ligands for most of the rhodopsin receptors bind within a cavity between the TM regions (Baldwin, 1994) with exceptions that include the glycoprotein binding receptors where the ligand-binding domain is within the N-terminus. Receptors of the secretin family have an N-terminus between ~60 and 80 amino acids long containing a conserved cysteine bridge that is particularly important for binding of the ligand to these receptors. Receptors subtypes belonging to the metabotropic glutamate receptor family also have a relatively long N-terminus (250-580 residues). The N-terminus is thought to form two distinct lobes separated by a cavity in which glutamate binds (a "venus fly trap") and causes the lobes to close around the ligand. However, the calcium-sensing receptor, which also belongs to family C also has a long cysteine-rich N-terminus but it is uncertain if it is involved in the binding of Ca^{2+} although it is crucial for mediating Ca^{2+} signalling. The N-terminal of the GABA receptors is long and contains the ligand-binding site but lacks the cysteine-rich domain found in other receptors of this family (Fredriksson *et al.*, 2003).

The extracellular domains of GPCRs (including both the N-terminus and the extracellular loops) and the pocket formed by the assembly of the seven-transmembrane helices form the ligand recognition sites for many small molecules. In contrast, peptides often bind to the N-terminal but may well make connections within the TM helices. The intracellular receptor surfaces of GPCRs, including the intracellular loops and the C-terminal domain are important for G-protein recognition and activation although the precise sites vary amongst

receptors (reviewed in Ulloa-Aguirre *et al.*, 1999; Wess, 1998; Gether, 2000; Klabunde and Hessler, 2002).

1.3.3 G protein coupled receptor (GPCR) signalling

Upon activation, GPCRs associate with distinct classes of heterotrimeric G proteins comprised of three subunits: the α subunit that has the guanine-nucleotide binding site and GTPase activity and the βγ complex (Neer, 1995; Surya *et al.*, 1998). Up to now, at least 23 α-subunits have been identified and are classified into four families ($G\alpha_{i/o}$, $G\alpha_{s}$, $G\alpha_{q/11}$, and $G\alpha_{12}$). At least 6 and 12 different molecular species of β- and γ-subunits have been described, respectively (Hur and Kim, 2002; Vanderbeld and Kelly, 2000).

In the classical model of GPCR signalling, stimulation of a GPCR leads to activation of heterotrimeric G-proteins, which promote the exchange of a molecule of GDP for a molecule of GTP within the active site of the α-subunit. This is then followed by dissociation of Gα-GTP and Gβγ (Gilman, 1987). Both Gα-GTP and Gβγ can regulate ion channels indirectly via second messengers and protein kinases or directly via physical interactions between G-protein subunits and the channel protein (Wickman and Clapham, 1995). Alternatively, these subunits activate effector molecules which are enzymes that generate second messengers (Hur and Kim, 2002). Two families of ion channels have been suggested to be directly regulated by G proteins: voltage-activated Ca^{2+} channels which are inhibited by Gβγ (Ikeda and Dunlap, 1999) and G-protein-activated inwardly rectifying K^{+} channels which are activated by Gβγ (Catterall, 2000) and Gα (Wickman and Clapham, 1995) . The activated subunits affect their target molecules until the intrinsic GTPase activity of Gα hydrolyses the GTP to inactivate the Gα and cause it to re-associate with Gβγ thus completing the cycle.

Among the Gα protein families, this project is particularly interested in those of the $G\alpha_{q/11}$ family and their roles in regulation of exocytosis since very little is known about the mechanisms underlying facilitation of stimulus-secretion coupling by receptors coupled to $G\alpha_{q/11}$. Activation of $G\alpha_{q/11}$-coupled receptors results in generation of important second messengers including inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which mobilizes intracellular Ca^{2+}, and diacylglycerol that alone or in combination with Ca^{2+} can activate Protein kinase C (PKC) (Figure 1.3.3). The receptor-dependent generation of $Ins(1,4,5)P_3$ and DAG is dependent on the activation of PLCβ mediated by the $G_{q/11}$ class of α subunits and the hydrolysis of a plasma membrane lipid, phosphatidylinositol 4,5 bisphosphate ($PtdIns(4,5)P_2$). $Ins(1,4,5)P_3$ activates the endoplasmic reticulum (ER) IP_3 receptor (IP_3R) to release Ca^{2+} and this is often followed by activation of Ca^{2+} release-activated Ca^{2+} channels (I_{crac}) or SOCs in the plasma membrane to promote Ca^{2+} entry and allow refilling of Ca^{2+} stores (Kiselyov *et al.*, 2002). Ca^{2+} and PKC have been reported to play a crucial role in regulation of exocytosis (refer to Section 1.5.6 for details).

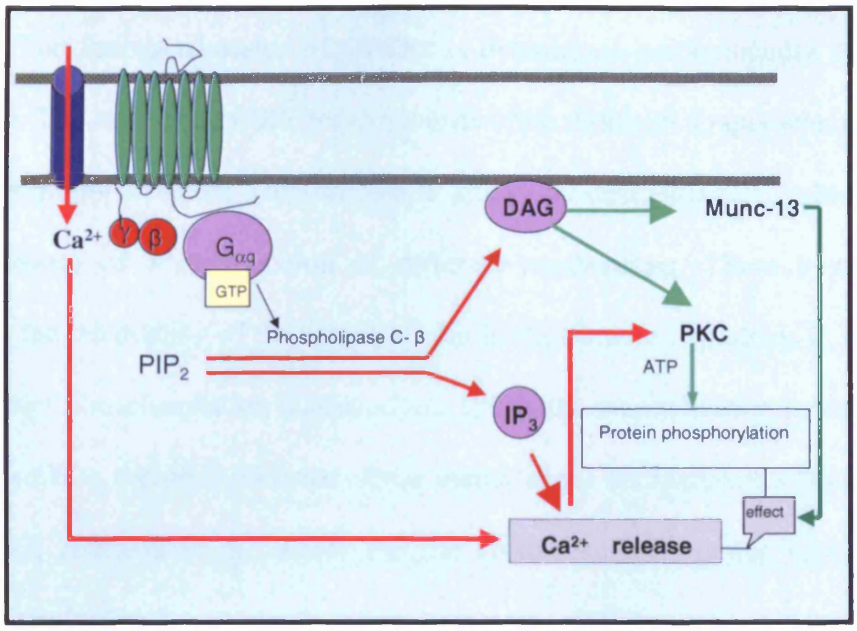


Figure 1.3.3. Activation of the $G\alpha_{q/11}$ –mediated signal transduction pathway. After ligand-induced receptor activation, the activated (GTP-bound) Gα- subunit of the heterotrimer dissociates from the Gβγ complex and stimulates the effector enzyme phospholipase Cβ (PLCβ) which in turn causes PtdIns(4,5)P_2 hydrolysis leading to formation of the second messengers Ins(1,4,5)P_3 which diffuses through the cytoplasm and releases Ca^{2+} from the endoplasmic reticulum, and DAG, which alone or in combination with Ca^{2+} activates PKC resulting in a cascade of protein phosphorylation (Ulloa-Aguirre *et al.*, 1999). Interestingly DAG may also activate Munc-13 directly which may be involved in mediating synaptic vesicle priming and fusion (Ashery *et al.*, 2000). This picture is modified from Ulloa-Aguirre *et al.* (1999).

1.3.4 Regulation of GPCR function

The functional status of GPCRs is determined predominantly by ligand binding. The exposure of GPCRs to agonists often results in a rapid attenuation of receptor responsiveness. This process is known as desensitisation, which is the consequence of a combination of different mechanisms. These mechanisms include the uncoupling of the receptor from heterotrimeric G proteins in response to receptor phosphorylation (Lohse *et al.*, 1990), the internalisation (endocytosis) of cell surface receptors to intracellular membranous compartments (Hermans *et al.*, 1997; Anborgh *et al.*, 2000) and the down-regulation of the total cellular complement of receptors due to reduced receptor mRNA and protein synthesis as well as both the lysosomal and plasma membrane degradation of pre-existing receptors (Pak *et al.*, 1999). The time frames over which these processes occur range from seconds (phosphorylation) to minutes (endocytosis) and hours (down-regulation). Phosphorylation of GPCRs occurs rapidly and involves phosphorylation of residues located in the intracellular loops and/or the COOH-terminal tail of the stimulated receptors by second messenger-dependent activated kinases (PKA or PKC) and/or by special class of serine/threonine-specific kinases called G protein-coupled receptor kinases (GRK1-7) (Freedman and Lefkowitz, 1996; Krupnick and Benovic, 1998; Hermans, 2003; Kristiansen, 2004). Morover, β- arrestin which belongs to a group of soluble inhibitory proteins binds to phosphorylated receptors leading to uncoupling from their respective G proteins. The binding of β-arrestin also targets some receptors to clathrin-coated pits from where they are internalised via endocytosis (Barak *et al.*, 1997). The β-arrestin mediates this process by interacting not only with agonist-occupied, phosphorylated receptor but also with a component of the clathrin-coated pit

machinery such as the heavy chain of clathrin itself and the β2-adaptin subunit of the clathrin adaptor protein AP-2. Interaction of β-arrestin with clathrin and AP-2, as well as phosphoinositides targets the GPCRs to punctate clathrin-coated pits at the cell surface. These pits are then pinched off the cell surface by the actions of dynamin, a GTPase. Following internalisation, receptors can be recycled to the plasma membrane or targeted for degradation in lysosomes. Internalisation can also occur in a β-arrestin-independent manner by, for example, association with caveolae. Caveolae are flask-shaped membrane invaginations that are rich in caveolin proteins as well as cholesterol. However, the mechanisms by which GPCRs targeted to caveolae are not well understood (reviewed in Pierce *et al.*, 2002). Receptor internalisation removes receptors from the cell surface whilst proteolytic degradation results in permanent loss of receptors and represents the predominant mechanism of down-regulation (Tsao and Von Zastrow, 2001). The pathway that mediates proteolytic down-regulation of GPCRs involves endocytosis followed by trafficking to lysosomes. Additional proteolytic machinery such as proteasomes or cell-associated endoproteases are also implicated in mediating down-regulation of certain GPCRs (Tsao and Von Zastrow, 2001).

The signalling by GPCRs is also subject to both acute and chronic regulation at sites other than the receptor, particularly by the regulation of the activity of downstream signalling molecules. These mechanisms are diverse and include, for example, the inactivation of Gα-subunits by regulators of G protein signalling (RGS proteins) which inhibit Gα by accelerating the rate of intrinsic GTPase activity of the subunits or alternatively by acting as effector antagonists

or guanine nucleotide dissociation inhibitors (Dohlman and Thorner, 1997; Hepler, 1999; Hollinger and Hepler, 2002; Hermans, 2003).

1.4 NICOTINIC ACETYLCHOLINE RECEPTORs (nAChRs)

1.4.1 nAChR subunits and subtypes

The nAChRs are pentameric ligand-gated ion channels (LGICs) and members of the superfamily of neurotransmitter receptors that includes gamma aminobutyric acid ($GABA_A$ and $GABA_C$ receptors), glycine and 5-hydroxytryptamine ($5\text{-}HT_3$) receptors (Stroud *et al.*, 1990; Gotti *et al.*, 1997). These receptors are known as Cys-loop receptors as all of them have a conserved sequence containing a pair of cysteines separated by 13 residues and linked by a disulfide bridge. nAChRs can be divided into two groups: muscle receptors which are found at the skeletal neuromuscular junction where they mediate neuromuscular transmission and neuronal receptors which are found throughout the peripheral and central nervous systems (Hogg and Bertrand, 2003).

To date, 17 nicotinic subunits have been cloned in vertebrates: α1, β1, γ, ε, and δ expressed in muscle tissue where they mediate transmission at the neuromuscular junction and twelve distinct "neuronal" α2-α10 and β2-β4 subunits widely expressed in the nervous system (McGehee and Role, 1995; Fucile, 2004). These subunits may constitute heteromeric or homomeric receptors. Neuronal subunits are also found in an increasing number of non-neuronal cells such as glial cells, endothelial cells, keratinocytes, macrophages, lymphocytes, ect (Sharma and Vijayaraghavan, 2002; Wang *et al.*, 2003). In addition, although early studies indicated that human small-cell lung carcinoma (SCLC) cells, which have a 'neuroendocrine' phenotype, express only muscarinic cholinergic receptors (Cunningham *et al.*, 1985), more recent studies have reported nicotinic receptor

expression (Codignola *et al.*, 1994). Most interestingly, SCLC cells express nicotinic receptors of the neuronal type. In particular, SCLC cells express α3 and β4 nicotinic receptor subunits. In addition, SCLC cells express α7 nicotinic subunit and consistent with this both α-bungarotoxin and α-conotoxin block the secretagogue and mitogenic effects of nicotine. Moreover, other neuronal nicotinic receptor subunits have also been found in SCLC cells, such as α5 and β2 subunits (Sher *et al.*, 2000). The distinct combinations of nAChR subunits produce receptors with different physiological properties and both α and β subunits seem to contribute to the functional diversity of the nAChRs as their molecular diversity arises from assembly of homomeric and heteromeric pentamers (Dajas-Bailader, 2002a).

Two different classes of neuronal nAChRs may be identified according to their Ca^{2+} permeability: (i) neuronal nAChRs containing subunits (α7-α9) able to bind the snake venom α-bungarotoxin (α-BTX) and to form homopentameric channels (α-BTX nAChRs) exhibiting highest Ca^{2+} permeability (Jagger *et al.*, 2000; Fucile *et al.*, 2003). (ii) heteropentameric non-α-BTX-sensitive nAChRs (non-α-BTX nAChRs) comprising at least one α (out of α2-α6) and one β (out of β2-β4) subunits which exhibit low Ca^{2+} permeability (Haghighi and Cooper, 2000; Lax *et al.*, 2002). The major brain subtype with high affinity for nicotine seems to be the α4β2 nAChR which is α-Bgt-insensitive (Flores *et al.*, 1992). The major ganglionic subtype is α3β4 and the major subtype with high affinity for α-Bgt found in brain and ganglia is composed of homomeric arrangements of α7 (Dominguez Del Toro *et al.*, 1994). In addition, assembling of α5 subunit either with α3β2 or α3β4 subunits yields nAChRs with high Ca^{2+} permeability

(Gerzanich *et al.*, 1998). The α-Bgt-sensitive nAChRs, especially the α7-containing subtype, have been the target of intense investigation as they show high Ca^{2+} permeability (Vijayaraghavan *et al.*, 1992; Séguéla *et al.*, 1993; Rathouz *et al.*, 1996). Pieces of evidence indicate a functional correlation between the activation of α7 nAChR and Ca^{2+}-dependent cellular processes such as neurotransmitter release, synaptic plasticity, cell growth, migration and survival (MacDermott, 1999; Belluardo *et al.*, 2000). Some studies have suggested an association of the α7 subunit with important neuropathologies such as Alzheimer's disease, scizophrenia and epilepsy (Leonard *et al.*, 2001). Ca^{2+} entering the neuron through α7 channels could act as a second messenger in several cellular processes including the regulation of neurite outgrowth, neurotransmitter release and synaptic plasticity (Torrão and Britto, 2002; Fucile, 2004).

1.4.2 Structure of the nAChR

Individual nAChR subunits consist of a number of distinct functional domains (Figure 1.4.2). The large extracellular N-terminal that participates in the formation of the ligand binding domain contains putative glycosylation sites, a disulphide-linked cysteine loop between residues homologous to 128 and 142 of the α1 subunit and the interface for agonist binding (loops A, B and C in α subunits and loops D, E and F in all subunits) (Figure 1.4.2, panel B) (Corringer *et al.*, 2000). The polypeptide chain of nAChR subunits contain four hydrophobic, putative transmembrane domains (M1-M4) (Sargent, 1993; Elgoythen *et al.*, 1994) (Figure 1.4.2, panel A). Evidence indicates that M2 is α-helical and lines the cation pore. M3 and M4 are separated by a large variable intracellular loop which contains putative phosphorylation sites for ser/thr kinases (Hogg *et al.*,

2003). As mentioned above, the twelve subunits have been classified into two subfamilies of eight α and three β subunits. The α subunits have two adjacent cysteines that are homologous to those present at positions 192 and 193 of the α subunits of the muscle-type nAChR. In contrast, the β subunits (β2-β4) lack the pair of adjacent cysteines and are considered structural subunits, although it has been clearly demonstrated that both α and β subunits contribute towards the pharmacological specificity of nAChR subtypes (Luetje and Patrick, 1991). Since nAChRs result from the assembly of five subunits, they constitute an excellent prototype of allosteric proteins in which the ligand binding is distinct from the "reaction site" that is, the ionic pore. Allosteric proteins have the particularity to undergo spontaneous transitions between different conformations (Buisson and Bertrand, 1998). Upon agonist binding, nAChRs undergo an allosteric transition from the closed, resting conformation to an open state which conducts the cations Na^+, K^+ and Ca^{2+}. In the active (open) conformation, the nAChR binds agonists with low affinity. The continued presence of agonist leads to ion channel closure and receptor desensitisation. In this condition, the nAChR is refractory to activation although it displays higher affinity for agonist binding. The rates of desensitisation and recovery differ between nAChR subtypes: for example, the α7nAChR displays very rapid desensitisation (Couturier *et al.*, 1990a; McGehee and Role, 1995; Leonard and Bertrand, 2001). Prolonged agonist exposure may produce an inactivated state, from which recovery is very slow. The α4β2 neuronal nAChR is prone to inactivation on chronic nicotine treatment (Kuryatov *et al.*, 2000). However, receptors composed of α3β4 or α3β4α5 display a slower time course of desensitisation than the α7 receptor (Couturier *et al.*, 1990b; Role, 1992). Transitions between resting, open and desensitised states are reversible and

different ligands may stabilise different receptor states: agonists initially stabilise the activate (open) state whereas competitive antagonists preferentially stabilise the nAChR in a closed state, either the resting or desensitised configuration (Buisson and Bertrand, 1998).

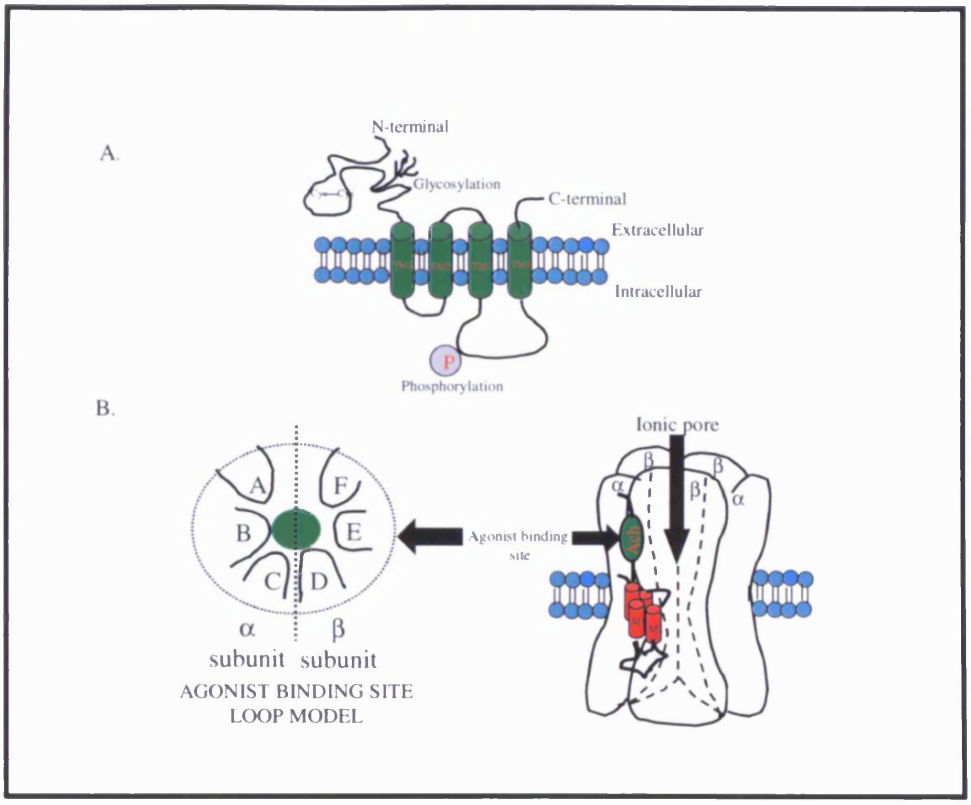


Figure 1.4.2. Schematic representation of neuronal nicotinic acetylcholine receptor structure. Panel A: shows a structure of a neuronal nAChR subunit. Panel B: structure overview of an nAChR and agonist binding site including the model of the agonist binding site loop (modified from Leonard and Bertrand, 2001).

1.4.3 Regulation of nAChRs

Phosphorylation is an important mechanism in the regulation of ligand-gated ion channels. This regulation includes modulation of receptor expression, subcellular localization and channel properties such as desensitisation and recovery from inactivation (Hogg and Bertrand, 2003). Both serine and tyrosine residues of the nAChR can be phosphorylated (Huganir, 1991) and this regulates receptor desensitisation (Mulle *et al.*, 1988). Investigations in cells from chick ciliary ganglia showed that α3, α4, α5, α7, β2, β3 and β4 subunits are phosphorylated by both PKA and PKC. Consensus sequences for PKA and PKC phosphorylation sites have been identified on the major intracellular loop between the M3 and M4 transmembrane segments of the rat, chick and human α7 and α4 subunits and two isoforms of the human α1 subunit. At each of these putative phosphorylation sites the phosphorylated residue is serine (Wecker *et al.*, 2001). The functional down-regulation of α4β2 nAChRs in permanently transfected HEK 293 cells in response to chronic nicotine exposure appears to be mediated by down-regulation of PKC activity (Eilers *et al.*, 1997). Furthermore, recovery from down-regulation is accelerated by using inhibitors of protein phosphatases 2A and 2B suggesting that nicotine-induced down-regulation of nAChRs involves dephosphorylation at PKC phosphorylation sites. Although, phosphorylation of α7 nAChRs *in vivo* remain to be investigated, the intracellular domain of neuronal nAChRs is the most divergent region of the molecule. However, the phosphorylation site is highly conserved suggesting that phosphorylation may be important in modulating α7 nAChR function (Hogg and Bertrand, 2003).

1.4.4 Function of nAChR

Neuronal nAChRs are gaining credence as significant players in the nervous system, particularly as a consequence of their relationship to a number of disease states in which they are perceived as novel drug targets (Decker *et al.*, 1998). These include Alzheimer's and Parkinson's diseases, schizophrenia, Tourette's syndrome and attention deficit hyperactivity disorder. In addition, neuronal nAChRs are also targets for analgesia, anxiolysis, neuroprotection and smoking cessation (Hogg *et al.*, 2003). A large body of evidence indicates that neuronal nAChRs are present presynaptically in the central nervous system where they facilitate neurotransmitter release, most likely through an increase in the intracellular $[Ca^{2+}]_i$ due to a substantial influx of Ca^{2+} through their channels. In addition to promoting exocytosis by opening of VOCCs through membrane depolarisation, nAChRs can also initiate exocytosis directly by virtue of their intrinsic Ca^{2+} permeability. Indeed, nAChRs have a pivotal role in the regulation of neurotransmitter release as presynaptic nAChRs facilitate the Ca^{2+}-dependent release of many neurotransmitter, which is consistent with activation of exocytotic mechanisms (Wonnacott, 1997). In addition to provoking Ca^{2+}-dependent exocytosis, presynaptic nAChRs also modulate transmitter release through Ca^{2+}-mediated cellular mechanisms for example, PKC which may have a role in the facilitation of catecholamine release (see Section 1.5.6) and is reported to contribute to the regulation of exocytosis in adrenomedullary cells (Soliakov and Wonnacott, 2001; Dajas-Bailador and Wonnacott, 2004).

The chromaffin cell of the adrenal medulla, which receives a major cholinergic input from the splanchnic nerve to release catecholamines into the bloodstream possesses neuronal nAChRs that comprise heterologous assemblies

of α3, α5, and β4 subunits (Criado *et al.*, 1992; Campos-Caro *et al.*, 1997) as well as a distinct homomeric α7 receptor inhibited by α-bungarotoxin (Garcia-Guzman *et al.*, 1994). More recently, by using a new protocol (i.e., consisting of short pulses of stimulation) and novel pharmacological tools (i.e., selective toxins to block nAChRs) it has been suggested that both α7 and α3β4 nAChR are involved in the generation of inward nAChR currents as well as in the triggering of Ca^{2+} entry and catecholamine release in response to stimulation of bovine chromaffin cells (López *et al.*, 1998; Maneu *et al.*, 2002).

1.5 PROTEIN KINASE C (PKC)

1.5.1 Protein Kinase C isoforms

The discovery of protein kinase C (PKC) in 1977 by Nishizuka and co-workers represented a major breakthrough in the signal transduction field (Takai *et al.*, 1977). PKC is regarded as a key triggering step in numerous cellular processes, from the regulation of gene transcription, mitogenesis, cell proliferation, apoptosis, remodelling of the actin cytoskeleton, to the modulation of ion channels and stimulus-secretion coupling in hormone and neurotransmitter release (Newton, 1995; Toker, 1998).

PKC represents a family of at least 12 serine / threonine kinases that participate in signal transduction in response to specific hormonal, neuronal and growth factor stimuli. Differences in their structures have permitted classification of mammalian PKC isoforms into three groups (Figure 1.5.1): the first group is the conventional PKCs (cPKCs) which includes α, βI, βII, and γ isoforms. This class is considered to be Ca^{2+}- dependent and activated by the second messenger DAG in the presence of phosphatidylserine (PS). The second group is the novel PKCs (nPKCs) which comprise the δ, ε, η, μ, and θ isoforms. Members of this

group are Ca^{2+}-independent and regulated by DAG and PS (Way *et al.*, 2000). The third group contains the atypical PKCs (aPKCs), namely ξ and ι/λ. PKCλ is a mouse homologue of the human ι isoenzyme. These protein kinases are Ca^{2+}-insensitive and do not respond to the tumor-promoting phorbol esters, which are pharmacological agents that are extensively used to mimic the action of DAG (Mellor and Parker, 1998). Finally, the recently discovered PKC-related kinases (PRKs) represent a fourth group consisting of at least three members, PRKs 1-3. These are similar to aPKCs in being Ca^{2+}-, DAG-, and phorbol ester-insensitive (Palmer *et al.*, 1995). The PKC isoforms differ in their structure, cofactor requirements, substrate specificity and tissue expression with specific subcellular distribution (Mochly-Rosen and Gordon, 1998). Moreover, the presence of more than one PKC isoform in a single cell type has led to the notion that each member of the PKC family plays a specific role in the processing of physiological and pathological responses to extracellular stimuli (Sena *et al.*, 2001). Today, based on biochemical and structural differences between the isoforms, selective PKC inhibitors and activators have been developed allowing the dissection of the different signalling pathways in which the members of this family participate (Casabona, 1997). Because of the importance of PKC isoforms in major cellular functions, they are considered as potential targets for therapeutic intervention. It is widely accepted that inhibitors of PKC isoforms may be useful in treating autoimmune diseases including rheumatoid arthritis, diabetes mellitus, multiple sclerosis, Alzheimer's disease or cardiovascular diseases (Gescher, 1992; Basu, 1993; Hofmann, 1997).

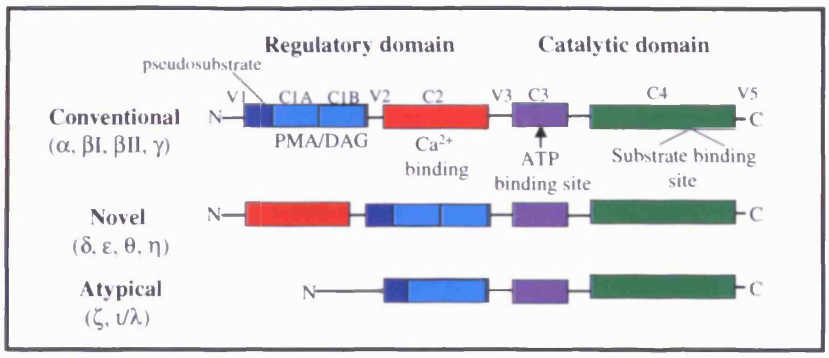


Figure 1.5.1. Schematic of primary structures of PKC family members indicating the domain composition. The PKC structure can be divided into an N-terminal (N) regulatory domain and a C-terminal (C) catalytic domain. The cPKC structure comprises four conserved regions (C1-C4) and five variable regions (V1-V5). The N-terminal moiety contains the regulatory modules: the pseudosubstrate; the C1A and C1B domains (not present in the atypical PKCs) binds DAG and phorbol esters; the C2 domain for conventional PKCs binds Ca^{2+}. The C-terminal moiety contains ATP and substrate binding sites. The figure is a modification of a figure from Nishizuka (1992).

PKCs can be considered as 'classical' transducers of signal from many extracellular agonists. The initial discovery of the enzyme as a Ca^{2+}-activated enzyme, was immediately followed by the realization that PKC was the major target of the lipid metabolite DAG (Inoue *et al.*, 1977; Kishimoto *et al.*, 1980). In addition, the observation that PKC is also the major receptor for tumor-promoting phorbol esters provided a key reagent for studying the mechanism of action of this enzyme (Castagna *et al.*, 1982).

Although, the receptor-mediated hydrolysis of inositol phospholipids was thought to be the sole mechanism leading to the activation of PKC, recent studies suggest the existence of several other routes to provide the DAG that is needed for enzyme activation (Farago and Nishizuka, 1990; Majewski and Iannazzo, 1998). For instance, phosphatidylcholine may be hydrolysed by PLD to produce DAG (Exton, 1988). In addition, both receptor-mediated, voltage-dependent and Ca^{2+} influx through non-selective cation channels may cause phospholipid degradation as it may be initiated by the activation of PLC and PLD and phospholipase A2 due to Ca^{2+} influx (Farago and Nishizuka, 1990).

1.5.2 Tissue distribution of PKC isoforms

PKC isoforms are widely distributed in mammalian tissues and some isoforms are localized to specific tissues. PKCα, βI, βII, δ, ε and ξ seem to be ubiquitous isoforms and are found in most or all tissues. PKCγ is largely restricted to the central nervous system (Nishizuka, 1988; Hug and Sarre, 1993). PKCη is strongly expressed in skin and lung but only slightly in brain. PKCθ is predominantly present in skeletal muscle and to a lower extent in lung, spleen, skin and brain (Osada *et al.*, 1992). PKCμ has been found in numerous tissues and is strongly expressed in thymus and lung. Furthermore, the distribution of these

isoforms among cell types has been studied extensively for neural tissues. Such studies reveal that the conventional isoforms are present in axon terminals at the endings of the sensory nerves and in post-synaptic dendrites. In contrast, PKCδ predominates in neuronal cell bodies and PKCε is concentrated in presynaptic terminals within the central nervous system. As most tissues are made up of several different cell types, it is also a challenge to learn what isoforms are present in specific cell types (Liu and Heckman, 1998). Moreover, within a single cell, PKC isoforms exhibit differences in their distribution before and after activation (Monchly-Rosen and Gordon, 1998). For example, within non-stimulated rat cardiomyocytes PKCβII is associated with fibrillar structures and on activation translocates to both the perinuclear region and cell periphery (Disatnik *et al.*, 1994). In these cells, PKCα and PKCζ translocate from the cytosol to the perinuclear membrane while PKCβI translocates from the cytosol and perinucleus to the nucleus on activation.

Increasing evidence suggests that individual PKC isoforms can translocate to subcellular locations other than the plasma membrane including other membrane vesicles, nuclear structures, and cytoskeletal components. The subcellular location of a specific isoform may directly control the potential of that isoform to perform distinct functions because the targeting of PKCs to discrete subcellular compartments would restrict their access to potential substrates (Keenan and Kelleher, 1998). This intracellular compartmentalization could reflect the presence of intracellular PKC receptors at cytosolic and particulate level, named "receptor for activated C kinase" (RACK) that are specific for each isoform; such receptors, which bind the different PKC isoforms in the active,

"open", conformation may play an important role in their subcellular distribution and dynamics of activation (Mochly-Rosen *et al.*, 1991a).

1.5.3 Structure of PKC isoforms

Members of the PKC family consist of a single polypeptide composed of an N-terminal regulatory region (approximately 20–40 kDa) and a C–terminal catalytic region (approximately 45 kDa). The regulatory region possesses the motifs that are involved in the binding of the phospholipid cofactors and Ca^{2+} and participates in protein-protein interactions that regulate PKC activity and localization. The carboxyl-terminal region is the kinase domain and includes motifs involved in ATP and substrate binding. The regulatory and catalytic domains are connected by a hinge region that is highly sensitive to proteolytic cleavage by cellular proteases (Ron and Kazanietz, 1999). Initial cloning of the first PKC group in the mid-1980s demonstrated that the polypeptide structure comprises four conserved (C1 – C4) and five variable regions (V1 – V5) (Coussens *et al.*, 1986). In the NH_2 terminal domain of the regulatory region, cPKCs contain two conserved sequences, C1 and C2 that are important for the regulation of the enzymatic activity. On the other hand, nPKCs and aPKCs are devoid of C2 regions. All isoforms have two conserved regions on the carboxyl terminal domain, C3 and C4 which are responsible for ATP binding and protein substrate binding respectively (Figure 1.5.1) (Azzi *et al.*, 1992; Dekker and Parker, 1994; Hug and Sarre, 1993; Tanaka and Nishizuka, 1994).

The C1 region is present in all PKC isoforms. It is a small globular structure (approx. 8 kDa) that has a binding site for DAG (Zhang *et al.*, 1995a; Newton, 1995). In addition, phorbol esters (non-hydrolysable analogues of the endogenous ligand) also bind to the C1 domain. Moreover, the C1 domain

specifically binds phosphatidylserine (Johnson *et al.*, 2000). This region is also characterized by the presence of the pseudosubstrate sequence and two repeats of cysteine-rich (cys1 and cys2) zinc finger-like motifs that form a "zinc butterfly"and binds DAG and phorbol esters (Ono *et al.*, 1989). Conventional and novel PKCs have two C1 domains but some evidence suggests that only one engages ligand *in vivo*. For some isoforms such as PKCδ, the C1B domain is primarily responsible for binding DAG (Szallasi *et al.*, 1996). For others, such as PKCα, the C1A and C1B domains have equivalent roles in targeting PKC to membranes (Bogi *et al.*, 1999). Atypical PKC and PRK isoforms are slightly different in that they have only one cysteine-rich region. As the ligand-binding pocket of the C1 domain is impaired in aPKCs and PRKs, these isoforms do not respond to either DAG or phorbol esters (Hurley *et al.*, 1997). The function of the single zinc finger in the aPKCs is still unknown (Mellor and Parker, 1998). The beginning of the N–terminal region has a motif that resembles the consensus sequence found in the phosphorylation sites of prominent PKC substrates but possesses alanine instead of serine or threonine (House and Kemp, 1987). As a result, this motif cannot be phosphorylated and appears to be a pseudo–substrate that serves an autoregulatory function by blocking the catalytic site. This autoinhibitory domain or pseudosubstrate binds to the substrate binding site in the catalytic domain and keeps the enzyme in an inactive state in the absence of cofactors and activators (Orr *et al.*, 1992).

Interestingly, the C1 domain is also present in several other mammalian proteins such as DAG kinase, protein kinase D (PKD), and the chimaerin family. Munc–13 also contains C1 and C2, which have been shown to interact with two synaptic vesicle proteins, namely DOC2 (double C2 domain) and syntaxin

(Mellor and Parker, 1998). Consequently, these proteins are considered other targets for DAG and phorbol esters. This lack of specificity in the stimulating action of phorbol esters raises the possibility that some of the effects attributed to PKC may in fact due to the interactions with these or other as yet not identified proteins (Kiley and Jaken, 1994).

In contrast, the C2 domain is present in conventional and novel PKCs (Nalefski and Falke, 1996; Ponting and Parker; 1996). In conventional PKCs, this 12 kDa domain serves as a membrane-targeting module that binds anionic phospholipids in a Ca^{2+}-dependent manner. The C2 domain of novel isoforms lack key residues involved in Ca^{2+} binding and as a result bind neither Ca^{2+} nor phospholipids (Newton, 2003). Like the C1 domain, the C2 domain has been found to be present in many other proteins including synaptotagmin, rabphilin–3A, phospholipases and GAPs (GTPase-activating proteins) (Ponting and Parker, 1996). Similarly, a class of proteins has been identified that interacts with the C2 domains of PKCs such as the receptors for activated PKCs (RACKs).

The C–terminal regions (C3 – V5) have been recognised in all PKC isoforms as the catalytic domain. The C3 region contains an ATP–binding sequence similar to that found in other protein kinases whereas the C4 domain, contains the sites required for the recognition of substrates (Way *et al.*, 2000). There is evidence that the V5 domain plays a critical role in the cellular localisation. Previous studies in the human U 937 monocytic cell line have shown that PKCβI is localised to the microtubules while PKCβII is localised in part to secretory granules. The only difference between these two proteins is the V5 region (Way *et al.*, 2000).

1.5.4 Sub-cellular targeting of PKC isoforms

Kraft *et al.* (1982) reported for the first time that PKC translocates from the soluble fraction of cells to the particulate fraction in response to phorbol esters. The translocation of PKC from cytoplasm to plasma membrane has become a hallmark for PKC activation (Kraft *et al.*, 1982). The activation of PKC is triggered by a large number of extracellular signals including hormones, neurotransmitters, and growth factors that act through cell surface receptors. The activation of these receptors regulates the intracellular levels of various PKC activators including DAG, Ca^{2+}, and many other lipid mediators (Feng *et al.*, 1998). The mechanisms by which the conventional PKC isoforms become membrane-associated and thus activated have been shown to involve two sequential steps. First there is an initial Ca^{2+}- and anionic phospholipid-dependent interaction of the C2 domain with the membrane followed by binding of DAG to the C1 domains (Newton and Johnson, 1998, Medkova and Cho, 1999; Bittova *et al.*, 2000). The interaction of DAG with the C1 domain also results in an increased specificity of both membrane-association and activation for phosphatidylserine over other anionic phospholipids (Orr and Newton, 1992; Newton and Keranen, 1994; Johnson *et al.*, 2000). The initial interaction of the C2-domain with the membrane brings the adjacent C1 domains into close opposition with the membrane head group region. This facilitates the binding of DAG, which promotes a penetration (Newton and Johnson, 1998; Johnson *et al.*, 2000). The combined interactions of the C1 and C2 domains with the membrane provides the free energy for structural rearrangements that lead to the dissociation of the N-terminal pseudosubstrate from the active site, which allows substrate binding. This process is further facilitated by a weak interaction of the released

pseudosubstrate with anionic head groups at the membrane surface (Mosior and McLaughlin, 1991). The regulatory domains of nPKC isoforms also contain C1 and C2 domains, but it is not known whether similar mechanisms of membrane-association and activation are involved (Slater *et al.*, 2002). In addition to the natural activators, including DAG, the enzyme is activated with high affinity and specificity by the tumor promoting phorbol esters (Blumberg, 1991). For this reason, phorbol esters are often used in the study of the mechanisms of PKC activation, based on the assumption that they compete directly with DAG for a common binding site on the enzyme (Sharkey and Blumberg, 1985).

The isoform-specific functions may result in part from their differential subcellular localization (Mochly-Rosen and Gordon, 1998), therefore, PKC subcellular localization has been extensively studied in cultured cells using antibody staining and immunofluorescent microscopy (Mochly-Rosen, 1995). However, many signal transduction events involving PKC are rapid, transient, and difficult to follow in fixed cells. Green fluorescent protein (GFP), because of its inherent bioluminescence and stoichiometric labelling, represents a sensitive optical reporter to follow the real time localization of many proteins in live cells.

1.5.5 Regulation of PKC

PKC is regulated by three distinct mechanisms. Firstly, by phosphorylation which 'primes' the enzyme for catalysis (Blumberg *et al.*, 1984). Previous studies have shown that PKC is inactive when it is synthesized but subsequent phosphorylation stabilizes the catalytically competent conformation of the kinase and causes release of the mature enzyme into the cytosol (Borner *et al.*, 1989). Indeed, PKC is processed by a series of ordered phosphorylations that are

required for the enzyme to gain catalytic competence and the correct intracellular localization (Keranen *et al.*, 1995; Tsutakawa *et al.*, 1995).

The first and rate-limiting step in the processing of PKC is phosphorylation on the activation loop by phosphoinositide-dependent kinase, PDK-1, which is regarded as the upstream kinase for PKC isoforms (Chou *et al.*, 1998; Dutil *et al.*, 1998). PDK-1 plays a pivotal role in signalling by turning on the catalytic function of diverse members of the AGC family kinases (Toker and Newton, 2000). These kinases require activation loop phosphorylation to gain catalytic competence and this therefore functions as a switch. Although this phosphorylation is required for the maturation of PKC, once the enzyme is phosphorylated at the C-terminal sites, the activation loop phosphate is dispensable (Keranen *et al.*, 1995). Thus, the activation loop phosphorylation is required to initiate the C-terminal processing, but once this event is completed, phosphate on the activation loop site is not necessary for activity. The importance of phosphate at the conserved segment of PKC comes from two things: it correctly positions residues for catalysis and it unmasks the entrance to the substrate binding cavity (Johnson and O'Reilly, 1996).

The second step in the processing of PKC is phosphorylation of the turn motif. Phosphorylation at the activation loop triggers the rapid phosphorylation of a motif in a pro-rich domain and this site corresponds to Ser338 in PKA which is present at the apex of a turn on the upper lobe of the kinase domain. Molecular modelling suggests that this site occupies a similar position in PKC, hence the name 'turn motif'. Several lines of evidence suggest that this site is modified by autophosphorylation. The phosphate at the turn motif is needed for catalytic function of the enzyme, whereas dephosphorylation at this position abolishes

activity (Keranen *et al.*, 1995). Biochemical studies indicate that phosphate at this position locks PKC in a catalytically competent, thermally stable, and phosphatase-resistant conformation (Edwards *et al.*, 1999). Following phosphorylation of the turn motif, PKC isoforms rapidly autophosphorylate at the hydrophobic motif (Behn-Krappa and Newton, 1999). The hydrophobic site has been proposed to be modified by its own upstream kinase, PDK-2 (Alessi *et al.*, 1997). The hydrophobic site has a second role in addition to stabilizing the structure of PKC: the sequence surrounding the hydrophobic phosphorylation motif forms a docking site for PDK-1 (Biondi *et al.*, 2000). This site is exposed for PDK-1 binding in the unphosphorylated forms of PKC but becomes masked in the phosphorylated (and inactive) conformation. It has been suggested that newly synthesized PKC localizes to the membrane where it adopts a conformation in which the pseudosubstrate is out of the active site, thus exposing the activation loop phosphorylation site. This conformation is essential to target PKC for phosphorylation by PDK-1 (Dutil and Newton, 2000).

The second mechanism of PKC regulation is by ligand binding that promotes the membrane association of PKC and this membrane interaction provides energy to release the pseudosubstrate from the substrate-binding cavity (Kraft *et al.*, 1982).

Initial studies on PKC focused on its requirement of cofactors for activation, and the three classical activators were identified: phosphatidylserine, Ca^{2+}, and DAG. Furthermore, biochemical and biophysical studies on PKC have established the central roles of the C1 and C2 domains in driving this spatial redistribution, which leads to activation.

Regulation of PKC activity by phosphatidylserine. The activity of all isoforms of PKC is regulated by phosphatidylserine, an aminophospholipid found exclusively on the cytoplasmic leaflet of membranes which typically comprises approximately 15 mol% of the total lipid (Newton, 1993). Binding studies revealed that in the absence of DAG, PKC displays little selectivity for phosphatidylserine. The accepted model of activation of PKC by lipids is that on binding DAG (or phorbol esters) in the presence of the phospholipid cofactor, a conformational change in PKC results in the removal of the pseudosubstrate from its binding site and in the activation of enzyme. It is believed that the cysteine-rich and C2 domains are not the only regions involved in phospholipid binding. Thus the pseudosubstrate domain, once it is removed from its binding site may also contribute to membrane binding (Orr *et al.*, 1992).

Regulation of PKC activity by diacylglycerol and phorbol esters. Phorbol esters and DAG cause a dramatic increase in the affinity of PKC for membranes. In another words it serves as a 'molecular glue' to recruit PKC to membranes. The nature of the 'molecular glue' could be a consequence of the occupancy of the ligand binding cavity in the C1 domain which results in the presentation of a contiguous hydrophobic surface. Thus, C1 domain ligands recruit PKC by altering the surface properties of the domain to favour membrane presentation (Zhang *et al.*, 1995a). Both phorbol esters and DAG regulate PKC by the same mechanism. However, they can have very different biological effects for two reasons: firstly, membrane recruitment and activation initiated by DAG is short lived because DAG is rapidly metabolized, whereas phorbol esters are not readily metabolized and cause sustained activation of PKC. Secondly, phorbol

esters are more potent than DAG in recruiting PKC to membranes (Newton, 2001).

Regulation of PKC activity by Ca^{2+}. Although the molecular events mediating Ca^{2+}-induced activation of cPKCs are not fully understood, there is evidence that this cation facilitates translocation by increasing the affinity of the C2 domain for anionic lipids. It is reported that increased intracellular Ca^{2+} is not essential for PKC translocation and activation especially if the C1 domain achieves sufficiently tight binding. The C2 domain does not need to be fully engaged on the membrane for activation. Therefore, phorbol ester stimulation can cause translocation of conventional PKC isoforms in the absence of changes in intracellular Ca^{2+}. DAG and Ca^{2+} synergize in activating PKC but this synergism does not result from allosteric interactions between the C1 and C2 domains. Rather, each ligand independently causes the affinity of PKC for anionic lipids to increase (Hannun *et al.*, 1986). In addition, the presence of one ligand dramatically decreases the amount of the second ligand required to activate the enzyme (Oancea and Meyer, 1998).

The third mechanism of PKC regulation is by anchoring proteins: a family of proteins called RACKs (for receptors for activated C kinase) anchor the active conformation of phosphorylated PKC at specific cellular locations (Mochly-Rosen *et al.*, 1991b). PKC-RACK interaction is mediated at least in part by the C2 region in cPKCs and the C2-like region (within the V1 region) in nPKCs (Csukai *et al.*, 1997). To date, two RACKs have been identified: RACK1, the selective RACK for PKCβII (Ron *et al.*, 1994), and RACK2, (previously identified as β`COP (Stenbeck *et al.*, 1993)), the selective RACK for PKCε (Csukai *et al.*, 1997). βIIRACK and εRACK bind their respective isoforms in a selective and

saturable manner (Csukai *et al.*, 1997). In addition, both RACKs bind PKC in its active conformation. Despite the differences in the sequences of RACK1 and RACK2, the two proteins share common features. Although, RACK1 and RACK2 are not PKC substrates, both increase PKC phosphorylation of substrates (Ron *et al.*, 1994). Other proteins named STICKs (for substrates that interact with C kinase) bind inactive, phosphorylated PKC and then release the activated kinase following their phosphorylation (Jaken, 1996).

Other mechanisms that regulate PKC activity include its proteolytic degradation. The hinge region at the border between the V3 and catalytic domains is thought to be a site sensitive to proteolytic cleavage by trypsin or by the Ca^{2+}-dependent neutral proteases calpain I and II (Saido *et al.*, 1992). Prolonged exposure to phorbol esters can cause loss of certain isoforms of PKC from cells (Majewski and Iannazzo, 1998 and Liu and Heckman, 1998). For instance, after PMA (phorbol-12-myristate-13-acetate) treatment, PKCα is depleted through a net increase in proteolysis without a change in the rate of synthesis. Thus, PKCα is only rarely found to be completely down-regulated (Young *et al.*, 1987). PKCβ is more susceptible to complete loss from many cells types (Hug and Sarre, 1993). It has been commonly observed that cPKCs are down-regulated the quickest, whilst the nPKCs are down-regulated more slowly, and aPKCs are not down-regulated (Huwiler *et al.*, 1991; Sena *et al.*, 1996) by prolonged exposure to phorbol esters.

1.5.6 Functions of PKC

A multiplicity of functions has been ascribed to PKC. Recurring themes are that PKC is involved in receptor desensitisation, the regulation of transcription, mediation of the immune response, regulation of cell growth and

modulation of ion channel activity (Newton, 1995). In addition, PKC plays a crucial role in exocytosis (Terbush and Holz, 1990). One aspect of this regulation is mediated through its effects on ion channels, which was first observed in the central nervous system (CNS) where PKC may modulate noradrenaline release. In addition, there is strong evidence that the facilitatory effects of phorbol esters on action-potential-induced transmitter release are mediated by PKC (Kotsonis and Majewski, 1996; Schroeder *et al.*, 1995). Furthermore, the effects of phorbol esters on noradrenaline release are attenuated by PKC inhibitors (Majewski *et al.*, 1997) as well as by PKC down-regulation (Schroeder *et al.*, 1995). In contrast, some studies revealed that phorbol esters modulate action-potential-induced transmitter release in most systems rather than induce release by themselves (Robinson, 1992). However, in some studies phorbol esters by themselves induce transmitter release in the absence of nerve stimulation such as in rat brain synaptosomes (Dekker *et al.*, 1991), SH-SY5Y cells (Vaughan *et al.*, 1995), PC12 cells and chromaffin cells (Burgoyne *et al.*, 1988; Mollard *et al.*, 1995; Teschemacher and Seward, 2000). Activation of PKC enhances Ca^{2+} channel activity and potentiates fast synaptic transmission as a result of the direct phosphorylation of the Ca^{2+} channel α subunits (Maeno-Hikichi *et al.*, 2003). The channels which are most relevant to facilitation of transmitter release are the voltage activated Ca^{2+} channel (N, L, P/Q) types in which PKC phosphorylation has been shown to increase Ca^{2+} conductance either directly or by preventing G-protein receptor mediated inhibition of the channel (Swartz, 1993; Zamponi *et al.*, 1997). Studies on PC12 cells indicate that Ca^{2+} influx through L-type channels is the major contributory route of Ca^{2+} influx in PKC-mediated catecholamine secretion (Taylor *et al.*, 2000). A strong coupling of secretion to L-type Ca^{2+}

channels following PKC activation might be considered surprising since this class of Ca^{2+} channels has not been known to associate with the SNARE complex (El Far *et al.*, 1995). The second channel family that may be involved are K^+ channels and PKC phosphorylation has been shown to inhibit K^+ outflow in neurons. Such inhibition results in a more persistent depolarization and enhanced Ca^{2+} entry (Majewski and Iannazzo, 1998; Colby and Blaustein, 1988; Takano *et al.*, 1995). Moreover, PKC may regulate influx of Ca^{2+} through nicotinic channels (Wakade *et al.*, 1986).

PKC may also have a key role in synaptic plasticity (Linden and Routtenberg, 1989) since treatment with phorbol ester leads to an increased recruitment of vesicles into the ready releasable pool (RRP) in hippocampal neurons (Stevens and Sullivan, 1998). Furthermore, this effect is blocked by PKC inhibition with bisindolylmaleimide. Similarly, in chromaffin cells, activation of PKC increases both the size and the rate of replenishment of the readily releasable vesicle pool (Gillis *et al.*, 1996). This effect of phorbol ester was originally attributed to activation of PKC and although several targets for PKC phosphorylation have been identified, the link to vesicle recruitment is not clear (Nagy *et al.*, 2002). A large number of studies on many different secretory cell types have implicated protein phosphorylation in the control of regulated exocytosis and many of these studies suggested a role for PKC (Rink *et al.*, 1983; Shapira *et al.*, 1987). The key proteins of the exocytotic machinery that have been identified as potential PKC substrates include, SNAP-25, VAMP, synaptotagmin I, rab3a and Munc18 (Barclay *et al.*, 2003), although it is not clear which is responsible for the effects of PKC. There is some evidence that phosphorylation of both SNAP-25 (Shimazaki *et al.*, 1996) and Munc18 (Fujita *et al.*, 1996) by

PKC reduces their affinity for syntaxin. In the case of SNAP-25, this could be due to inhibition of SNARE complex formation and thereby exocytosis. In contrast, phosphorylation of Munc18 would allow it to dissociate more readily from syntaxin allowing frees syntaxin to participate in the assembly of the SNARE complex. Another substrate for PKC is the putative Ca^{2+} sensor synaptotagmin I. However, the physiological significance of the phosphorylation of these proteins is currently unknown (Burgoyne and Morgan, 2003). The role of these PKC substrates in catecholamine secretion from chromaffin cells has received considerable attention. However, the role of MARCKS and another DAG/phorbol ester target, Munc13, has been less well explored and the current study therefore examined their involvement in catecholamine secretion (See Chapters 5 and 6).

It is proposed that changes in the extent of release due to a switch to kiss- and -run exocytosis can be controlled by activation of PKC (Graham *et al.*, 2000). In other words, PKC activation may speed up the release kinetics. This hypothesis is based on data derived from studies on chromaffin cells in which phorbol esters modified single granule release events such that release was initially faster and terminated more rapidly. This rapid termination of release is consistent with the activation of kiss- and -run exocytosis. Furthermore, the PKC substrate Munc18 is implicated as a potential target for the control of kiss- and -run exocytosis through PKC-mediated phosphorylation (Barclay *et al.*, 2003). Synaptotagmin I is also a substrate for PKC (Hilfiker *et al.*, 1999) and overexpression of this protein has been shown to modify fusion pore kinetics measured using amperometry in PC12 cells (Wang *et al.*, 2001).

Role of PKC in the reorganization of the actin cytoskeleton. The actin cytoskeleton is a dynamic network composed of actin polymers and a large

variety of associated proteins (Geeraert *et al.*, 2003). Most cell types possess actin filaments organized into a variety of structures including a cortical actin network found beneath the plasma membrane as in for example chromaffin cells (Burgoyne and Cheek, 1987). The idea that the cortical actin network could form a barrier to exocytosis and hence act as a site of regulation of exocytosis was first proposed by Orci and co-workers (1972). The dynamics of the cytoskeleton during exocytosis have been intensively studied in adrenal chromaffin cells (Trifaró, 1990; Trifaró and Vitale, 1993). It has been suggested that the cortical F-actin cytoskeleton is involved in regulated secretion controlling the number of secretory vesicles present at release sites in the subplasmalemmal region (Trifaró and Vitale, 1993; Vitale *et al.*, 1995). In resting chromaffin cells, 1%-3% of the total chromaffin vesicles are either docked to the plasma membrane or within 50 nm of it (Vitale *et al.*, 1995). This population of vesicles constitutes the release-ready vesicle pool (Neher and Zucker, 1993; Vitale *et al.*, 1995). The rest of the chromaffin vesicles (97%-99%) form a reserve pool and remain behind a barrier of cortical F-actin (Vitale *et al.*, 1995). The stimulation of chromaffin cells is accompanied by a focal and transient disassembly of the cortical F-actin network (Cheek and Burgoyne, 1986; Vitale *et al.*, 1991, 1995). This allows the movement of additional secretory vesicles from the reserve pool to release sites on the plasma membrane in preparation for exocytosis (Vitale *et al.*, 1995). Therefore, the dynamics of the cortical actin network control the size of the release-ready vesicle pool and consequently, the initial rate of exocytosis (Vitale *et al.*, 1995).

At least two pathways control the cortical F-actin network in chromaffin cells during secretion, namely the Ca^{2+}-scinderin pathway and the PKC-MARCKS pathway (Trifaró *et al.*, 2000; Cuchillo-Ibãñez *et al.*, 2004). Previous

work has demonstrated that scinderin, an F-actin severing protein, has a major role in the release of chromaffin vesicles by controlling disassembly of the cortical F-actin cytoskeleton through a Ca^{2+}-dependent and PtdIns(4,5)P_2 regulated mechanism (Zhang *et al.*, 1996a). However, several lines of research suggest that PKC can regulate catecholamine secretion without modifying scinderin activity (Rodríguez del Castillo *et al.*, 1992; Rosē *et al.*, 2001). For this reason, extensive studies were performed to investigate the PKC substrates that are responsible for its potentiating effect on secretion. In recent years, a substrate of PKC, MARCKS has been investigated. MARCKS has been implicated in several cellular processes such as motility, mitosis, cytoskeletal dynamics, phagocytosis, and transformation (Aderem, 1992; Allen and Aderem, 1995). MARCKS has a basic domain of 25 amino acids, located in the middle of the primary structure, which is the phosphorylation site domain (PSD) (Aderem, 1992; Blackshear, 1993). MARCKS PSD domain, in addition to containing four serines that are possible substrates for PKC, can also interact with calmodulin, phospholipids, and actin in a complex manner (Graff *et al.*, 1989; Arbuzova *et al.*, 1997). Phosphorylation of MARCKS in the PSD domain by PKC inhibits F-actin cross-linking (Hartwig *et al.*, 1992) and it has also been reported that the effect of PKC on catecholamine release from chromaffin cells is mediated through MARCKS phosphorylation (Rosē *et al.*, 2001). Other proteins believed to be involved in the regulation of the cytoskeleton that are also substrates for PKC include annexin I (Wang and Creutz, 1992), and annexin II (Johnstone *et al.*, 1992). It is generally accepted that the pathways that trigger disassembly of the cortical F-actin could increase the size of the release-ready vesicle pool with a

consequent enhancement in the initial rate of vesicular exocytosis (Vitale *et al.*, 1995).

Although many physiological roles for PKC have been identified, it is hard to define the physiologically relevant functions of individual PKC isoforms (Mellor and Parker, 1998). These difficulties are due to the ubiquitous and potentially redundant expression of PKC isoforms, the large number of identified PKC regulators and substrates and the presence of alternative DAG and Ca^{2+} receptors that are also activated by commonly used PKC activators (Brose and Neher, 2002). Therefore, the elucidation of the precise roles of specific PKC isoforms may open a new field in which neurotransmitter release could be pharmacologically manipulated (Majewski and Iannazzo, 1998).

1.6 AIMS OF THE PROJECT

Modulation of neurotransmitter release by either ionotropic nicotinic receptors or $G\alpha_{q/11}$-coupled receptors is a key feature of information processing of the mammalian nervous system and may be essential for events such as regulation of cardiovascular function and the stress response. Moreover, there is evidence that some of the modulation by either ionotropic nicotinic receptors or $G\alpha_{q/11}$-coupled receptors is via PKC and changes in the exocytotic machinery. The overall aim of this project is explore the role of PKC, in particular the role of specific PKC isoforms in catecholamine secretion mediated by ionotropic nicotinic receptors and $G\alpha_{q/11}$-coupled receptors and investigate the mechanisms underlying PKC activation. Previous studies, particularly using phorbol esters have indicated a facilitating role of PKC in exocytosis. However, the circumstances in which PKC plays a role, the extent of regulation and the

mechanisms underlying both PKC activation and its regulation of exocytosis are unclear.

The initial aim was to explore the use of either PC12 or bovine chromaffin cells as a model system, confirming the expression of both ionotropic nicotinic receptors and a range of $G\alpha_{q/11}$-coupled receptors. The aim was then to investigate which PKC isoforms are expressed in these cells and to study the impact of PKC activation and particularly the role of specific PKC isoforms on catecholamine secretion evoked by either ionotropic nicotinic receptors or $G\alpha_{q/11}$-coupled receptors. Finally, the aim was to consider the mechanism of PKC activation and the way in which PKC was able to influence exocytosis.

2: MATERIALS AND METHODS

2.1 CHEMICALS AND MATERIALS

Adrenal glands from 18- to 24-month-old cows were obtained from a local abattoir. Collagenase type 2 was supplied by Worthington Biochemical (Lakewood, NJ, USA) and DNase I was supplied by Böehringer Mannheim (Mannheim, Germany). All other cell culture reagents including PBS (phosphate buffer saline) Dulbecco's were obtained from Gibco Life Technologies (Paisley, UK) and Sigma Aldrich (Poole, UK). Rat tail collagen used for culturing of chromaffin cells was purchased from Roche Pharmaceuticals Division (Basel, Swizerland) and Sigma (Poole, UK), respectively.

The cells were cultured in plasticware supplied by NUNC (Bibby Sterilin, Stafford, UK). Glass coverslips, both 22mm and 16mm diameter, were obtained from Chance Proper (Warley, UK) and the cuvettes used for spectrofluorimetry were obtained from Sarstedt (Leicester, UK).

Fluo-3-acetoxymethyl ester (fluo-3-AM), pluronic acid F-127, rhodamine-phalloidin, and jasplakinolide were supplied by Molecular Probes Ltd (Leiden, Holland). Adenosine triphosphate disodium salt (ATP), acetylcholine, methacholine, bradykinin, angiotensin II, atropine (sulphate salt), thapsigargin, histamine, epinephrine bitartrate, apyrase, nicotine, (+)-tubocurarine chloride hydrate, pertussis toxin (PTX), hexamethonium, cytochalasin D, BSA (bovine serum albumin), goat serum, HRP-conjugated anti-mouse and anti-rabbit (whole molecule) IgG antibodies, goat anti-mouse and anti-rabbit IgG (whole molecule) FITC (fluorescein isothiocyanate) conjugate and Bradford reagent were obtained from Sigma Aldrich (Poole, Dorset, UK). Phorbol-12-myristate-13-acetate (PMA), 4α-phorbol-12,13-didecanoate (4α-PMA) (inactive isomer), wortmannin,

myristoylated protein kinase C inhibitor, calphostin C, Ro31-8220 (1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)-maleimide, methane sulphonate], HBDDE (2,2`,3,3`,4,4`-hexahydroxy-1,1`-biphenyl-6,6`-dimethanol dimethyl ether), and protease inhibitor cocktail set 1 containing (500μM AEBSF (4-(2-aminoethyl)-benzolsulfonylfluoride-hydrochloride), 500μM EDTA, 1μM E-64 ((2s,3s)-3-(n,{(s)-1-[n-(4-guanidinobutyl)carbamoyl]3-methylbutyl}carbamoyl)oxirane-2-carboxylic acid), 1μM leupeptin, and 1μg/ml aprotinin) were supplied by Calbiochem (CN Biosciences Ltd, Nottingham, UK). LY333531 hydrochloride ((S)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9,16,21-dimetheno-1H,13H-dibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazcyclohexadecene-1,3(2H)-dione hydrochloride) was obtained from A.G. Scientific, Inc. (San Diego, CA, USA). U-73122 (1-(6-((17β-3-methoxyestra-1, 3, 5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione), U-73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione), myristoylated PKCβC2-4 inhibitor (N-myristoyl-Ser-Leu-Asn-Pro-Glu-Trp-Asn-Glu-Thr), and myristoylated PKCεV1-2 inhibitor (N-myristoyl-Glu-Ala-Val-Ser-Leu-Lys-Pro-Thr) were supplied by BIOMOL Research Laboratories Inc. (Waidmannstrabe, Hamburg, Germany). Myo-[^{3}H]-inositol, muscarinic antagonist 1-[N-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS, ECL plus enhanced chemiluminescent Western blotting reagent and ECL hyperfilm were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Precision plus pre-stained protein standards for Western blots and the Mini-PROTEAN II electrophoresis gel equipment were obtained from Bio-Rad Laboratories Ltd. (Hertfordshire,UK). Nitrocellulose (0.45μm pore size) for protein transfer was supplied by Schleicher and Schuell (London, UK).

Emulsifier-Safe scintillation cocktail was supplied by Packard (Pangbourne, Berks, UK). The NucleoBond Plasmid Maxi Kit was supplied by Clontech Laboratories (Palo Alto, CA, U.S.A) and transfections were carried out using either Nucleofector transfection reagent from Amaxa Biosystems (Würzburg, Germany) or Lipofectamine 2000 from Invitrogen Life Technologies (Paisley, UK). Vectashield medium was purchased from Vector Laboratories Inc. (Burlingame, CA, USA). The viral constructs containing EGFP-PKCβII and EGFP-PKCδ were generous gift of Dr. Guy Rutter (Department of Biochemistry, University of Bristol). The vectors for the $Ins(1,4,5)P_3$ (eGFP-$PH_{PLC\delta1}$) and diacylglycerol (eGFP-$PKC\gamma C1_2$) biosensors were provided by Professor T. Meyer (Stamford University, Stanford, CA). MARCKS-eGFP was kind gift from Dr. Hideo Mogami (Hamamatsu University, Japan). Munc13-eGFP was provided by Dr. Nils Brose (Max-Planck Institute for Experimental Medicine, Molecular Neurobiology Group, Göttingen, Germany). Bovine brain extract was a generous gift from Dr. Bazbek Davletov (Medical Research Council of Molecular Biology, Cambridge, UK). DNA encoding the human muscarinic M_3 receptor that had been cloned into plasmid pcDNA3 was supplied from Invitrogen Life Technologies (Paisley, UK).

For details of all primary antibodies, please refer to Table 2.1. These commercial antibodies were purchased from Transduction Laboratories (BD Bioscience, Oxford, UK), Sigma (Poole, UK), Santa Cruz Biotechnology Inc. (California, CA, USA), and Upstate Biotechnology Inc. (Lake Placid, NY, USA).

All other standard chemical reagents of analytical grade were supplied by either Sigma Aldrich (Poole, U.K) or Fisher Scientific (Loughborough, U.K).

2.2 CELL CULTURE

2.2.1 Preparation of bovine chromaffin cells

Adult bovine chromaffin cells were prepared as described previously (Marley and Livett *et al.*, 1987; Vitale *et al.*, 1991; Seward and Nowycky, 1996). Adrenal glands from 18- to 24-month-old cows were obtained from a local abattoir. Excess fat was removed and glands placed in ice cold Locke's buffer (154.2mM NaCl, 2.6mM KCl, 2.2 mM K_2HPO_4, 0.85mM KH_2PO_4, 1mM glucose, 0.5mM HEPES, 1mM phenol red, pH 7.2) containing antibiotics (100μg/ml penicillin/streptomycin, 50μg/ml gentamycin). Upon arrival at the laboratory, the adrenal vein was cannulated and the gland perfused with the same buffer. The outer capsule and part of cortex were dissected from the gland taking care not to puncture the medulla. The gland was suspended in a 50ml tube and perfused at 25ml/min for 30 min at 37°C with the digestive enzymes 0.03% collagenase type 2 and 0.01% DNase I added to a Locke's buffer. Residual cortex was scraped off using a scalpel blade and the medulla minced and placed in a trypsinisation jar containing 25ml of the Locke's with added enzymes for 30 min at 37°C. This suspension was filtered through a 40μm nylon mesh and diluted with 25ml of enzyme free Locke's buffer. The cells were centrifuged at 5000g for 5 min at room temperature. A further 25ml of Locke's buffer were used to resuspend the cells. The cells were counted and the solution diluted to $2x10^5$ cells/ml. Cells were plated on collagen-coated coverslips in 6 or 24 well plates at densities of $5x10^5$ and $1\text{-}2x10^5$ cells/well, respectively in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% foetal calf serum, 100μg/ml penicillin/streptomycin, 50μg/ml gentamycin, 10μM 5-fluro-deoxy-uridine (5-FDU), 10μM cytosine-β-γ-arabino-furanoside (cyto-sar) and 0.5mM glutamine.

The cells were incubated for 48 h at 37°C in 5% CO_2 humified atmosphere and used for 5-7 days post gland collection.

2.2.2 Cell counts

Cells were diluted with medium (1:5 dilution) then mixed with 50µl Trypan blue dye which is one of several stains recommended for use in dye exclusion procedures for viable cell. This method is based on the principle that viable cells do not take up certain dyes, whereas non-viable cells stain. Cells were counted after addition of small amount of Trypan blue-cell suspension mixture to both chambers of the hemocytometer (Superior Marienfeld, Cupertino, CA., USA), and then total cells per ml were counted by multiplying the count times dilution factor times 10^4 (count 10 squares).

2.2.3 Collagen coating procedure

Collagen (50mg) was dissolved in 10ml of 0.1% sterile glacial acetic acid and mixed by stirring for 1 h at room temperature. Aliquots (each 200µl) were then stored at -20°C until use. A frozen aliquot (200µl) of this stock was defrosted then dissolved by adding 8ml of 60% sterile ethanol. Collagen solution was then added to cover the well or coverslip (300-500µl). Collagen coating of the well or coverslip was facilitated by incubation at room temperature until the ethanol completely evaporated and collagen coated the entire well or coverslip. Cells were then plated at the required concentration and incubated for 24 h to allow firm adherence.

2.3 WESTERN BLOTTING (IMMUNOBLOTTING)

2.3.1 Preparation of whole cell extract for immunodetection of PKC isoforms

The method was based on that previously described (Willars *et al.*, 1999; 2001; Tovey and Willars, 2004). Multidishes (6 well) were coated with collagen

and seeded with approximately 1-2x10^6 cells. After overnight culture to allow the cells to adhere to collagen, the monolayers were washed with 1ml non-sterile PBS Dublecco's and then solubilised with 300µl solubilization buffer (10mM Tris, 10mM EDTA, 500mM NaCl, 1% ($^v/_v$) ethylphenylpolyethylene glycol (Nonidet P40), 0.1% sodium-dodecyl-sulphate (SDS), 0.5%$^w/_v$ deoxycholic acid, 1mM phenylmethylsulphonyl fluoride (PMSF), 100µg/ml iodoacetamide, 100µg/ml benzamidine, and 5µl/ml protease inhibitor cocktail, pH 7.4) and left on ice for 30 min with gentle rocking. Finally, the wells were scraped gently with a fine pipette tip in order to ensure complete removal of cellular materials. The solubilised cells were then transferred to a microfuge tube and stored at -20°C until use.

2.3.2 Cell fractionation for studying the translocation of PKC isoforms

The method was based on that previously described (Willars *et al.*, 1996; 2001). Confluent monolayers of cells in a 6 well plate were washed with 1ml of Krebs'/ HEPES buffer (KHB) (composition: 10mM HEPES, 4.2mM $NaHCO_3$, 11.7mM D-glucose, 1.2mM $MgSO_4$,1.2mM KH_2PO_4, 4.7mM KCl, 118mM NaCl, and 1.3mM $CaCl_2$, pH 7.4). Cells were then incubated at 37°C for 5 min with 1ml KHB in the presence or absence of agonist and lysed in 200µl of ice-cold lysis buffer (20mM Tris-HCl, 5mM EGTA, 2mM EDTA, 1mM dithiothreitol (DDT), 0.5mM PMSF, 10µM benzamidine hydrochloride, 5µM iodoacetamide, pH 7.4) and left on ice for 30 min.

Separation of cytosolic and membrane fractions was performed by centrifugation (20,800g, 15 minutes, 4°C). A 100µl aliquot of supernatant, which represents the cytosolic fraction, was transferred to a 1.5ml microfuge tube and the insoluble pellet, which represents the membrane fraction, was resuspended in

200µl of solubilization buffer. An equal volume of 2X sample buffer (100mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% ($^{v}/_{v}$) glycerol, 200mM dithiothreitol (DDT)) was added to the cytosolic and membrane fractions. Aliquots were denatured by placing in a Grant Boekal heating block at 100°C for 5 min. Proteins within the samples (30µl, 30µg.lane^{-1} of proteins within the sample) were separated by SDS-PAGE (SDS polyacrylamide gel electrophoresis) (Bio-Rad mini-Protean II electrophoresis cell) using a 10% running gel (prepared using: 5.9ml H_2O, 5.0ml of 30% acrylamide mix, 3.8ml of 1.5M Tris (pH 8.8), 0.15ml of 10% SDS, 0.15ml of 10% ammonium persulphate and 0.006ml of TEMED) at 200 V for 45 min using a Tris and glycine-based running buffer (25mM Tris-base, 250 mM glycine, 0.1% SDS ($^{w}/_{v}$)). Proteins were then transferred to nitrocellulose using a semi-dry blotter (buffer: 39mM glycine, 48mM Tris-base, 0.037% SDS ($^{v}/_{v}$), 20% methanol ($^{v}/_{v}$), pH 8.3) at 0.65mA/cm^2 for 65min. The nitrocellulose was then blocked with 5% ($^{w}/_{v}$) powdered milk in low salt TTBS (100mM NaCl, 20mM Tris- base, 0.05% Tween 20 ($^{v}/_{v}$), pH 7.5) either overnight at 4°C or for 1 h at 20°C. The nitrocellulose was then incubated with the commercially available primary antibodies against PKC isoforms at the required dilution (see Table 2.1) for 1 h at room temperature. The excess antibodies were removed by washing three times in TTBS with each wash lasting for 10 min. Subsequently, secondary antibody (HRP-linked anti-mouse IgG-peroxidase antibody) was added (1:1000) and incubated for 1 h at room temperature. The excess secondary antibody was then removed by washing three times in TTBS with each wash lasting for 10 min.

Western blots were developed with ECL plus Western blotting detection reagent for 5 min, then exposed to Amersham ECL hyperfilm for 1-10minutes.

Immunoblots were analysed by densitometer where appropriate using Scion image analysis software (v4.02 beta, Frederick, Maryland, USA). Local background subtraction was applied to images and data either normalized to basal (nonstimulated) or calculated as percentage total cellular protein content for studying PKC translocation.

2.4 IMMUNOCYTOCHEMISTRY

2.4.1 Immunocytochemistry for studying PKC localization

Cells cultured on collagen-coated coverslips at a density of $5x10^5$ cells per coverslip were washed three times with PBS. Cells were then incubated for the required period in the absence (control) or presence (stimulated) of agonists. Following the incubation, the solution was quickly removed and replaced with 4% paraformaldehyde in PBS (pH 7.4) and maintained for 30 min. After permeabilization for 10 min in PBS containing 0.2% Triton X-100, the cells were incubated for 1 h in 10% normal goat serum in PBS to block non-specific binding, then, washed three times (each 5 min) with PBS. This step was followed by overnight incubation with PKC isoform specific antibody (Transduction Laboratories) (diluted 1:100 in PBS containing 10% normal goat serum) at 4°C. Coverslips were then washed three times with PBS each for 5 min. A goat FITC-conjugated anti-mouse secondary antibody (1:512 in PBS containing 10% normal goat serum) was applied for 1 h in the dark at room temperature. Coverslips were then rinsed three times with PBS each for 5 min and mounted in Vectashield medium to reduce photobleaching.

Controls were obtained by omitting the primary antibody in the incubation. No staining of cells was observed under these conditions. Cells were observed under confocal microscopy using an excitation wavelength of 488nm

and emitted light collected above 510nm with a narrowband pass dichroic mirror and filter and detected by a CCD camera.

2.4.2 Confocal microscopy for F-actin detection

Cells were plated on collagen-coated coverslips at a density of $5x10^5$ cells per coverslip. Cultured cells were rinsed three times with PBS (130mM NaCl, 100mM sodium phosphate, at pH 7.4). Cells were then incubated for the required period in the absence (control) or presence (stimulated) of agonists as described in the figure legends. Following incubation, cortical F-actin integrity was determined by adaptation of the method of Lee and Trifaró (1981). Briefly, cultured cells were fixed in 4% paraformaldehyde-PBS for 30 min. Excess paraformaldehyde was quenched with 50mM NH_4Cl-PBS for 15 min (Li *et al.*, 2003). The cells were permeabilized by three successive exposures of 5 min each to 50, 100, and 50% acetone and washed several times with PBS. F-actin was stained with 0.1μM rhodamine-phalloidin for 40 min in the dark at room temperature. Finally, the coverslips were rinsed several times with PBS and mounted in Vectashield.

Slides were observed with an Ultra*VIEW* confocal microscope. Cells were excited at 568nm and emitted light collected with a broad band RGB emission filter. The effect of several treatments on the percentage of cells showing cortical F-actin disassembly was determined by examining two coverslips for each experimental condition in each experiment. One hundred single-rounded chromaffin cells were examined per coverslip. Each cell was classified as having either a "continuous" or "discontinuous" cortical rhodamine (F-actin) fluorescent ring as described previously (Vitale *et al.*, 1991). The percentage of chromaffin cells showing cortical F-actin disassembly (discontinuous rhodamine fluorescent ring) was calculated for each experimental condition. To avoid personal bias, code

numbers were given to coverslips. The cells were examined and classified without knowing whether they were from control or treated preparations. The codes were revealed to identify the experimental conditions used, only after all results were recorded (single-blind design).

Three dimensional image analysis was performed either by using Ultra*VIEW* confocal software where the maximum intensity of the fluorescent ring was set to 4096 arbitrary units or by using the public domain program "ImageJ" developed at the U.S. National Institutes Of Health downloaded from the internet (http://rsb.info.nih.gov/nih-image/) and the maximum intensity of fluorescent ring was set to 250 arbitrary units.

2.5 MEASUREMENT OF INTRACELLULAR ($[Ca^{+2}]_i$)

2.5.1 Confocal imaging of $[Ca^{+2}]_i$

Confocal imaging of $[Ca^{2+}]_i$ was performed as described previously (Werry *et al.*, 2002; Tovey and Willars, 2004). Cells were plated onto 22 mm diameter collagen coated glass coverslips and cultured for 24-48 hours. Cells were incubated in the dark for 45 min at room temperature in 2ml Krebs'/HEPES buffer containing 6μM fluo-3-acetoxymethyl ester (fluo-3-AM) and 0.044% pluronic acid F-127.

Coverslips were transferred into 2ml fresh Krebs'/HEPES buffer and left for 15 minutes at room temperature to allow for hydrolysis of the ester group. Coverslips were mounted in a chamber on the stage of an Olympus 1X70-S1F inverted microscope with a 40X oil emersion objective. The chamber was continuously perfused with Krebs'/HEPES at a rate of 5ml per minute pre-heated to 37°C with a Peltier unit. Using an Ultra*VIEW* confocal imaging system (PerkinElmer Life Sciences, Cambridge, U.K.) cells were excited with a

krypton/argon laser at 488nm and emitted light collected at 510nm with a narrowband pass dichroic mirror and filter and detected by a CCD camera at rate of 3-4 frames per second. Agonists were perfused and the images were recorded as a single section through the cell monolayer and averaged from 5-10 cells in a field of view.

Ultra*VIEW* LCI software was used to analyse fluorescence intensities. The images were analysed by selecting circular regions of interest in cytoplasm of all cells in a field of view and then calculating the average fluorescence intensity for this region over time. The raw data was exported as an ASCII file for further analysis and plotted against time using Origin version 6.0 (Microcal). Data are represented graphically as fold change in fluorescence intensity relative to basal levels.

2.6 BRADFORD PROTEIN ASSAY (BCA PROTEIN DETERMINATION PROCEDURE)

Protein determination procedure is an adaptation of Bradford method (Bradford, 1976). Briefly, duplicate protein standards were created by serial dilution of 1mg/ml BSA to give 0,1,2,5,10,20, and 50μg/ml. The total liquid volume in each tube was adjusted to 1ml by the addition of deionized water. The unknown samples with an approximate concentration of protein between 1-50μg/ml were prepared and the total volume also adjusted to 1ml with deionized water. Thereafter, to each tube, 2ml of Bradford Reagent was added and vortexed. Both standards and samples were incubated at room temperature for 5 min and then transferred to polystyrene cuvettes. The absorbance at 595nm was measured using a spectrophotometer (BU-65Spectrophotometer, memory PAC module).

The net absorbance versus the protein concentration of each standard was plotted and the protein concentration of the unknown sample was determined

from the standard curve plotted using GraphPad Prism (GraphPad Software, 3rd order polynomial, San Diego, CA, USA).

2.7 DETERMINATION OF PHOSPHOLIPASE C ACTIVITY

2.7.1 Measurement of [^{3}H]-inositol phosphate accumulation

Agonist-induced accumulation of [^{3}H]inositol mono- and polyphosphates ([^{3}H]-InsP_x) was determined as described previously (Willars *et al.*, 1999). In brief, cells were plated at equivalent density ($5x10^5$ cells/well) in collagen-coated 24 well multidishes for 24 h before the assay. Then, cells were prelabelled with 3μCi.ml^{-1} of myo-[^{3}H]-inositol (86 Ci.mmol^{-1}) in 1ml of growth media for 48 h at 37°C to ensure equilibrium labelling (Willars and Nahorski, 1995b; Willars *et al.*, 1998a).

Cells were washed once with a Krebs'/HEPES buffer containing 10mM LiCl (KHB/Li^+) to inhibit inositol monophosphatase activity, then incubated for 10 min at 37°C with 200μl KHB/Li^+. Agonists (made up at twice the desired final concentration in KHB/Li^+) were added in a 200μl volume at the appropriate time. Reactions were stopped by the addition of 400μl of ice-cold 1M trichloroacetic acid (TCA). After 15 min extraction on ice, a 400μl aliquot from each well was removed and added to 100μl of 10mM EDTA in a 1.5ml microfuge tube. Following this, 1ml of a freshly-prepared 1:1 (v/v) mixture of tri-n-octyl-amine and 1,1,2-trichloro-trifluoroethane was added and the tube vortexed to ensure thorough mixing. Samples were stood for approximately 5 min to allow separation of samples into aqueous and organic phases. A 400μl aliquot of the upper aqueous phase was removed and added to 25μl of 250mM $NaHCO_3$. This sample was applied to a Dowex chloride anion exchange column (8% cross linkage; 100-200 dry mesh; AG1-X8; Sigma), which was then washed with 10ml H_2O, and then

12ml of 25mM ammonium formate. Elution of [^{3}H]inositol phosphates ([^{3}H]-$InsP_x$) from the columns was performed by washing the columns through with 10ml 1M HCl and collected into scintillation vials. A 3ml aliquot of this eluant was mixed with 15ml of Emulsifier-Safe Scintillation Cocktail (Baird *et al.*, 1989) and radioactivity determined by scintillation counting. Dowex chloride columns were regenerated by washing with 10ml of 2M HCl followed by 10ml H_2O.

2.7.2 Single-cell measurement of Ins (1,4,5)P_3 formation using a biosensor

Chromaffin cells were seeded onto 22mm collagen coated coverslips at a density of $5x10^5$ cells per coverslips and cultured for 48 h before transient transfection with the eGFP-tagged pleckstrin homology domain of PLCδ1 (eGFP-$PH_{PLC\delta1}$) plasmid cDNA (1μg) (Nash *et al.*, 2001; Tovey and Willars, 2004) using lipofectamine 2000 as described in manufacturer' instructions (Invitrogen Life Technologies, Paisley, UK) 48 h prior to the experiment. Using an Ultra*VIEW* confocal imaging system (PerkinElmer Life Science, Cambridge, UK) as described previously (Nash *et al.*, 2001; Nahorski *et al.*, 2003; Tovey and Willars, 2004), in brief, eGFP was excited with an krypton/argon laser at 488nm and emitted light collected above 510nm with a narrowband pass dichroic mirror and filter and detected by a CCD camera.

Ultra*VIEW* LCI software was used to analyse fluorescence intensities. The images were analysed by selecting circular regions of interest in cytoplasm of responding cells and then calculating the average fluorescence intensity for this region over time. The raw data was exported as an ASCII file for further analysis in the Microsoft Excel and plotted against time using Origin version 6.0 (Microcal). Data are represented graphically as fold change in cytosolic fluorescence intensity relative to basal levels.

2.7.3 Single-cell measurement of diacylglycerol (DAG) formation using a biosensor

Chromaffin cells were transiently transfected with eGFP-tagged with C1 domain of PKCγ (eGFP-PKCγCl_2) (Oancea and Meyer, 1998; Tovey and Willars, 2004) using lipofectamine 2000 as described in manufacturer' instructions (Invitrogen Life Technologies, Paisley, UK) 48 h prior to the experiment as described by. Imaging experiments were performed 48 h after transient transfection using an Ultra*VIEW* confocal imaging system as described above (see sections 2.5.1 and 2.9.3).

2.8 CATECHOLAMINE ASSAY

The catecholamine assay was based on the oxidation of catecholamine to produce a fluorescent derivative. The method was performed as described by Von Euler and Lishajko (1961). Chromaffin cells were plated on collagen coated 24-well plates at a density of $5x10^5$ cells/well. The medium was removed and cells washed three times using Krebs'/HEPES buffer. Cells were stimulated with agonist in 200-300 µl Kreb's buffer at 37°C. Then, 130µl of the buffer over the cells was transferred into pre-chilled microfuge tubes and spun (16,000g for 1 min) at 4°C. Duplicate aliquots of 50µl were taken for assay. Each duplicate was mixed with 2ml of 1M sodium acetate buffer (pH 6) and 100µl of 0.25% potassium ferricyanide. The solution in each tube was mixed by vortexing briefly and incubated for 5 min at room temperature. The reaction was stopped by adding 1ml of a 1:10 dilution of 2% ($^{w}/_{v}$) freshly prepared ascorbic acid in 5M NaOH. The solution was thoroughly mixed and transferred to a cuvette. The absorbance was measured using a Perkin-Elmer spectrofluorimeter (model: LS-50B) at 416nm excitation and 517nm emission wavelengths.

For determination of the cellular catecholamine content, 30μl of 10% NP-40 was added to the remaining Krebs'/HEPES buffer over the cells and duplicate aliquots of 50μl were taken for assay.

The concentration of catecholamines was calculated by using the equation obtained from a standard curve generated using epinephrine.

Calibration with standard epinephrine was performed by preparing various concentrations of epinephrine bitartrate (0-200μM). A duplicate (50μl) of each concentration was taken for the assay. The concentrations of epinephrine were plotted against the intensity and the data fitted with a linear function using GraphPad Prism (GraphPad Software, 3rd order polynomial, San Diego, CA, USA):

$$Y = A + BX$$

This equation was then used to calculate the concentration of catecholamines in samples taken from the cells where A is the intercept, B is the slope, Y is the absorbance and X is the unknown concentration of catecholamine.

2.9 DETECTION OF MARCKS TRANSLOCATION AND PHOSPHORYLATION

2.9.1 Single-cell imaging of MARCKS-eGFP

Chromaffin cells were seeded onto 22mm collagen-coated coverslips at a density of $5x10^5$ cells per coverslip and cultured for 48 h before transient transfection with the cDNA (1μg/well) encoding MARCKS-eGFP (Ohmori *et al.*, 2000) using Lipofectamine 2000 as described in the manufacturer's standard protocol 48 h prior to the experiment. The MARCKS-eGFP was then viewed using an Ultra*VIEW* confocal imaging system (PerkinElmer Life Science, Cambridge, UK) as described previously (Nash, *et al.*, 2001; Nahorski *et al*, 2003; Tovey and Willars, 2004). In brief, eGFP was excited with a krypton/argon laser

at 488nm and emitted light collected above 510nm with a narrowband pass dichroic mirror and filter and detected by a CCD camera.

Ultra*VIEW* LCI software was used to analyse fluorescence intensities. The images were analysed by selecting circular regions of interest in the cytoplasm of cells that showed membrane localization of MARCKS-eGFP and then calculating the average fluorescence intensity for this region over time. The raw data were exported as an ASCII file for further analysis in Microsoft Excel and plotted against time using Origin version 6.0 (Microcal). Data are represented graphically as fold changes in cytosolic fluorescence intensity relative to basal levels (F/F_0).

2.9.2 Immunoblotting for studying phosphorylation of endogenous MARCKS

Cells were seeded onto collagen-coated wells in 6-well culture dishes at a density of $2x10^6$ cells per well. Cells were then incubated at 37°C for 20 min in 1ml Krebs'/HEPES buffer in the presence or absence of agonist and lysed in 300µl of ice-cold phospho-MARCKS solubilization buffer (65mM PIPES, 25mM HEPES, 8mM EGTA, 1mM EDTA, 1%$^{v}/_{v}$ Triton X-100, 1mM DDT, 40mM β-glycerolphosphate, 1mM Na_2VO_4, 10mM NaF, 1mM PMSF, pH 7.5) and left on ice for 5 min with gentle rocking. Solubilized cells were centrifuged (13000g, 5min, 4°C) to yield supernatant. The supernatants were denatured by boiling in an equal volume of sample buffer and subject to SDS/PAGE as described above (see Section 2.3.2). Proteins were transferred onto nitrocellulose membranes, blocked, and probed with phospho-MARCKS antibody overnight at 4°C. Blots were then stripped and re-probed for MARCKS for 1 h at room temperature. Immunoreactive bands of either phospho-MARCKS or MARCKS were detected by enhanced chemiluminescence (ECL) reagents and exposure to Hyperfilm-ECL

(see Section 2.3.2 for details of immunoblotting and see Table 2.1 for details of antibodies).

2.10 DETECTION OF MUNC13-1 LOCALIZATION

2.10.1 Single-cell imaging of Munc13-1-eGFP

Chromaffin cells were seeded onto 22mm collagen-coated coverslips at a density of $5x10^5$ per coverslip and cultured for 48 h before transient transfection with cDNA (1μg/well) encoding Munc13-1-eGFP (Ashery *et al.*, 2000; Stevens *et al.*, 2005) using Lipofectamine 2000 according to the manufacturer's instructions. After 48 h, coverslips were mounted on the stage of a Perkin-Elmer Ultra*VIEW* confocal microscope, maintained at 37°C with a Peltier unit and perfused with with Krebs'/HEPES buffer (5ml min^{-1}) in a chamber volume of approximately 0.5ml. Visualization of changes in cytosolic fluorescence in real time was performed as described previously (Werry *et al.*, 2002; Tovey and Willars, 2004) alone or in the presence of test agent. In brief, eGFP was excited with a krypton/argon laser at 488nm and emitted light collected above 510nm with a narrowband pass dichroic mirror and filter and detected by a CCD camera. Ultra*VIEW* LCI software was used to analyse fluorescence intensities. The images were analysed by selecting circular regions of interest in the cytoplasm of responding cells and then calculating the average fluorescence intensity for this region over time. The raw data were exported as an ASCII file for further analysis in Microsoft Excel and plotted against time using Origin version 6.0 (Microcal). Data are represented graphically as fold change in cytosolic fluorescence intensity relative to basal levels (F/F_0).

2.10.2 Immunocytochemical localisation of Munc13-1

Chromaffin cells were seeded on 22mm collagen-coated coverslips and the subcellular localisation of Munc13-1 was determined by immunocytochemical methods using either non-transfected cells (endogenous Munc13-1) or cells transiently expressing Munc13-1-eGFP (see above). Where required, cells were challenged with test agents (37°C in Krebs'/HEPES buffer) before being fixed and processed for immunocytochemistry as described above (see Section 2.4.1 and Table 2.1).

Membrane localization was analysed by calculating the percentage of cells showing membrane localization in control and treated preparations. To avoid personal bias, code numbers were given to coverslips and cells were examined and classified in a single-blind fashion. A total of three hundred cells from three different cell cultures were examined for each experimental condition.

2.11 DATA ANALYSIS

Data from three or more identical experiments are presented as the mean ± SEM (standard error of the mean). Where statistical analysis was required, the format of the data was taken into account in the selection of an appropriate test. Two-tailed, unpaird Student's *t*-test was used for direct comparison of a test value with a control, with $P<0.05$ accepted as statistical significance. For comparison of multiple data sets, one-way analysis of variance (ANOVA) was used. If $P<0.05$ using ANOVA, data was further analysed using an appropriate post-hoc tests (either Dunnet's which is designed to restrict the number of comparisons by only comparing each group with a specified control group or Bonferroni's test which is designed to compare only selected pairs of means). Concentration-response curves were fitted by GraphPad Prism (GraphPad Software, 3rd order polynomial, San

Diego, CA, USA) using a standard four-parameter logistic equation. EC_{50} mean values and standard errors of the mean were generated from the mean of values generated from separate curves. EC_{50} values are presented as log10 M mean ± SEM (n).

Table 2.1: Primary antibody working concentrations

Antibody	Source	Raised against	Cross-reactivity	Supplier	Working concentration ($\mu g.ml^{-1}$)
PKCα	mouse	mouse	human, dog, rat, chick, frog	Transduction Laboratories	0.25
PKCβ	mouse	mouse	human, rat, chick	Transduction Laboratories	1.0
PKCγ	mouse	mouse	rat	Transduction Laboratories	0.25
PKCδ	mouse	mouse	human, rat	Transduction Laboratories	0.5
PKCε	mouse	mouse	human, dog, rat, chick	Transduction Laboratories	0.25
PKCη	mouse	mouse	none	Transduction Laboratories	1.0
PKCθ	mouse	mouse	none	Transduction Laboratories	1.0
PKCι	mouse	mouse	human, dog, rat, chick	Transduction Laboratories	1.0
PKCλ	mouse	mouse	human, dog, rat, chick	Transduction Laboratories	1.0
β-actin	mouse	mouse	human, bovine, sheep, pig, cat, dog, rat, guinea pig, chicken, carp	Sigma	0.5
MARCKS	mouse	human, bovine	rat, mouse, chicken	Upstate Biotechnology	0.25
p-MARCKS	rabbit	goat	human, mouse, rat	Santa Cruz	0.2
Munc13-1	mouse	mouse	mouse, rat	Synaptic Systems	2.0

3: CHARACTERIZATION OF RECEPTOR-MEDIATED ELEVATION OF $[Ca^{2+}]_i$ IN MODEL NEUROSECRETORY cells

3.1 INTRODUCTION

This chapter details preliminarily work on two neurosecretory models to select a system that expresses a range of $G\alpha_{q/11}$-coupled receptors with the aim of comparing Ca^{2+} signalling evoked by different $G\alpha_{q/11}$-coupled receptor agonists with ionotropic receptors such as the nicotinic receptor. To identify the presence of $G\alpha_{q/11}$-coupled receptors, $[Ca^{2+}]_i$ was measured in either cell populations (PC12 cells) or single cells (chromaffin cells). Initially, the work of this project was conducted using a PC12 cell line, which was originally isolated by Green and Tischler (1976) from a rat pheochromocytoma. This clonal cell line resembles an adrenal chromaffin cell under normal conditions (see main Introduction). Following initial studies the project then focused on adrenal chromaffin cells, which are derived embryonically from the neural crest and generally regarded as modified sympathetic neurons (Burgoyne *et al.*, 1993b; 1996; Burgoyne and Morgan, 1998b) (see main Introduction).

In the last few decades these neurosecretory models (PC12 cells and chromaffin cells) have been used extensively as model systems to study Ca^{2+}-triggered exocytosis (Kilpatrick *et al.*, 1982). It has been suggested that in cell types where a rise in $[Ca^{2+}]_i$ is the main trigger of regulated exocytosis, Ca^{2+} can be derived from entry of external Ca^{2+}, by Ca^{2+} release from intracellular Ca^{2+} stores or both. The importance of these two Ca^{2+} sources varies considerably between cell types (Cheek and Burgoyne, 1985; Cheek *et al.*, 1989; Burgoyne and Morgan, 2003). Adrenal chromaffin cells and PC12 cells have been reported to express a wide variety of $G\alpha_{q/11}$-coupled receptors, many of which elicit

exocytosis from chromaffin cells. These include the histamine H_1 receptor, muscarinic receptor, angiotensin AT_{1A} receptor, bradykinin B_2 receptor, prostaglandin EP_3 receptor and PAC_1 receptors for pituitary adenylate cyclase-activating polypeptide (PACAP) (Marley, 1987; O'Sullivan and Burgoyne, 1989; Marley, 2003). Activation of these $G\alpha_{q/11}$-coupled receptors increases the activity of inositol phospholipid-specific phospholipase C and subsequently produce an elevation of $[Ca^{2+}]_i$ (Plevin and Boarder, 1988; Sasakawa *et al.*, 1989; Bunn *et al.*, 1990). In addition, activation of either ionotropic receptors (nicotinic receptors) or depolarisation mediated by high K^+ result in a rise of $[Ca^{2+}]_i$ (Burgoyne, 1991; Zhou and Neher, 1993; Harkins and Fox, 1998; Gueorguiev *et al.*, 1999). Ca^{2+} signalling can, therefore, be used as a valuable means for identification of $G\alpha_{q/11}$-coupled receptors and nicotinic receptors in these cells.

Various receptor agonists of either metabotropic or ionotropic receptors are thought to exhibit different Ca^{2+} signalling profiles. Such differences might also influence their ability to trigger secretion. Furthermore, different receptors may differentially activate or use varying components of the signalling pathways (for example, different PKC isoforms), which may impact on their ability to regulate exocytosis. The aim was therefore, to select a model cell system that expresses a variety of receptors with different Ca^{2+} signalling profiles.

3.2 RESULTS

Ca^{2+} signalling in chromaffin cells

Identification of $G\alpha_{q/11}$-coupled receptors expressed in bovine chromaffin cells by studying Ca^{2+} signalling

In this study, single-cell confocal imaging of cells loaded with the Ca^{2+}-sensitive dye fluo-3 were used to identify which receptors are expressed in bovine chromaffin cells.

Stimulation of histaminergic (Figure 3.2.1), cholinergic (Figure 3.2.2), bradykinin (Figure 3.2.3) and nicotinic (Figure 3.2.4) receptors, respectively with histamine (100μM), acetylcholine (100μM), bradykinin (10μM) and nicotine (100μM) respectively in the presence of 1.3mM extracellular Ca^{2+} evoked peak and sustained Ca^{2+} signals. However, removal of extracellular Ca^{2+} markedly reduced the peak Ca^{2+} responses and abolished the sustained phases of Ca^{2+} signalling (Figures 3.2.1-3.2.4). There were significant differences in the peak Ca^{2+} responses evoked by these agonists in the presence of extracellular Ca^{2+} from that evoked in absence of extracellular Ca^{2+} (** $P<0.01$, unpaired Student's *t*-test).

Activation of AT_{1A} angiotensin receptor by angiotensin II (100nM) (Figure 3.2.5) in the presence of 1.3mM extracellular Ca^{2+} evoked only a slight peak Ca^{2+} response and no sustained plateau of Ca^{2+} signalling (Figure 3.2.6: Panel A). The peak Ca^{2+} response was slightly attenuated in the absence of extracellular Ca^{2+} (Figure 3.2.6: Panel B). The mean data for agonist-mediated alterations in $[Ca^{2+}]_i$ are shown in Figure 3.2.6.

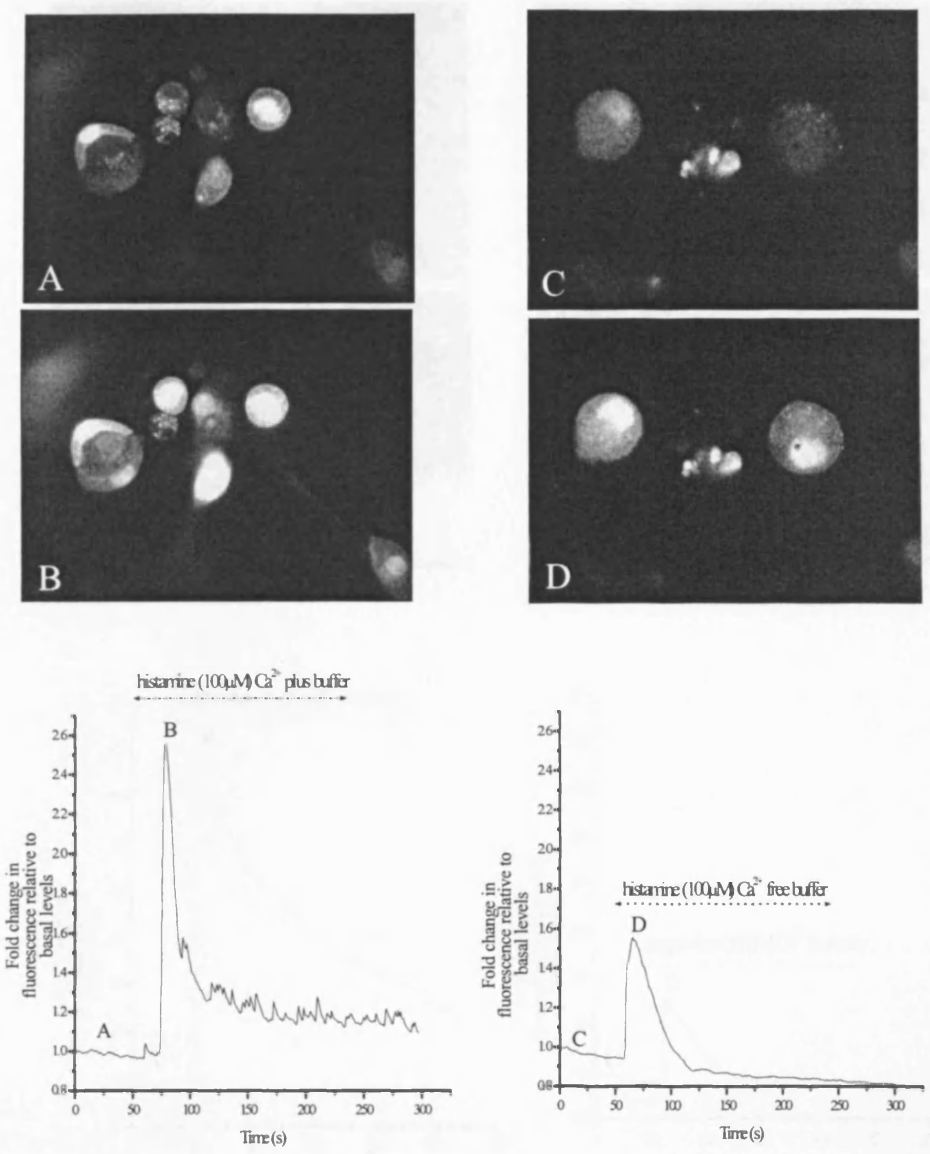


Figure 3.2.1. **Single cell imaging of histamine-mediated Ca^{+2} signalling in chromaffin cells.** Cells were cultured on glass coverslips loaded with fluo-3-AM, excited at λ488 and imaged on the confocal microscope. Cells were challenged with 100μM histamine via perfusion after 50 seconds in the presence or absence of 1.3mM $[Ca^{2+}]_e$. Dotted lines indicate the presence of agonist. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Images A and B were taken at time points indicated on the graph and demonstrate the effect of agonist in the presence of extracellular Ca^{2+} while images C and D show the effect of agonist in the absence of extracellular Ca^{2+}. Data are representative of three independent experiments (three adrenal gland preparations, approximately 280 cells in total).

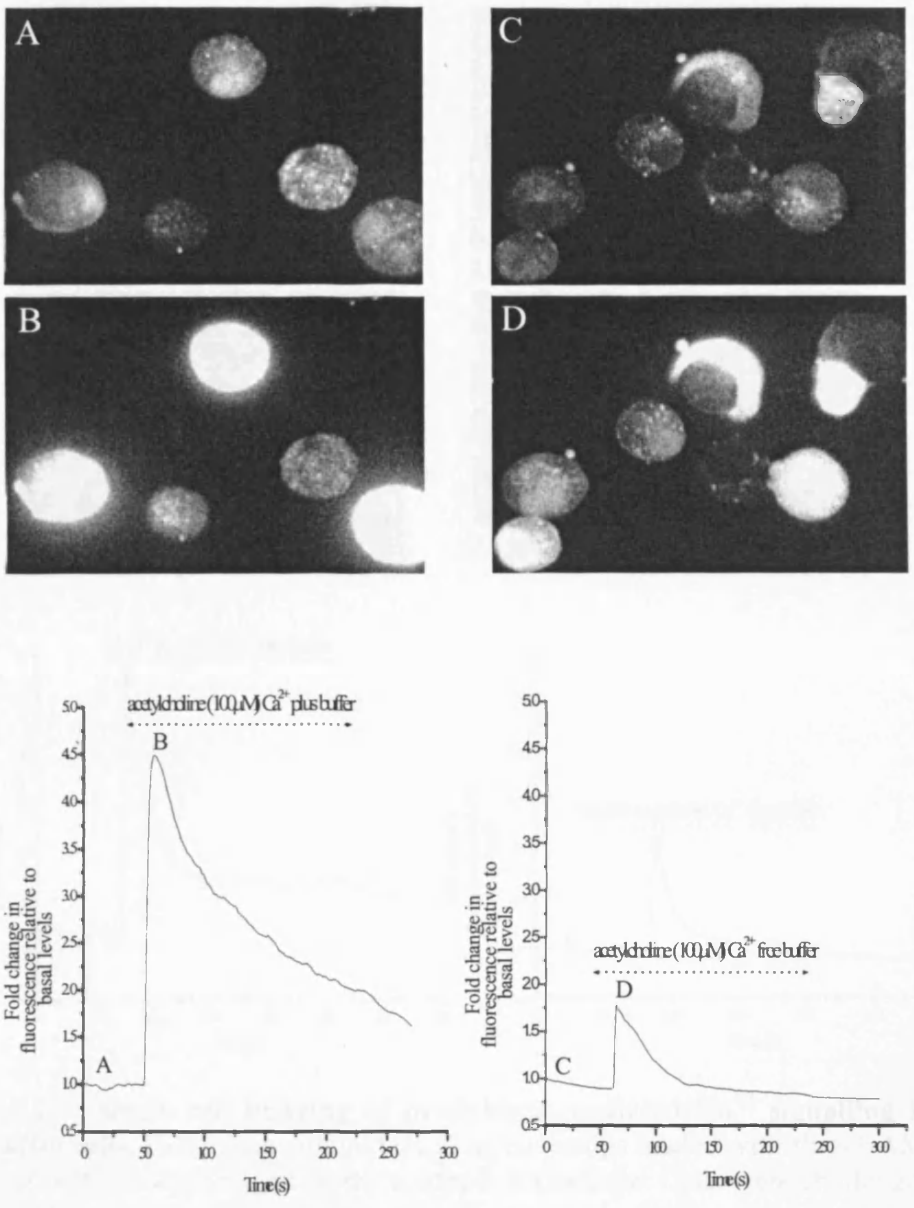


Figure 3.2.2. **Single cell imaging of acetylcholine-mediated Ca^{+2} signalling in chromaffin cells.** Cells were cultured on glass coverslips loaded with fluo-3-AM, excited at λ488 and imaged on the confocal microscope. Cells were challenged with 100μM acetylcholine via perfusion after 50 seconds in the presence or absence of 1.3mM $[Ca^{2+}]_e$. Dotted lines indicate the presence of agonist. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Images A and B were taken at time points indicated on the graph and demonstrate the effect of agonist in the presence of extracellular Ca^{2+} while images C and D shown the effect of agonist in absence of extracellular Ca^{2+}. Data are representative of three independent experiments (three adrenal gland preparations, approximately 180 cells in total).

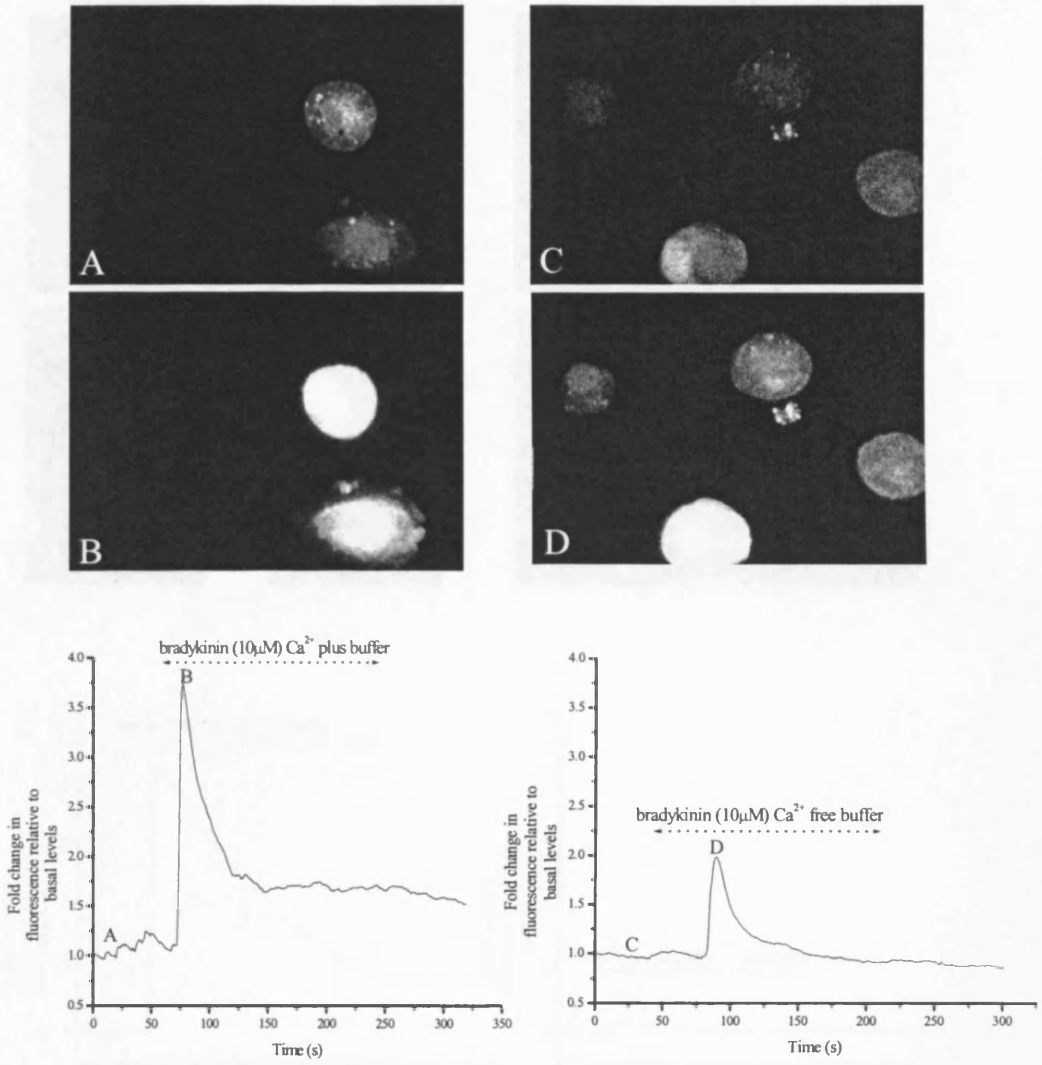


Figure 3.2.3. **Single cell imaging of bradykinin-mediated Ca^{+2} signalling in chromaffin cells.** Cells were cultured on glass coverslips loaded with fluo-3-AM, excited at λ488nm and imaged on the confocal microscope. Cells were challenged with 10μM bradykinin via perfusion after 50 seconds in the presence or absence of 1.3mM $[Ca^{2+}]_e$. Dotted lines indicate the presence of agonist. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Images A and B were taken at time points indicated on the graph and demonstrate the effect of agonist in the presence of extracellular Ca^{2+} while images C and D show the effect of agonist in the absence of extracellular Ca^{2+}. Data are representative of three independent experiments (three adrenal gland preparations, approximately 160 cells in total).

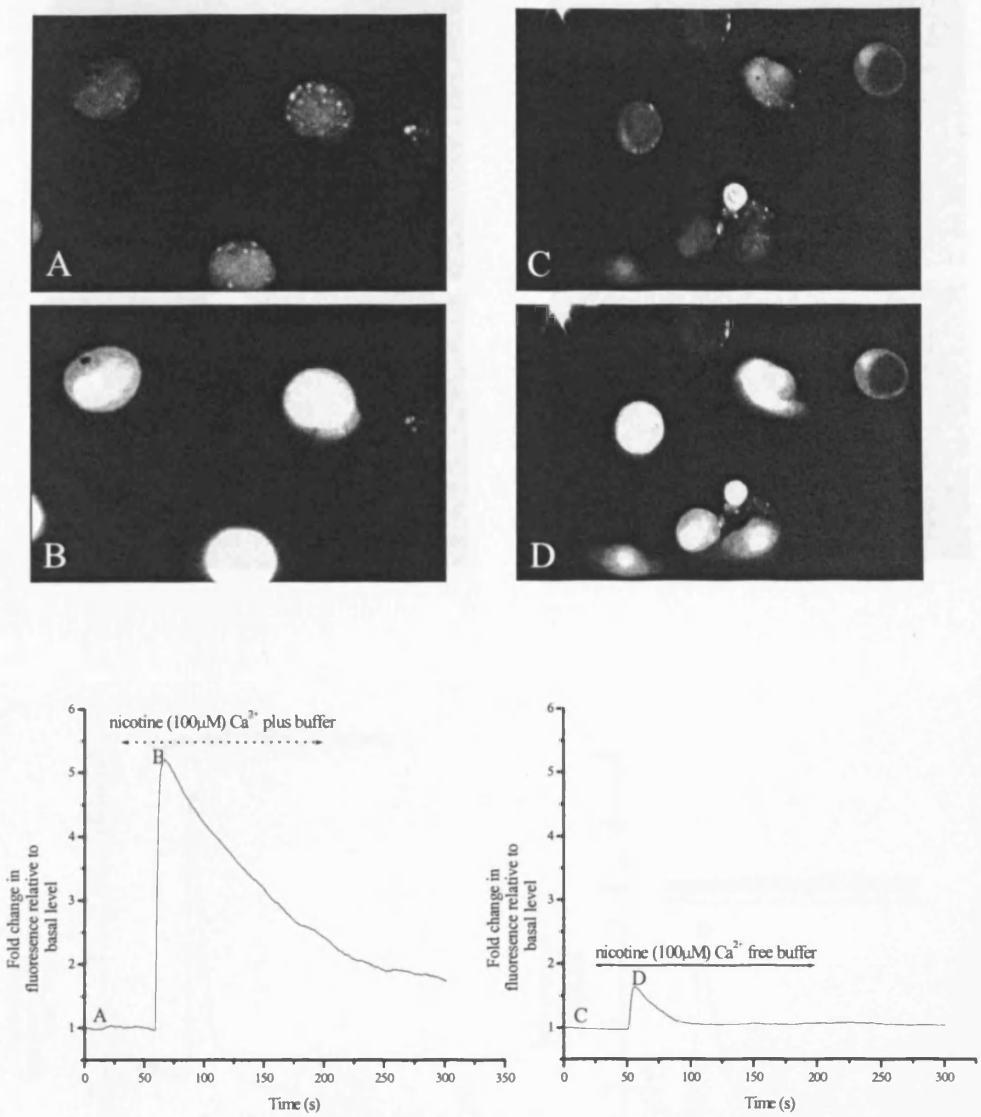


Figure 3.2.4. **Single cell imaging of nicotine-mediated Ca^{+2} signalling in chromaffin cells.** Cells were cultured on glass coverslips loaded with fluo-3-AM, excited at λ488nm and imaged on the confocal microscope. Cells were challenged with 100μM nicotine via perfusion after 50 seconds in the presence or absence of 1.3mM $[Ca^{2+}]_e$. Dotted lines indicate the presence of agonist. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Images A and B were taken at time points indicated on the graph and demonstrate the effect of agonist in the presence of extracellular Ca^{2+} while images C and D show the effect of agonist in the absence of Ca^{2+}. Data are representative of three independent experiments (three adrenal gland preparations, approximately 240 cells in total).

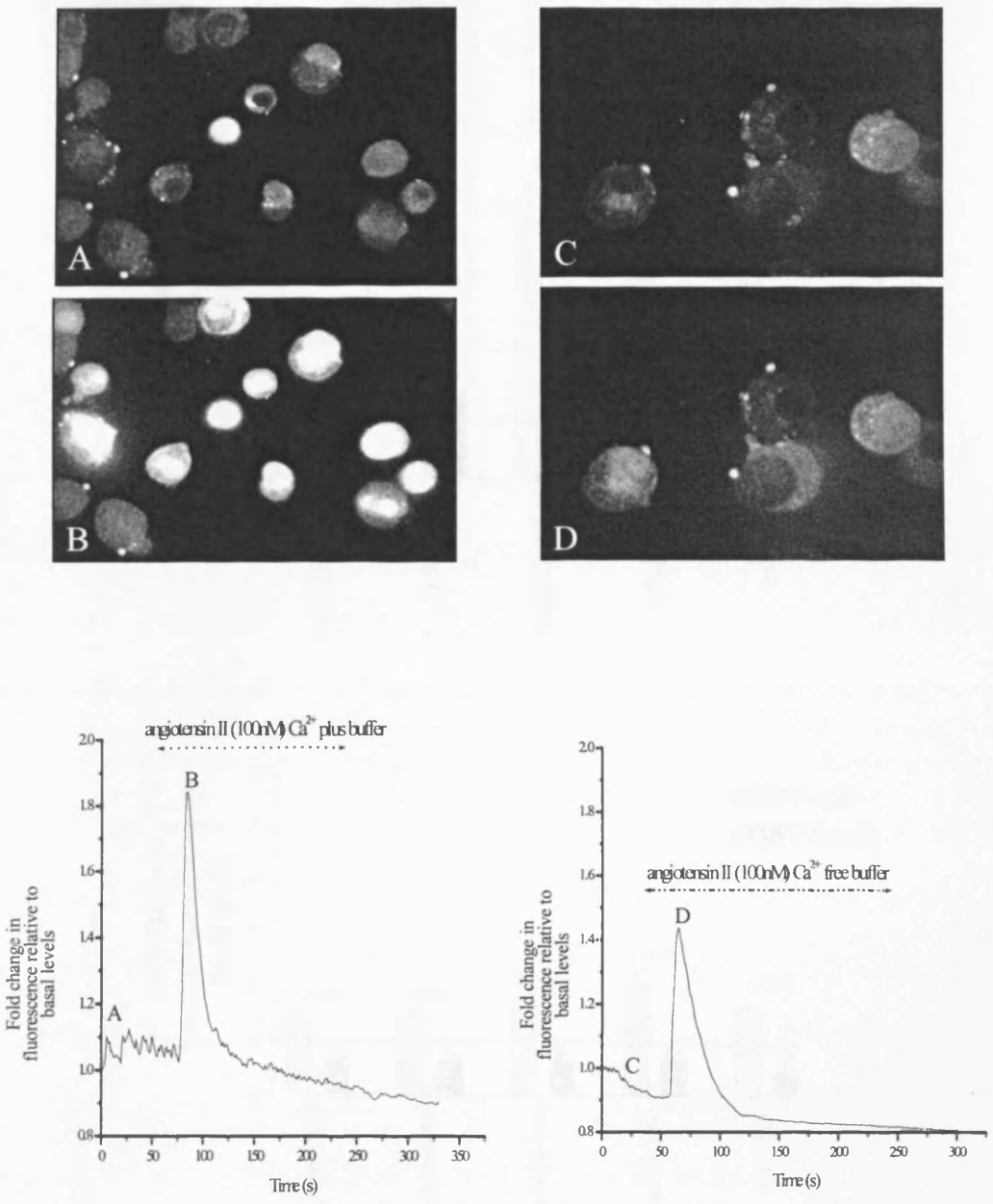
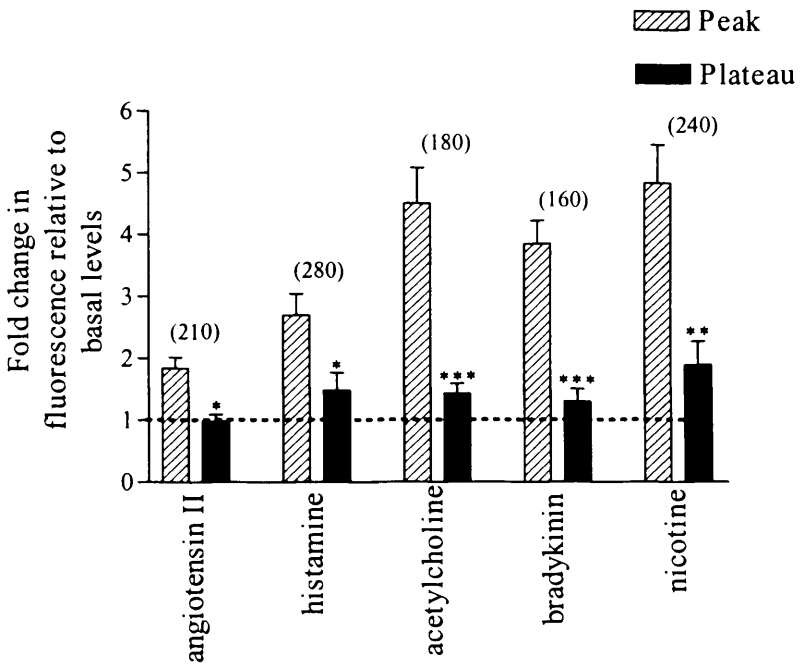


Figure 3.2.5. **Single cell imaging of angiotensin II-mediated Ca^{+2} signalling in chromaffin cells.** Cells were cultured on glass coverslips loaded with fluo-3-Am, excited at λ488 and imaged on the confocal microscope. Cells were challenged with 100nM angiotensin II via perfusion after 50 seconds in the presence or absence of 1.3mM $[Ca^{2+}]_e$. Dotted lines indicate the presence of agonist. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Images A and B were taken at time points indicated on the graph and demonstrated the effect of agonist in the presence of extracellular Ca^{2+} while images C and D show the effect of agonist in the absence of extracellular Ca^{2+}. Data are representative of three independent experiments (three adrenal gland preparations, approximately 210 cells in total).

A.



B.

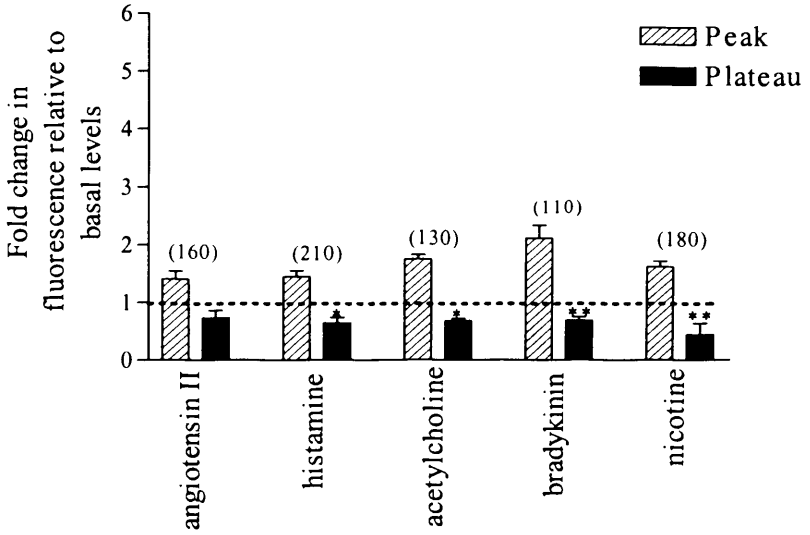


Figure 3.2.6. Summary data of single cell imaging of agonist-mediated Ca^{2+} signalling in chromaffin cells. Data are the summary data from Figures 3.2.7 to 3.2.11. Cells were cultured on coverslips, loaded with fluo-3 and imaged using the confocal microscope. A) Cells were challenged with agonists in the presence of 1.3mM $[Ca^{2+}]_e$. B) Cells were challenged with agonists in the absence of extracellular Ca^{2+}. The hatched bars represent the average maximal change in fluorescence relative to basal levels after agonist perfusion while the black bars represent the change in fluorescence above basal 200 seconds following agonist perfusion. The dotted lines indicate the basal fluorescence. Data are mean + SEM, n=3 (* $P<0.05$, ** $P<0.01$, ***$P<0.001$ vs. peak value of control, as determined by paired Student's *t*-test).

3.3 DISCUSSION

3.3.1 Summary of data

Initial work on the PC12 cell line indicated that the clone of PC12 cells used in this study did not express receptors coupled to $G\alpha_{q/11}$ that had previously been identified on these cells. Therefore, chromaffin cells were used as an alternative choice. Data demonstrated that bovine chromaffin cells express a wide range of receptors that elevate $[Ca^{2+}]_i$ most likely through activation of $G\alpha_{q/11}$ including those for: angiotensin II, histamine, acetylcholine, and bradykinin. In addition, chromaffin cells increase $[Ca^{2+}]_i$ in response to activation of ionotropic nicotinic receptors. The sustained phases of the Ca^{2+} responses mediated by either $G\alpha_{q/11}$-coupled receptors or ionotropic nicotinic receptors were dependent on extracellular Ca^{2+} whilst the peak responses to these agonists also showed some dependence on extracelular Ca^{2+}. Histamine and angiotensin II exhibited different Ca^{2+} profiles and they were therefore selected to study their regulatory role in exocytosis (see subsequent Chapters). Specifically, histamine induced a peak and sustained plateau of $[Ca^{2+}]_i$ elevation. In contrast, angiotensin II evoked only a peak Ca^{2+} response. As seen in Chapters 4 and 5, the subsequent focus of this work will be on comparing the effects of ionotropic receptors on catecholamine release with those of either histamine or angiotensin II $G\alpha_{q/11}$-coupled receptors.

3.3.2 Ca^{2+} signalling-mediated by $G\alpha_{q/11}$-coupled receptors and ionotropic receptors in bovine chromaffin cells

The results presented in this chapter indicate that bovine adrenal chromaffin cells express a wide variety of receptors coupled to $G\alpha_{q/11}$ that mediate Ca^{2+} signalling, including those for angiotensin II, histamine,

acetylcholine, and bradykinin. This is in agreement with previous findings that indicated the presence of a wide range of $G\alpha_{q/11}$-coupled receptors many of which also elicit exocytosis. This includes AT_1 angiotensin II receptors, H_1 histamine receptors, cholinergic receptors, and B_2 bradykinin receptors (Feldberg and Lewis, 1964; Wilson and Kirshner, 1977; Plevin and Boarder, 1988; Sasakawa *et al.*, 1989; Bunn *et al.*, 1990; Bottari *et al.*, 1993; Herrington *et al.*, 1995; Roberts-Thomson *et al.*, 2000; Marley, 2003). In addition, this study highlights differences in Ca^{2+} signalling profiles evoked by these receptors. Thus, histamine receptors, cholinergic receptors and bradykinin receptors evoked a peak followed by sustained plateau Ca^{2+} response, whereas activation of angiotensin II receptors mediated only a peak Ca^{2+} signal. It has been proposed that $G\alpha_{q/11}$-protein-mediated activation of PLC with subsequent generation of the Ca^{2+}-mobilising signal $Ins(1,4,5)P_3$ and thereby mobilization of intracellular Ca^{2+} stores contributes substantially to the peak Ca^{2+} response to $G\alpha_{q/11}$-coupled receptor agonists in chromaffin cells (Zimlichman *et al.*, 1987; O'Sullivan and Burgoyne, 1989; Plevin and Boarder, 1988; Noble *et al.*, 1988; Forsberg *et al.*, 1986; Sasakawa *et al.*, 1989; Bunn *et al.*, 1990; Stauderman *et al.*, 1990; Roberts-Thomson *et al.*, 2000; Kiselyov *et al.*, 2002). It has been known for some considerable time that chromaffin cells have an $Ins(1,4,5)P_3$-sensitive Ca^{2+} store (Stoehr *et al.*, 1986) located in the endoplasmic reticulum (Bayerdorffer *et al.*, 1984; Prentki *et al.*, 1984; Cheek and Burgoyne, 1985; Cheek *et al.*, 1993). It was suggested previously that localization of endoplasmic reticulum at/or near one pole of the chromaffin cells might contribute to the observed localization of Ca^{2+} signals to one pole of the cells (O'Sullivan *et al.*, 1989). Furthermore, the $Ins(1,4,5)P_3$-sensitive stores in chromaffin cells were localized away from the

bulk of the secretory granules in the area between the plasma membrane and nucleus where the endoplasmic reticulum is located (Burgoyne *et al.*, 1989; O'Sullivan *et al.*, 1989). The sustained phase of the Ca^{2+} response may be due to Ins(1,4,5)P_3 generation, the depletion of intracellular Ca^{2+} stores and the subsequent activation of capacitative Ca^{2+} entry through store-operated Ca^{2+} channels (SOCs) (Putney, 1986; 1990; Putney and McKay, 1999; Barritt, 1999; Fomina and Nowycky, 1999). This study revealed the contribution of capacitative Ca^{2+} entry to the sustained Ca^{2+} responses (see Chapter 6). In addition, there is evidence that activation of voltage-operated calcium channels (VOCCs) might also be involved in Ca^{2+} signalling evoked by $G\alpha_{q/11}$-coupled receptor agonists (Zerbes *et al.*, 1998, Marley, 2003). Previous studies have demonstrated the presence of L, N, P and Q-type on bovine adrenal chromaffin cells (Artalejo *et al.*, 1994; López *et al.*, 1994a; 1994b; Lara *et al.*, 1998).

The Ca^{2+} signalling data also suggests that sustained Ca^{2+} responses mediated either by histamine, acetylcholine or bradykinin are markedly dependent on extracellular Ca^{2+} influx through either VOCCs or SOCs, since elimination of extracellular Ca^{2+} completely abolished the sustained phase induced by these stimuli. In contrast, the peak Ca^{2+} responses evoked by these agonists were only partially dependent on extracellular Ca^{2+} influx since removal of extracellular Ca^{2+} partially reduced the peak Ca^{2+} signalling. It has been previously proposed that placing cells in low extracellular Ca^{2+} might itself cause some Ca^{2+} store depletion particularly from stores close to the membrane which contribute to Ca^{2+} release (Wheldon *et al.*, 2001; Willars *et al.*, 1995a; 1998b). The present finding is consistent with previously published work that Ca^{2+} responses evoked by these agonists were more transient in Ca^{2+} free buffer than

in the presence of external Ca^{2+} (O'Sullivan et al., 1989; Stauderman and Pruss, 1990; Robinson and Burgoyne, 1991; Nassar-Gentina *et al.*, 1997; Zerbes *et al.*, 1998).

Data presented indicate that cholinergic receptor activation with acetylcholine in the presence of extracellular Ca^{2+} evoked the greatest peak Ca^{2+} response compared with the $G\alpha_{q/11}$-coupled receptors examined in this study, but its effect was comparable to that evoked by ionotropic receptors. Since acetylcholine is the physiological secretagogue for chromaffin cells (Douglas and Rubin, 1961), similarities in Ca^{2+} signalling evoked by acetylcholine and nicotine might be attributed to the fact that acetylcholine activates both types of cholinergic receptors, nicotinic and muscarinic (Wilson and Kirshner, 1977). Although the biggest Ca^{2+} signalling might be due to the former receptor (i.e., nicotinic receptors), experiments performed in this study in the absence of extracellular Ca^{2+} showed some Ca^{2+} response mediated by acetylcholine. This suggests that some of Ca^{2+} response is likely to be via $G\alpha_{q/11}$-coupled receptors. Another explanation for the greatest Ca^{2+} response evoked by acetylcholine could be due to the nature of the cholinergic receptors coupled to $G\alpha_{q/11}$ that triggers Ca^{2+} mobilization from the intracellular stores and thereby activates capacitative Ca^{2+} entry.

The present study demonstrated that angiotensin II exhibited a unique $[Ca^{2+}]_i$ response profile that was different from that mediated by other $G\alpha_{q/11}$-coupled receptor agonists in that it did not have a sustained plateau. Thus, Ca^{2+} signalling data directed the attention to select histamine and angiotensin II in order to compare their signalling pathways specifically their impact on exocytosis. Acetylcholine was excluded because it acts simultaneously on nicotinic and

muscarinic receptors present in chromaffin cells. The possible explanation for different Ca^{2+} signalling profiles mediated by histamine and angiotensin II is that angiotensin type 1A receptor rapidly desensitises (Olivares-Reyes *et al.*, 2001). Indeed, previous studies have suggested that PKC-induced phosphorylation promotes desensitisation of the AT_{1A} receptor (Barker *et al.*, 1995; Olivares-Reyes *et al.*, 2001).

It is widely accepted that histamine and angiotensin II both trigger secretion of catecholamines in bovine chromaffin cells and this depends upon external Ca^{2+}, with histamine being a much better secretagogue than angiotensin II (O'Sullivan *et al.*, 1989). It has been proposed that beside the capacitative Ca^{2+} entry discussed above, the nature of the channels through which histamine and possibly angiotensin II stimulate Ca^{2+} entry and the mechanism of their opening are unknown (Robinson and Burgoyne, 1991).

Nicotinic receptor-mediated elevation of $[Ca^{2+}]_i$ was extremely dependent on external Ca^{2+} in agreement with previously published work (Burgoyne, 1991; Nassar-Gentina *et al.*, 1997). Activation of nAChRs is known to depolarise cells, which activates VOCCs and allows Ca^{2+} influx (Vijayaraghavan *et al.*, 1992; Rathouz and Berg, 1994). In addition to this, nAChRs themselves can serve as Ca^{2+} entry pathways (Miledi *et al.*, 1980; Decker and Dani, 1990; Zhou and Neher, 1993; Zhang *et al.*, 1996b). Ca^{2+} permeability of $\alpha3\beta4$ subunits of nAChRs is quite high but less than $\alpha7$ that exhibits high relative permeability to Ca^{2+} (Costa *et al.*, 1994; Rathouz and Berg, 1994; McGehee and Role, 1995; Ragozzino *et al.*, 1998). Chromaffin cells express the neuronal type of nAChR, which is represented by pentametric complexes (eight α ($\alpha2$-$\alpha9$) and three β ($\beta2$-$\beta4$) neuronal nAChR subunits) (Sargent, 1993). Pharmacological studies indicate

the presence of multiple populations of adrenal nAChRs such as α3, α5, α7, and β4 nAChR subunits (Garcia-Guzman *et al.*, 1995; Wenger *et al.*, 1997). The subunit combination of nAChRs associated with the catecholamine secretion from chromaffin cells was reported to be α3β4 or α3β4α5 (Tachikawa *et al.*, 2001). Besides Ca^{2+} influx through nAChR channels (Zhou and Neher, 1993; Harkins and Fox, 1998) and VOCCs (Kilpatrick *et al.*, 1981; Knight and Kesteven, 1983), activation of nAChR can also induce Ca^{2+} release from ryanodine- and Ins(1,4,5)P_3-sensitive intracellular stores (Eberhard and Holz, 1987; 1988; Nakaki *et al.*, 1988; Robinson and Burgoyne, 1991; Roberts-Thomson, 2000; Dajas-Bailador *et al.*, 2002a). The release of Ca^{2+} from these stores and activation of capacitative Ca^{2+} entry might contribute to the sustained Ca^{2+} response triggered by nicotinic receptor activation. The sustained elevations of intracellular Ca^{2+} in response to nicotinic receptor stimulation have been observed previously in hippocampal neurones (Dajas-Bailador *et al.*, 2000b), chick cilliary ganglia neurones (Shoop *et al.*, 2001) and PC12 cells (Gueorguiev *et al.*, 2000) and consistent with observations obtained from the present study on chromaffin cells. In addition, the present study shows evidence of some contribution of thapsigargin-sensitive Ca^{2+} stores to sustained Ca^{2+} response mediated by nicotinic receptor activation (see Chapter 6).

It is worthwhile to note that not all of the cells examined responded with elevated $[Ca^{2+}]_i$ to most agonists. Indeed, the reason for this may be due to the fact that chromaffin cultures might be not entirely pure and may contain other cell types including bovine adrenal medulla endothelial cells (BAMEC) and fibroblasts (Bossu *et al.*, 1992; Vinet and Vargas, 1999). In addition, these contaminated cells might divide in culture and can exert major distorting effects

on biochemical measurements of older cultures. Therefore, chromaffin cells should be used after only short culture periods and where possible the results confirmed with freshly isolated cells. Another explanation for failure of some chromaffin cells to respond to agonist is that the rise in $[Ca^{2+}]_i$ might be spatially restricted often to one pole of cell which might hinder Ca^{2+} diffusion in cytoplasm.

4: CATECHOLAMINE RELEASE EVOKED BY ACTIVATION OF EITHER LIGAND GATED ION CHANNELS OR $G\alpha_{q/11}$-COUPLED RECEPTORS

4.1 INTRODUCTION

The aim of the work described in this chapter was to examine the effect of the stimulation of either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors on catecholamine release from cultured bovine adrenal chromaffin cells.

Triggered exocytosis is the most common cellular mechanism for secretion. It consists of fusion of an intracellular vesicle with the plasma membrane and provides a highly regulated system to release a variety of substances, including neurotransmitters, as discrete packages called "quanta" (Katz, 1971). The exocytosis process is a chain of complex mechanisms that involve vesicle docking, priming, and fusion (Borges *et al.*, 2002) (see Chapter 1). Adrenomedullary chromaffin cells are terminally differentiated secretory cells of neural crest origin, dedicated to the synthesis, storage, and release of catecholamines (Livett, 1984). Catecholamines are stored within chromaffin granules at very high concentrations (0.5-1M) (Borges *et al.*, 2002). At least three types of chromaffin cells are present in the adrenal medulla: adrenaline (85%), noradrenaline (14-15%) and dopamine (1%) containing cells (Eaton and Dulphan, 2004). In addition, a number of different neuropeptides and proteins are co-stored and co-released with the catecholamines from the adrenal medulla (Takiyyuddin et al., 1990) including enkephalins, neuropeptide Y (NPY), dynorphin, galanin, vasoactive intestinal peptide (VIP), substance P, neurotensin, somatostatin, dopamine β-hydroxylase (DBH), chromogranins A and B (CgA, CgB), and secretogranin II (SgII) (Winkler *et al.*, 1986; Fischer-Colbrie *et al.*, 1995) (see Chapter 1 for details of different vesicles and differential release). In response to a

variety of stimuli, catecholamines are released and this cell model has been widely used in the study of neuroendocrine function and in investigations of the mechanisms involved in secretion.

The adrenal medulla receives cholinergic innervation from the sympathetic nervous system via the splanchnic nerve (Burgoyne, 1984a). Catecholamine secretion from chromaffin cells is evoked by acetylcholine (ACh) released from the splanchnic nerve which activates nicotinic acetylcholine receptors (nAChRs) on chromaffin cells. The influx of cations through the receptor channel causes plasma membrane depolarisation and opening of voltage-gated Ca^{2+} channels. The rapid influx of extracellular Ca^{2+} through these channels initiates a number of different processes that lead to exocytosis including docking of secretory vesicles to the plasma membrane prior to fusion and fusion of the vesicles with the plasma membrane. Following this, the emptied readily releasable pool is replenished due to breakdown of the cortical cytoskeleton and migration of secretory granules to the cell surface, and fusion of the granule membranes with the plasma membrane (Cox and Parsons, 1997; Mollard *et al.*, 1995). In fact, both the rapid exocytotic release of catecholamines from docked vesicles (Douglas and Rubin, 1961) and the refilling of the readily releasable pool (Von Rűden and Neher, 1993) are dependent on the local rise of the $[Ca^{2+}]_i$. It is widely accepted that catecholamine secretion from the bovine adrenal medulla is evoked largely by nicotinic receptor activation. However, the bovine adrenal medulla also expresses a number of G protein-coupled receptors (GPCRs) that stimulate catecholamine secretion. This includes AT_1 angiotensin II receptors, H_1 histamine receptors, B_2 bradykinin receptors, EP_3 prostaglandin (PG) receptors, PAC_1 receptors for pituitary adenylate cyclase-activating polypeptide (PACAP), and in some species

muscarinic cholinoceptors and P2Y purinoceptors (Marley, 2003; Marley *et al.*, 2002; Aguilar *et al.*, 1992). The efficacy of several of these receptors, including those for angiotensin II, bradykinin, ATP and prostaglandins is very low; however, receptors for histamine and PACAP are highly effective and comparable to powerful secretagogues such as nicotinic receptor agonists and K^+ depolarization (Donald *et al.*, 2002). Indeed, there is considerable evidence that these $G\alpha_{q/11}$-coupled receptors play substantial physiological roles in driving adrenal catecholamine secretion in parallel to and in cooperation with nicotinic receptors. Like the secretory responses to nicotinic receptor activation and K^+-evoked depolarisation, the secretion in response to $G\alpha_{q/11}$-coupled receptor agonists depends on the influx of extracellular Ca^{2+} (Marley, 2003).

Several pieces of evidence link actin to the dynamic regulation of secretion and neurotransmission (Viviani *et al.*, 1996). It has been proposed that the actin microfilament network localized underneath the plasma membrane of chromaffin cells (Lee and Trifaró, 1981; Trifaró *et al.*, 1984; Cheek and Burgoyne, 1986) acts as a barrier to the movement of secretory granules, blocking their access to exocytosis sites at the plasma membrane (Trifaró *et al.*, 1982; Burgoyne, 1991). Thus, removal of the actin barrier would allow the free movement of granules and their subsequent interaction with the plasma membrane (Lelkes *et al.*, 1986). Prior to the disruption of the F-actin network very few secretory vesicles are found between it and the plasma membrane. This region, 0-50nm wide, is occupied by 1.2-2.4% of the total number of secretory vesicles present in chromaffin cells (Vitale *et al.*, 1995). It has been shown that nicotinic receptor stimulation of chromaffin cells induces the disassembly of cortical filamentous actin (F-actin) (Cheek and Burgoyne, 1986 and Rodríguez Del

Castillo *et al.*, 1992) allowing the free movement of secretory vesicles to release sites at the plasma membrane (Vitale *et al.*, 1991). There is evidence that secretion mediated by H_1 histamine receptors is a consquence of redistribution of subplasmalemmal scinderin (an F-actin severing protein) concomitantly with cortical F-actin disassembly (Zhang *et al.*, 1995b). Two pathways are known to be involved in the control of chromaffin cell cortical F-actin networks during secretion: the Ca^{2+}-scinderin and PKC-MARCKS pathways (Cuchillo-Ibãñez *et al.*, 2004). Several lines of research suggest the involvement of PKC in the regulation of actin cytoskeleton organization and dynamics and this may contribute to facilitating catecholamine secretion (Rosē *et al.*, 2001). This contributory effect of PKC may be attributed to the phosphorylation of cytoskeletal proteins and the disruption of cortical F-actin near the plasma membrane. This would then allow an increase in the size of readily releasable pool of secretory granules (Gillis *et al.*, 1996). Acute treatment of chromaffin cells with PMA disrupts the F-actin network in some cortical areas and causes a 2-3 fold increase in the number of secretory vesicles within 0-50nm of the plasma membrane (Trifaró *et al.*, 2000). In addition, PMA does not change the basal catecholamine secretion but enhances the initial rate of secretion in response to nicotine.

In the basal (unstimulated) state, F-actin can be visualized with rhodamine-labeled phalloidin as a strong cortical fluorescent ring (Cheek and Burgoyne, 1986; Vitale *et al.*, 1991; 1995). Upon stimulation this cortical fluorescent ring is disrupted. Since phalloidin is a probe for F-actin, the disappearance of rhodamine fluorescence indicates disassembly of actin filaments at specific subplasmalemmal areas (Vitale et al., 1991). Disassembly of cortical F-

actin in response to stimulation is thought to allow the movement of vesicles from the reserve compartment to replenish the readily releasable pool in preparation for exocytosis to occur (Vitale *et al.*, 1991; 1995).

The aims of the experiments described in this chapter were to characterize the effect of activation of either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors on secretion of catecholamines from cultured bovine adrenal chromaffin cells. In this study KCl was used as control depolarizing agent. Catecholamine release from populations of chromaffin cells was monitored by a spectrofluorimetric assay of extracellular catecholamines and referenced (as %) to the total cellular content determined after cell lysis (see Chapter 2). The effect of activation of either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors on cytoskeletal cortical F-actin integrity was then investigated and the impact of chemical stabilization of cortical F-actin filaments on catecholamine secretion determined.

4.2 RESULTS

Time-dependent increase in catecholamine secretion-evoked by nicotinic receptors

Release (at 37°C) from monolayers of cultured (3 days) cells was monitored by a spectrofluorometric assay of extracellular catecholamines and referenced (as %) to the total cellular content determined after cell lysis as described in "Materials and Methods". Figure 4.2.1 shows a time-dependent increase in the release of catecholamines evoked by nicotine (100μM). At the zero time, the basal release was 2.6 ± 0.8%. Catecholamine release during incubations of 30s, 1 min, 3 min, and 5 min were 4.0 ± 0.3%, 6.0 ± 0.5%, 10.3 ± 0.3%, and 17.7 ± 0.6% of the cell content, respectively. The maximal release (24.70%) was observed at 10 min stimulation with nicotine. With 10 min and 20 min treatments, the extent of release was similar (24.7% and 24.7%, respectively) release was approximately similar. Therefore, a 10 min incubation was selected as suitable time for incubation throughout the catecholamine assays.

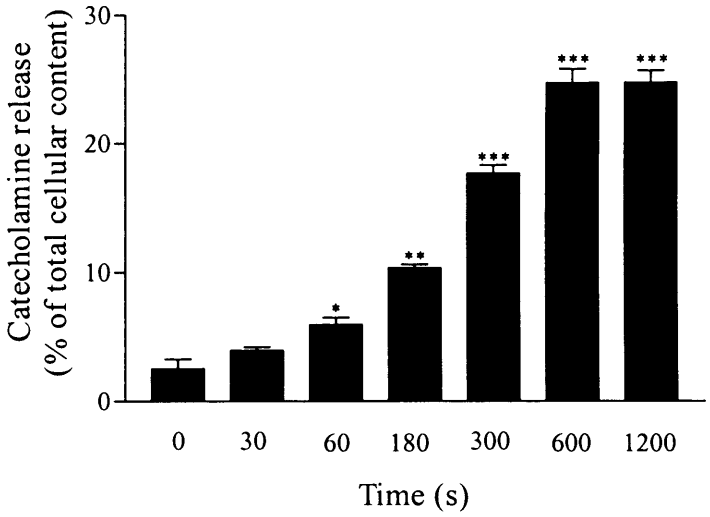


Figure 4.2.1. Time course of nicotine-mediated secretion of catecholamines from bovine adrenal chromaffin cells. Chromaffin cells were incubated with 100μM nicotine for various periods as indicated. The amount of catecholamines released into the buffer was measured as described in "Materials and Methods" and is expressed as a percentage of total cellular content. Data show mean + SEM from three different cell preparations. * $P<0.05$. ** $P<0.01$, *** $P<0.001$ by one-way ANOVA followed by post-hoc Dunnett's test vs. zero time.

Effect of the activation of either ligand gated ion channels or $G\alpha_{q/11}$-coupled receptors on secretion of catecholamines

As shown in Figure 4.2.2, either nicotine (100μM) or KCl (40mM) caused a significant release of catecholamines that was abolished by removal of 1.3mM extracellular Ca^{2+} (i.e. no added Ca^{2+} to Krebs'/HEPES buffer) (Figure 4.2.2). Catecholamine secretion evoked by nicotine was significant greater than that mediated by KCl (Figure 4.2.2). Activation of $G\alpha_{q/11}$-coupled receptors, particularly histamine (100μM) and angiotensin II (100nM) evoked catecholamine secretion although considerably less than that evoked by either nicotine or KCl (Figure 4.2.3)

Both nicotine and KCl evoked a concentration-dependent increase in catecholamine release, with pEC_{50} values of 5.40 ± 0.75 for nicotine and 1.64 ± 0.65 for KCl (Figure 4.2.4: panel A and B). Although there was evidence of concentration-dependent increases in response to either histamine or angiotensin II, sigmoidal curves could not be fitted to the concentration-response curves making accurate determination of EC_{50} values problematic (Figure 4.2.4: panel C and D) (Table 4.2.1).

To test whether either nicotine or KCl potentiate the secretion of catecholamines evoked by $G\alpha_{q/11}$-coupled receptors, chromaffin cells were treated with histamine (100μM) in combination with either nicotine (100μM) or KCl (40mM) for 10 min. Histamine had no additional impact on catecholamine secretion as it did not enhance the release evoked by either nicotine alone or KCl alone. Similarly, angiotensin II (100nM) did not influence the secretion evoked by either nicotine or KCl (Figure 4.2.3).

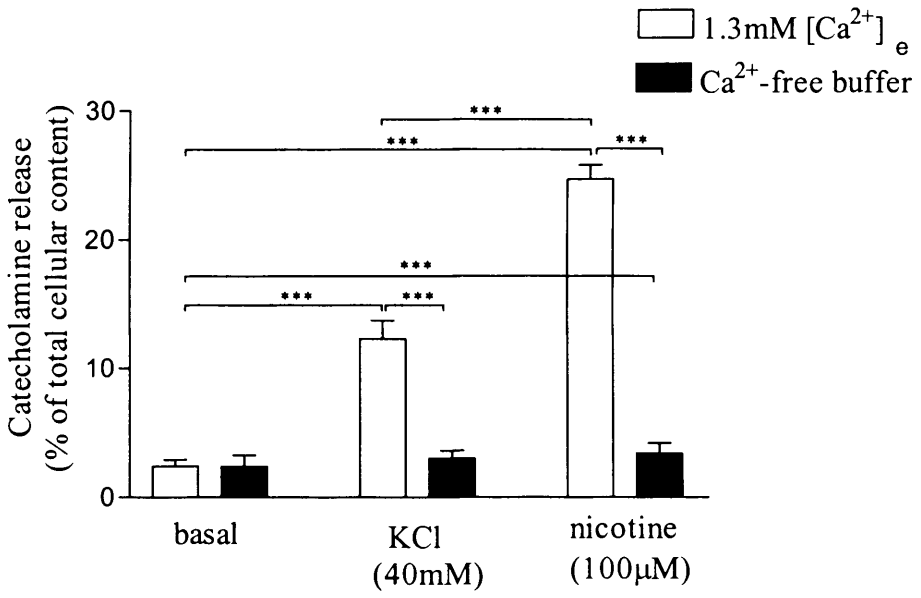


Figure 4.2.2. The influence of extracellular Ca^{2+} on either nicotine or K^+-evoked secretion of catecholamines from bovine adrenal chromaffin cells. Chromaffin cells were challenged with nicotine (100μM) or KCl (40mM) for 10 min at 37°C either in the presence of 1.3mM external Ca^{2+} or in Ca^{2+}-free buffer (i.e. no added Ca^{2+}). Catecholamine release was measured as described in "Materials and Methods" and is expressed as a percentage of total cellular content. (Basal release is that from unstimulated cells). Data are mean + SEM from three different cell preparations (each in duplicate). *** $P<0.001$ by one-way ANOVA followed by post-hoc Bonferroni's test. (Not all comparisons are shown on the graph).

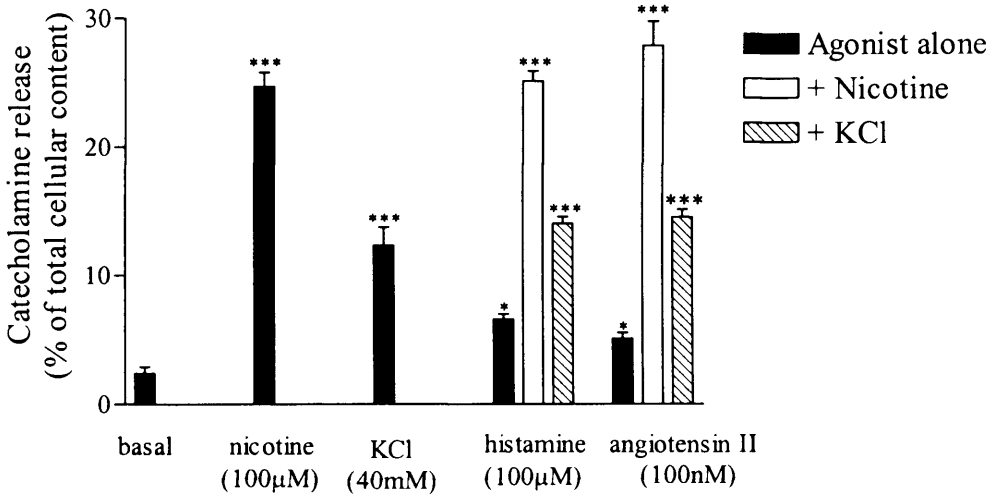


Figure 4.2.3. Effect of $G\alpha_{q/11}$-coupled receptors on secretion of catecholamines from bovine adrenal chromaffin cells. Chromaffin cells were challenged with either agonists of $G\alpha_{q/11}$-coupled receptors alone (i.e. histamine (100μM) or angiotensin II (100nM)) or a combination of activators of $G\alpha_{q/11}$-coupled receptors and ligand gated ion channels (i.e. nicotine (100μM) and KCl (40mM)) for 10 min at 37°C. Catecholamine release was measured as described in "Materials and Method" and is expressed as a percentage of total cellular content. Data show mean + SEM from three different cell preparations. * $P<0.05$, *** $P<0.001$ vs. basal (unstimulated cells) by one-way ANOVA followed by post-hoc Dunnett's test.

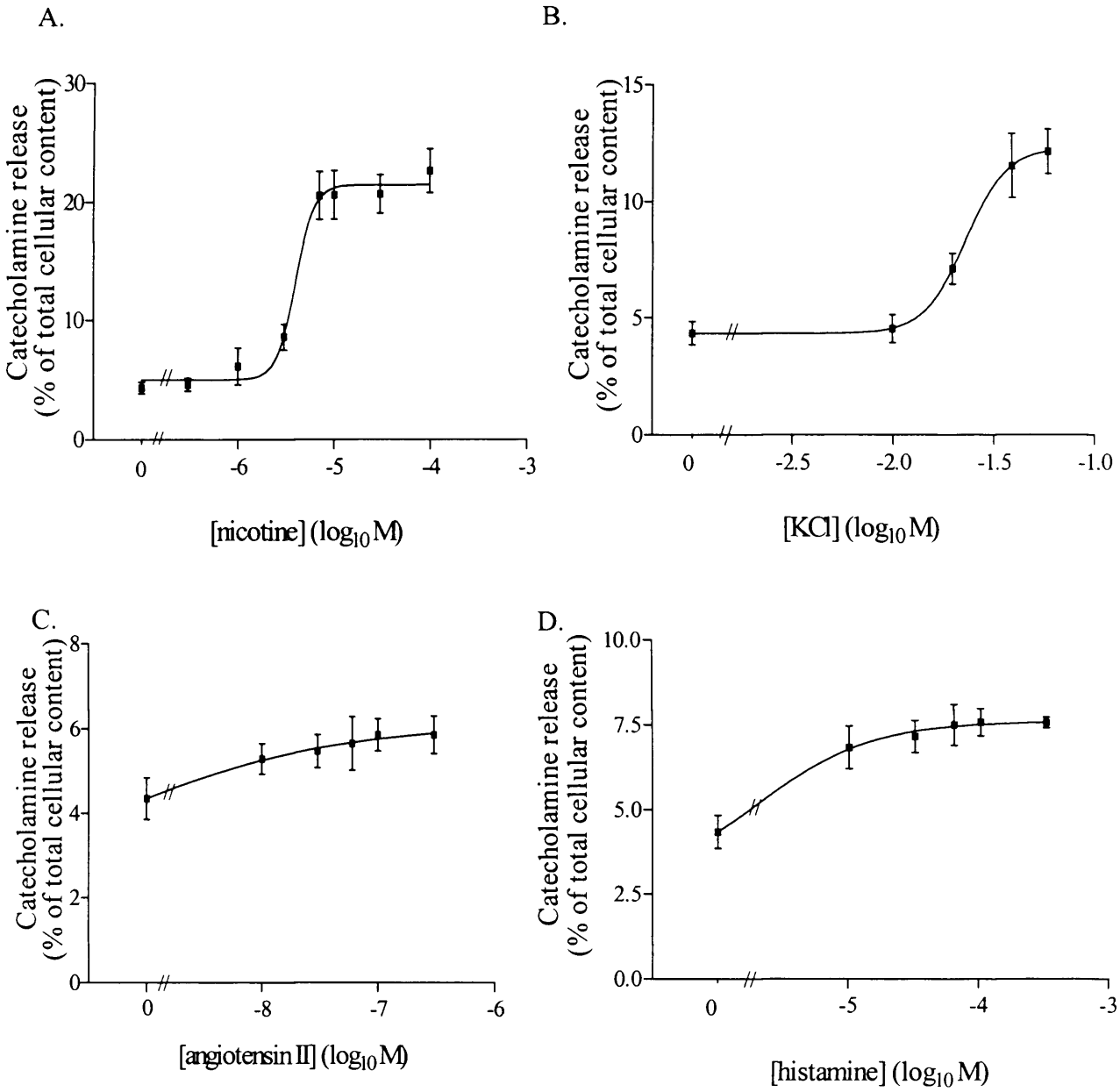


Figure 4.2.4. Concentration-dependent effects of nicotine, K^+, angiotensin II and histamine on secretion of catecholamines from bovine adrenal chromaffin cells. Chromaffin cells challenged with various concentrations of nicotine (panel A, pEC_{50} 5.4 (~4.0μM)), KCl (panel B, pEC_{50} 1.64 (~23mM)), histamine (panel C, pEC_{50} 5.25 (~5μM)) and angiotensin II (panel D, pEC_{50} 8.25 (~5nM)). Full details of the pEC_{50} values are given in Table 4.2.1. Catecholamine release was measured as described in Materials and Methods and is expressed as percentage of total cellular content. Data are mean ± SEM from three different cell preparations.

Table 4.2.1: Summary of pEC_{50} values for nicotine, KCl, histamine and angiotensin II evoked secretion of catecholamines from bovine adrenal chromaffin cells.

	nicotine	KCl	histamine	angiotensin II
pEC_{50} ± SEM	5.40 ± 0.75	1.64 ± 0.65	5.25 ± 0.56	8.25 ± 0.43
Hill slope	4.51	4.19	0.64	0.39

- Sigmoidal curves could not be fitted to the concentration-response curves for either angiotensin II or histamine. The pEC_{50} values are therefore very approximate values based on the curves.

Cortical F-actin disassembly mediated by nicotinic and $G\alpha_{q/11}$-coupled receptor activation

Disassembly of cortical F-actin may allow access of exocytotic vesicles from a 'reserve' pool to the plasma membrane (Vitale *et al.*, 1991; 1995). Confocal microscopy of rhodamine-phalloidin stained cells was used as described in "Materials and Methods" to assess the integrity of cortical F-actin.

In resting cells, staining of F-actin with rhodamine-labelled phalloidin revealed a weak and diffuse cytoplasmic staining and a continuous and bright cortical fluorescent ring (Figure 4.2.5a panels a and a`). The percentage of cells showing disruption of cortical F-actin in unstimulated cells was 7.0 ± 2.5% (Figure 4.2.5b.). Stimulation of chromaffin cells with nicotine (100μM, 40s) induced the fragmentation of the cortical F-actin ring (Figure 4.2.5a panels d and d`) (65.9 ± 4.6% of stimulated cells (n=600 cells)) (Figure 4.2.5b.). Incubation of cells with either cytochalasin D (2μM, 20 min), an F-actin destabilizer (Cooper, 1987), PMA (1μM, 10 min), histamine (100μM, 10 min), or angiotensin II (100nM, 10 min) also evoked disruption of the cortical F-actin ring (Figures 4.2.5a and 4.2.5b).

The effect of jasplakinolide, an agent that prevents F-actin depolymerization and stabilizes the actin network (Oheim and Stuhmer, 2000) was examined in chromaffin cells. Stimulation of cells with either nicotine (100μM, 40s) or cytochalasin D (2μM, 10 min) alone caused significant disassembly of cortical F-actin, which was almost completely prevented by pretreatment of cells with jasplakinolide (10μM, 30 min) (Figures 4.2.6a and 4.2.6b).

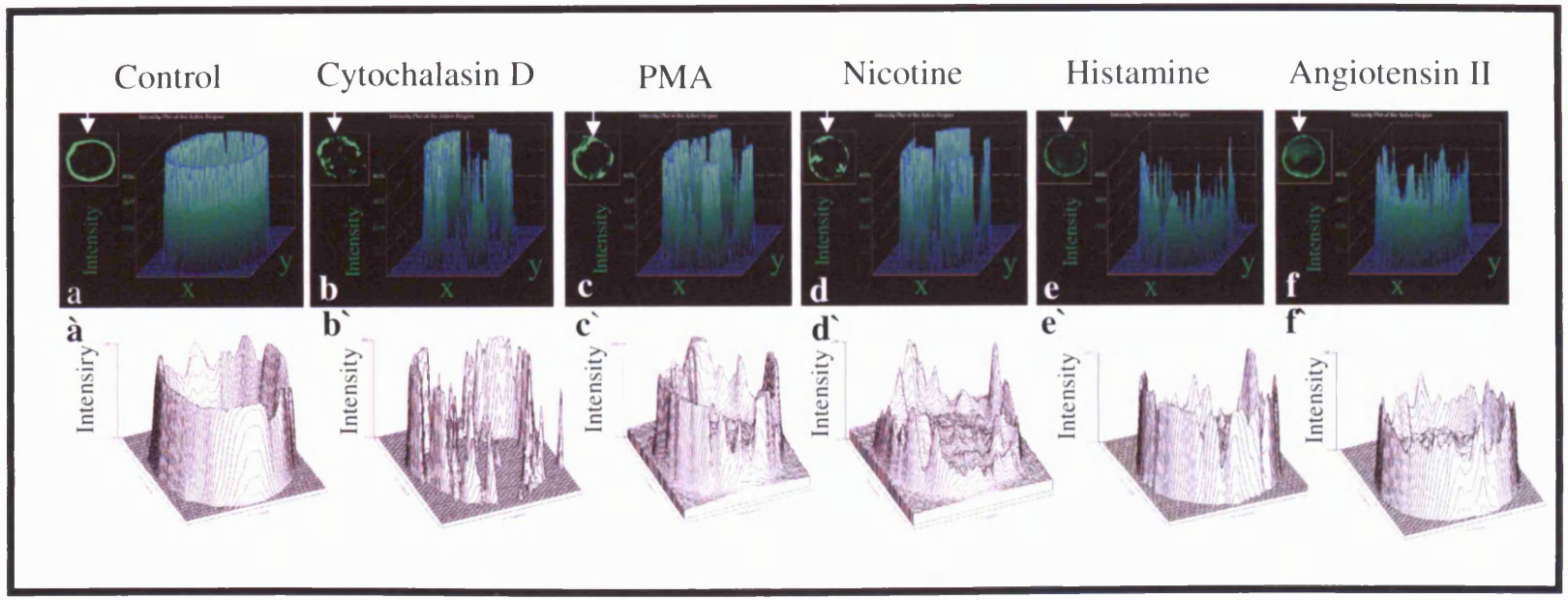


Figure 4.2.5a. Effect of either cytochalasin D, PMA, nicotine, histamine, or angiotensin II on the cortical F-actin network. Chromaffin cells were incubated in the absence of test agents or with cytochalasin D (2μM, 20 min), PMA (1μM, 10 min), nicotine (100μM, 40s), histamine (100μM, 10 min), and angiotensin II (100nM, 10 min) at 37°C. Following these treatments, the cells were fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under "Materials and Methods. The arrows in the left hand corner of the upper panels point out the confocal image of the cell which were then subject to image analysis. Three-dimensional image analysis were performed using either "UltraView software'' (Perkin-Elmer Life Science, Cambridge, UK) (a, b, c, d, e, and f) where the maximum intensity of the fluorescent ring was set to 4096 arbitrary units or using the public domain program "Image J" (a`, b`, c`, d`, e`, and f`). Images are representative of approximately six hundred cells from three different cell preparations. Control cells show an intact cortical ring (a, a`) with a uniform fluorescence intensity pattern. Cytochalasin D, PMA, nicotine, histamine, and angiotensin II treated cells show a disrupted cortical ring (b, c, d, e, and f) with irregularities such as patches of fluorescence.

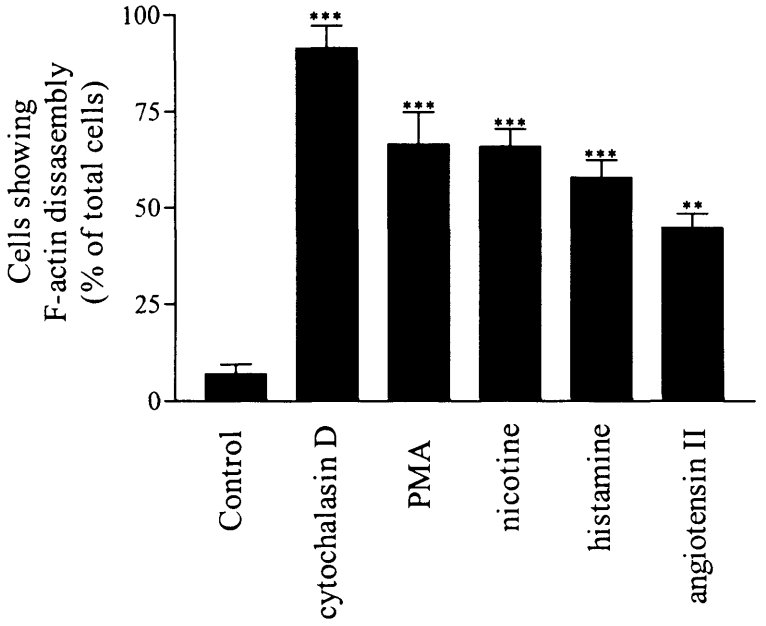


Figure 4.2.5b. Effects of cytochalasin D, PMA, nicotine, histamine, and angiotensin II on the integrity of cortical F-actin. Chromaffin cells were incubated in the absence of test agents or with cytochalasin D (2μM, 20 min), PMA (1μM, 10 min), nicotine (100μM, 40s), histamine (100μM, 10 min), or angiotensin II (100nM, 10 min) at 37°C. Following these treatments, the cells were fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under "Materials and Methods". The rhodamine cortical staining of chromaffin cells was analysed and classified as being either continuous or discontinous (see Figure 4.2.5a) and the percentage of cells displaying cortical F-actin disassembly (disrupted cortical rhodamine staining) calculated. Six hundred cells from a total of three different cell cultures were examined. Data shown are mean + SEM. ** $P<0.01$, *** $P<0.001$ vs. control by one-way ANOVA followed by post-hoc Dunnett's test.

.

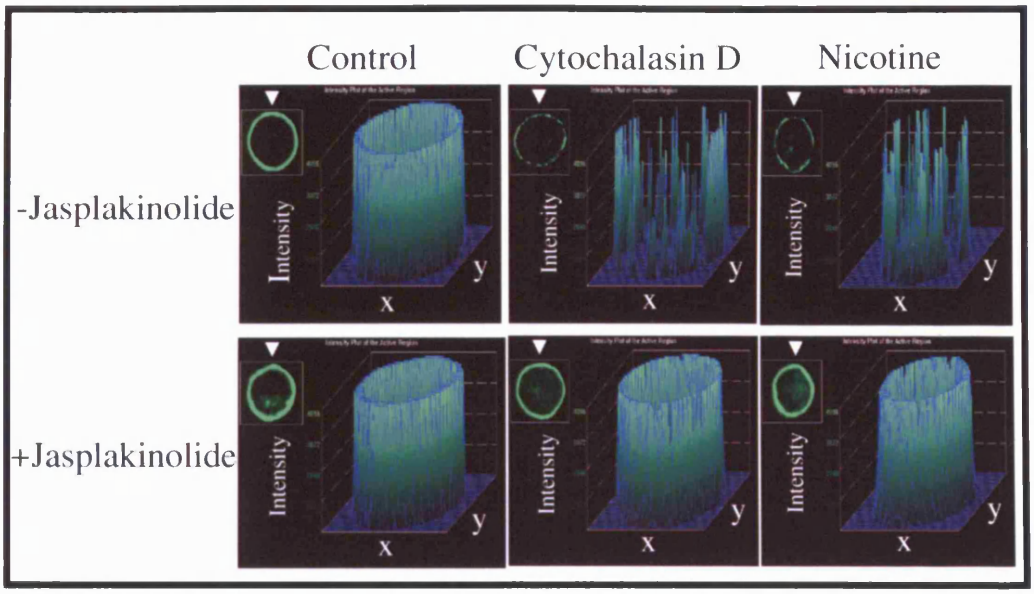


Figure 4.2.6a. Effect of a cortical F-actin stabilizer on cortical F-actin disassembly mediated by either cytochalasin D or nicotine. Chromaffin cells were incubated with or without jasplakinolide (10μM, 30 min) at 37°C before stimulation with either buffer control, cytochalasin D (2μM, 20 min) or nicotine (100μM, 40s). Following these treatments, the cells were fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under "Materials and Methods". The arrows at the left corner of panels point out the confocal image of the cell. Three-dimensional image analysis was performed using "Ultra*VIEW* software" (Perkin-Elmer Life Science, Cambridge, UK) where the maximum intensity of the fluorescent ring was set to 4096 arbitrary units. Images are representative of approximately six hundred cells from three different cell culture preparations. Control cells show an intact cortical ring with a uniform fluorescence intensity pattern. In the absence of jasplakinolide, either nicotine or cytochalasin D show a disrupted cortical ring with irregularities such as patches of fluorescence. However, preincubation of the cells with jasplakinolide essentially prevents the disruption of cortical F-actin by either nicotine or cytochalasin D. Mean data are shown in Figure 4.2.6b.

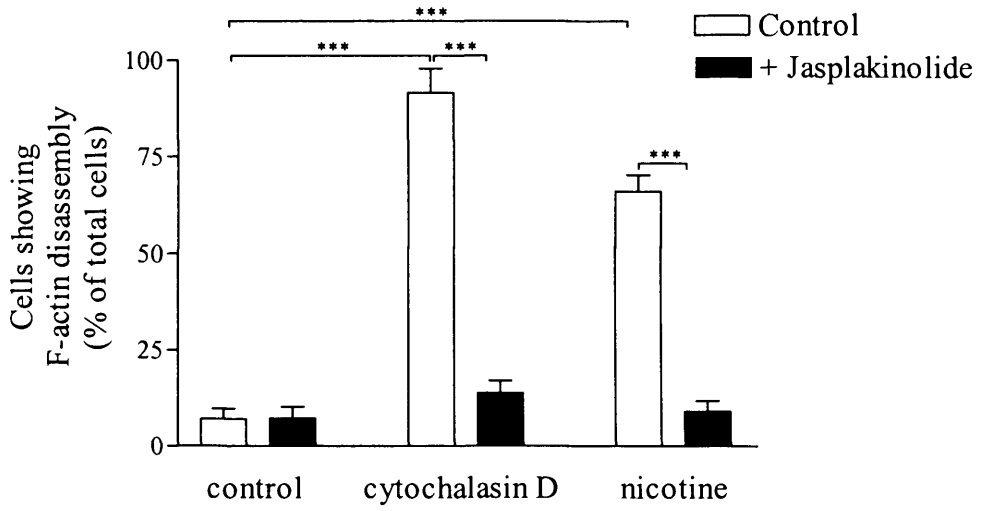


Figure 4.2.6b. Effect of jasplakinolide on cortical F-actin disassembly mediated by either cytochalasin D or nicotine. Chromaffin cells were incubated with either buffer control, cytochalasin D (2μM, 30 min) or nicotine (100μM, 40s) following preincubation with jasplakinolide (10μM, 30 min) at 37°C. Following these treatments, the cells were fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under "Materials and Methods". The rhodamine cortical staining of chromaffin cells was analysed and classified as being either (see Figure 4.2.6a) continuous or discontinuous and the percentage of cells displaying cortical F-actin disassembly (disrupted cortical rhodamine staining) calculated. Six hundred cells from a total of three different cell cultures were examined. Data shown are mean + SEM. *** $P<0.001$ by one-way ANOVA followed by post-hoc Bonferroni's test. (Not all comparisons are shown on the graph).

Effect of the cortical F-actin stabilizer jasplakinolide on secretion of catecholamines evoked by either nicotinic or $G\alpha_{q/11}$-coupled receptors

Prevention of cortical F-actin disassembly by jasplakinolide (10μM, 30 min) (see Figures 4.2.6a and 4.2.5b) significantly reduced secretion of catecholamines mediated by nicotine (100μM, 10 min) (10.5 ± 0.6% vs. 21.0 ± 3.3%, *** $P<0.001$) (Figure 4.2.7). In contrast, catecholamine release induced either by PMA (1μM), histamine (100μM) or angiotensin II (100nM) was not affected by stabilization of cortical F-actin with jasplakinolide (Figure 4.2.7).

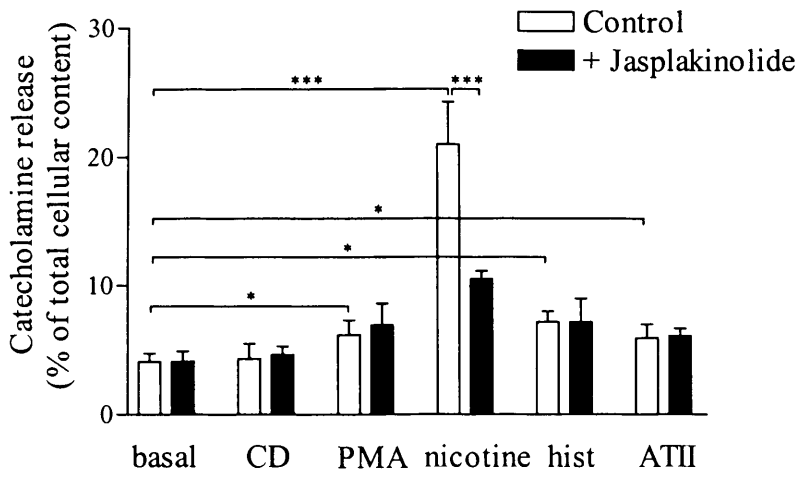


Figure 4.2.7. Effect of the cortical F-actin stabilizer jasplakinolide on catecholamine secretion evoked by either PMA, nicotine, histamine or angiotensin II. Chromaffin cells were incubated with or without jasplakinolide (10μM, 30 min) at 37°C before stimulation with either buffer control, cytochalasin D (2μM, 20 min) (CD), PMA (1μM, 10 min), nicotine (100μM, 10 min), histamine (100μM, 10 min) (hist), or angiotensin II (100nM, 10 min) (ATII). Catecholamine release was measured as described in "Materials and Methods" and is expressed as percentage of total cellular content. Data are mean + SEM from three different cell preparations. ** $P<0.001$, *** $P<0.001$ by one-way ANOVA followed by post-hoc Bonferroni's test. (Not all comparisons are shown on the graph).

4.3 DISCUSSION

4.3.1 Summary of data

The work described in this chapter demonstrates that nicotinic receptor activation caused release of catecholamines that was abolished by removal of extracellular Ca^{2+} and was both time- and concentration-dependent. Activation of $G\alpha_{q/11}$-coupled receptors with either histamine or angiotensin II was able to evoke catecholamine secretion and this was also concentration-dependent. Nicotinic receptor activation caused fragmentation of the cortical F-actin ring whereas stabilization of F-actin with an F-actin stabilizer (jasplakinolide) inhibited both nicotine-mediated fragmentation and catecholamine secretion and this strongly suggests that nicotinic receptor-mediated catecholamine secretion is crucially dependent on the reserve pool to replenish the readily releasable pool with secretory granules. Although, activation of $G\alpha_{q/11}$-coupled receptors with either histamine or angiotensin II mediated disruption of the cortical F-actin filaments, catecholamine secretion evoked by either histamine or angiotensin II was not affected by stabilization of cortical F-actin. Thus, this gives an indication that $G\alpha_{q/11}$-coupled receptor-mediated catecholamine release is independent of the reserve pool.

4.3.2 Effect of K^+ depolarization or the activation of either ligand-gated ion channels or $G\alpha_{q/11}$-coupled receptors on secretion of catecholamines

The bovine adrenal chromaffin cell has been widely used as model to study the dynamics of exocytosis and pools of neurotransmitter-storing vesicles (Burgoyne and Morgan, 1998a; Gillis and Chow, 1997; Morgan and Burgoyne, 1997). It is generally agreed that the adrenal medulla releases catecholamines into the blood stream in response to both splanchnic nerves and humoral stimulation

(Douglas, 1968; Alamo *et al.*, 1991). It has been suggested that although secretion is mainly triggered by nicotinic stimulation, other receptors such as H_1 histamine receptors and AT_1 angiotensin II receptors can also promote secretion of catecholamines from adrenal medullary cells (Marley, 1987; O'Sullivan and Burgoyne, 1989). This study demonstrates that activation of either nicotinic receptors or K^+ evoked depolarisation causes a much greater release of catecholamines than stimulation of $G\alpha_{q/11}$-coupled receptors and this is consistent with previous published work (Marley, 2003). The results presented in this chapter also indicate that nicotine was a more effective secretagogue than KCl. This is in agreement with previous findings (Cox and Parsons, 1997).

It is widely recognized that nicotinic receptor activation results either in membrane depolarisation with the opening of VOCCs in the plasma membrane or Ca^{2+} entery through the nicotinic receptor channel itself (ionotropic receptors) (Zhou and Neher, 1993; Harkins and Fox, 1998). Thus, the additional Ca^{2+} entry through the nicotinic receptor channel itself or the spatial relationship between nicotinic receptors and the secretory machinery could explain why nicotine was a more effective secretagogue than KCl.

This study highlights the importance of external Ca^{2+}, as secretion due to either nicotinic receptor activation or K^+-depolarization was abolished by removal of Ca^{2+}_e. This is in agreement with many other studies demonstrating an essential role of Ca^{2+} in triggering exocytosis from chromaffin cells either in population or single cell studies (Augustine and Neher, 1992; Burgoyne, 1984b; Cheek *et al.*, 1989; Cobbold *et al.*, 1987; Kao and Schneider, 1986; Kim and Westhead, 1989; Stauderman and Pruss, 1989; Stauderman *et al.*, 1990).

A rise in $[Ca^{2+}]_i$ is the main trigger of regulated exocytosis in many cell types. The Ca^{2+} can be derived from entry of external Ca^{2+}, by Ca^{2+} release from intracellular stores, or both. The importance of these two Ca^{2+} sources varies considerably between cell types. This ranges from cells in which Ca^{2+} entry is the only effective stimulus for exocytosis to cells in which Ca^{2+} stores are the predominant source. In adrenal chromaffin cells the release of Ca^{2+} from intracellular stores triggers only a very low level of secretion (Cheek and Burgoyne, 1985; Cheek *et al.*, 1989; Cheek and Thastrup; 1989; O'Sullivan *et al.*, 1989) and this may in part be due to the capacitative (store-operated) Ca^{2+} entry that accompanies emptying of intracellular Ca^{2+} stores (Robinson *et al.*, 1992; Fomina and Nowycky, 1999). Moreover, Ca^{2+}-dependent mechanisms triggered by nicotinic receptor stimulation include activation of PKC (Messing *et al.*, 1989; Vainio *et al.*, 1998; Soliakov and Wonnacott, 2001; Mahata *et al.*, 2002), which may then influence the exocytosis process (see Chapter 5).

Although, the ionotropic nicotinic receptors play a major role in eliciting catecholamine secretion, adrenal chromaffin cells also express $G\alpha_{q/11}$-coupled receptors such as histamine receptors and angiotensin II receptors (see Chapter 3) which also evoke a comparatively small release of catecholamines as shown in this chapter. This finding is supported by evidence in the literature that various GPCR agonists can trigger different amounts of release although nicotinic receptor stimulation evokes the biggest response (Ali and Burgoyne, 1990; O'Sullivan and Burgoyne, 1989; Marley, 2003). Some studies demonstrated that the efficacy of several of these agonists including angiotensin II, bradykinin, ATP and prostaglandins is very low. However, histamine and pituitary adenylate cyclase-activating polypeptide (PACAP) are highly effective and comparable to

powerful secretagogues such as nicotinic agonists and K^+-depolarization (Burgoyne, 1991; Watanabe *et al.*, 1992; Isobe *et al.*, 1993). This is somewhat in contrast to the data in this study, which indicated that stimulation of either histamine receptors or angiotensin II receptors elicited a very low secretory effect compared to the powerful exocytotic effects evoked by either nicotinic receptors or K^+-depolarization. This different amount of release triggered by different secretagogues may be attributed to the spatial organization of Ca^{2+} signals (Cheek *et al.*, 1993) as cytosolic Ca^{2+} concentrations may not be raised in a uniform manner throughout the cytoplasm by secretagogues and the pattern of the Ca^{2+} wave may depend on the pathways through which extracellular Ca^{2+} enter the cells (Kuwashima *et al.*, 2000). Another contributing factor to the low amount of catecholamine secretion evoked angiotensin II is likely to be that angiotensin AT_1 receptors rapidly desensitise (Olivares-Reyes *et al.*, 2001).

The secretory effects of histamine on chromaffin cells are mediated exclusively by H_1 receptors which are highly expressed in the adrenal medulla (Chang *et al.*, 1979). In chromaffin cells as in many other cells, stimulation of H_1 receptors activates PLC via a pertussis toxin-insensitive G-protein and causes the production of $Ins(1,4,5)P_3$ (Noble *et al.*, 1986; Plevin and Boarder, 1988) and the subsequent release of Ca^{2+} from intracellular stores (Stauderman and Pruss, 1990; Stauderman and Murawsky, 1991; Challiss *et al.*, 1991). Such Ca^{2+} released from intracellular stores can cause secretion of catecholamines but this release is transient, lasting less than 1 min and is quantitatively small (Bunn and Boyd, 1992). In contrast, the substantial release of catecholamines evoked by sustained application of histamine is abolished in Ca^{2+}-free solution and reduced by 70-80% following inhibition of VOCCs (O'Farrell and Marley, 1999; Bunn and Boyd,

1992). These studies indicate that histamine H_1 receptors on chromaffin cells are coupled to the activation of VOCCs, predominantly L-, N-, and P/Q-type VOCCs, leading to the influx of extracellular Ca^{2+} and that this responsible for mediating the majority of the secretory effect (Wallace *et al.*, 2002; Marley *et al.*, 2002).

It is well established that angiotensin II type 1 receptors (AT1Rs) are coupled to a plethora of different types of G-protein (Richards *et al.*, 1999). It has been proposed that the stimulatory effects of angiotensin II on secretion are associated with activation of PLC, a store-dependent rise in $[Ca^{2+}]_i$ and activation of PKC (Teschemacher and Seward, 2000).

Although catecholamine secretion evoked by $G\alpha_{q/11}$-coupled receptors would be expected to be concentration-dependent, sigmoidal curves could not be fitted to the concentration-response curves for either histamine or angiotensin II. The concentration-dependence of histamine-evoked catecholamine secretion has been studied by several groups but no consistent EC_{50} value has been reported. The EC_{50} values are approximate for angiotensin II (EC_{50} ~5nM) and consistent with other reported values (Teschemacher and Seward, 2000), whereas the determined EC_{50} value for histamine (EC_{50} ~5μM) was slightly higher than the reported EC_{50} values (100nM-1.9μM) (Houchi *et al.*, 1997; Livett and Marley, 1986; Bunn and Boyd, 1992; Choi *et al.*, 1995; Noble *et al.*, 1988).

The activation of $G\alpha_{q/11}$-coupled receptors did not augment the catecholamine release mediated by either nicotine or KCl suggesting that they may share components of the secretory pathway.

4.3.3 Cortical F-actin disassembly mediated by nicotinic and $G\alpha_{q/11}$-coupled receptor activation

The exocytotic vesicles are present in chromaffin cells in at least two compartments: the release ready vesicle pool and reserve pool (Heinemann *et al.*, 1993; Vitale *et al.*, 1995). The traffic of vesicles between these compartments is subject to fine regulation. Previous studies have suggested that an F-actin microfilament network plays an important role in this regulation (Vitale *et al.*, 1991; 1995). It is evident that in chromaffin cells, F-actin forms a cortical network which excludes the large majority of secretory vesicles from plasma membrane docking (Burgoyne *et al.*, 1982; Vitale *et al.*, 1995). Therefore, the F-actin network acts as a barrier (negative clamp) blocking the access of secretory vesicles to exocytotic sites at the plasma membrane. In resting chromaffin cells, 1%-3% of the total chromaffin vesicles are either docked to the plasma membrane or within 50nm of it (Vitale *et al.*, 1995). This population of vesicles constitutes the release-ready vesicle pool (Neher and Zucker, 1993; Vitale *et al.*, 1995). The rest of the chromaffin vesicles (97%-99%) form a reserve pool and remain behind a barrier of cortical F-actin (Vitale *et al.*, 1995). Stimulation of chromaffin cells is accompanied by disassembly of the cortical F-actin network (Cheek and Burgoyne, 1986; Vitale *et al.*, 1995). This allows the movement of additional secretory vesicles from the reserve pool to release sites on the plasma membrane (Vitale *et al.*, 1995). Thus, the cortical actin network controls the size of the release-ready vesicle pool and consequently, the initial rate of exocytosis (Vitale *et al.*, 1995).

In this study confocal microscopy of rhodamine-phalloidin-stained cells was used to assess the integrity of cortical F-actin. Rhodamine-labelled phalloidin

was used as a probe for F-actin (Vitale *et al.*, 1991) and disappearance of cortical sub-plasmalemmal rhodamine fluorescence was considered as an index of disassembly of actin filaments. As demonstrated in this study, disassembly was evoked by nicotinic receptor stimulation and this finding is consistent with a previous study showing that the number of secretory vesicles close to the membrane increased following nicotinic receptor stimulation (Burgoyne *et al.*, 1982). Thus, disruption of cortical F-actin would result in an increase in the size of the readily releasable pool of secretory granules (Vitale *et al.*, 1995) allowing enhanced exocytosis of catecholamines. This study highlights the importance of cortical F-actin disassembly in the secretory process mediated by nicotinic receptors since jasplakinolide stabilizes F-actin (Oheim and Stuhmer, 2000) and prevents its disruption leading to inhibition of the recruitment of secretory granules to the site of exocytosis. These data strongly suggest that nicotinic receptor-mediated catecholamine secretion is dependent on the reserve pool to replenish the readily releasable pool with secretory granules to allow exocytosis to occur and this was consistent with previous findings (Cheek and Burgoyne, 1986; Vitale *et al.*, 1991; 1995; Trifaró *et al.*, 2000). In addition, this also could be a logical explanation for the large amount of catecholamine secretion evoked by nicotinic receptor activation. This study shows that although activation of either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors mediates disassembly of cortical F-actin, this is apparently only crucial for secretion mediated by nicotinic receptors, since stabilization of cortical F-actin failed to prevent secretion of catecholamines evoked by either histamine or angiotensin II. This could be attributed to the ability of $G\alpha_{q/11}$-coupled receptors to evoke release from the pre-docked vesicles in the readily releasable pool. This could also explain the low

amount of catecholamine secretion triggered by $G\alpha_{q/11}$-coupled receptors. Similarly, this study demonstrated that F-actin disassembly mediated by either cytochalasin D, an F-actin destabilizer (Brenner and Korn, 1979, Flanagan and Lin, 1980; Schliwa, 1982) and PMA failed to trigger secretion of catecholamines and this was in agreement with previous findings that cortical F-actin disassembly modifies the extent of secretion but is itself not sufficient to activate exocytosis (Burgoyne and Cheek, 1987; Vitale *et al.*, 1995; Cuchillo-Ibãñez *et al.*, 2004).

It has been suggested that in chromaffin cells, the cortical actin network is controlled by scinderin, a Ca^{2+}-dependent F-actin severing protein (Rodríguez Del Castillo *et al.*, 1990). In addition to scinderin, PKC-MARCKS pathway may also control the cortical actin network (Trifaró *et al.*, 2000) (see Chapter 5).

The link between nicotinic-mediated catecholamine release and that mediated by $G\alpha_{q/11}$-coupled receptors is the elevation of $[Ca^{2+}]_i$. Increased cytosolic Ca^{2+} plays several roles including driving granule fusion with the plasma membrane through a Ca^{2+} sensor possibly a member of the synaptotagmin family (Burgoyne and Morgan, 1998b). Additionally, activation of Ca^{2+} entry at the plasma membrane may trigger fusion of vesicles docked close to the Ca^{2+} channels or promote vesicle priming through effects on Ca^{2+}-binding proteins such as DOC2, rabphillin or CAPS (Benfenati *et al.*, 1999; Elhamdani *et al.*, 1999). Furthermore, Ca^{2+} may disrupt the F-actin network located below the plasma membrane (Vitale *et al.*, 2000; Doussau and Augustine, 2000) as Ca^{2+} released from $Ins(1,4,5)P_3$-sensitive stores may act locally to produce actin disassembly through activation of Ca^{2+}-sensitive actin-severing proteins, leading to vesicle recruitment from the reserve pool to the release-ready vesicle pool (Zhang *et al.*, 1995b). Moreover, generation of DAG at the plasma membrane

may increase vesicle docking and priming by activation of PKC which leads to phosphorylation of cytoskeletal proteins controlling vesicle recruitment to the release-ready vesicle pool (Vitale *et al.*, 1995) (see Chapters 5 and 6), and/or phosphorylation of proteins that regulate SNARE complex formation (Turner *et al.*, 1999). Additionally, DAG may also activate Munc-13 directly to regulate SNARE complex formation (Turner *et al.*, 1999).

Since the role of PKC in neurotransmitter evoked by nicotinic receptors and $G\alpha_{q/11}$-coupled receptors is still a matter of debate, the role of PKC in exocytosis mediated by either nicotinic receptors and $G\alpha_{q/11}$-coupled receptors will be investigated in the next chapter.

5: THE ROLE OF PKC IN AGONIST-MEDIATED RELEASE OF CATECHOLAMINES FROM CHROMAFFIN CELLS

5.1 INTRODUCTION

Within this chapter the major aims were to determine the specific PKC isoforms present in chromaffin cells and activated in response to stimulation of the different receptors. Further, these studies were designed to explore the roles of the different isoforms in the regulation of exocytosis and determine the potential nicotinic receptor-mediated activation of MARCKS as a possible link between PKC activation, disruption of the cortical F-actin cytoskeleton and PKC-dependent enhanced catecholamine release (see Chapter 1 for details).

More than one PKC isoform is usually expressed in a single cell type, thus leading to the notion that each member of the PKC superfamily plays a specific role in the processing of physiological and pathophysiological responses to extracellular stimuli (Sena *et al.*, 2001). PKC is generally in the cytosol in the inactive state but after cell stimulation translocates to the plasma membrane where it becomes activated in the presence of specific lipid co-factors. Translocation of PKC to the plasma membrane is considered a hallmark for activation (Mochly-Rosen *et al.*, 1990). In addition, it is evident that specific PKC isoforms can also translocate to other subcellular locations including the membrane of other vesicles, nuclear structures and cytoskeletal components.

Translocation of PKC from the cytosolic to membrane fraction cell has been observed following activation of $G\alpha_{q/11}$-coupled receptors that mediate PtdIns(4,5)P_2 breakdown (Terbush and Holz, 1986; Willars *et al.*, 1996; Sena *et al.*, 1996; Teschemacher and Seward, 2000; Bartlett *et al.*, 2005). Although, the traditional view that $G\alpha_{q/11}$-coupled receptor activation is required for PKC

activation, there is evidence for activation of PKC by ligand-gated ion channels including nicotinic receptors (Mollard *et al.*, 1995; Vainio *et al.*, 1998; Soliakov and Wonnacott, 2001; Roberts-Thomson *et al.*, 2000). There have been a number of approaches to defining precisely which PKC isoforms are activated in response to particular stimuli including the determination of cytosol to membrane translocation by Western blotting of cell fractions. Similarly, immunocytochemical studies with isoform-specific antibodies have been used to determine the subcellular localization of PKC isoforms (Goodall *et al.*, 1997; Buchner *et al.*, 1999). In addition, cells can be transfected to express fusion constructs of enhanced green fluorescent protein (eGFP) and PKC, which allows the dynamics of PKC translocation in response to different stimuli to be monitored in live cells in real time (Sakai *et al.*, 1997; Shirai *et al.*, 1998). Studies described within this chapter use this range of techniques to address the issue of which PKC isoforms are activated. (Cubitt *et al.*, 1995; Sakai *et al.*, 1997). Although, a great many previous studies have used phorbol esters (Blumberg *et al.*, 1984; Terbush and Holz, 1990; Ryves *et al.*, 1991; Tanaka and Nishizuka, 1994; Nishizuka, 1995; Sena *et al.*, 1996; Sakai *et al.*, 1997; Mochly-Rosen and Gordon, 1998), this may lack specificity and result in a massive and uncontrolled stimulation of PKC that may not be physiologically relevant. The current study specifically seeks to determine receptor/agonist-dependent PKC activation.

The work described within this chapter focus on the role of PKC in nicotinic receptor- and $G\alpha_{q/11}$-coupled receptor-mediated secretion of catecholamines in chromaffin cells. In order to pursue this there were three specific aims. Firstly, to identify the PKC isoforms expressed in bovine chromaffin cells; secondly, to identify which PKC isoforms are activated in

response to stimulation of either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors. Thirdly, to determine the impact of PKC activation on the integrity of cortical F-actin and finally to assess whether there was any evidence for an involvement of PKC-mediated phosphorylation of MARCKS in PKC-mediated effects on the cortical F-actin cytoskeleton.

5.2 RESULTS

Identification of PKC isoforms in bovine chromaffin cells

Immunoblotting of whole cell extracts for PKC isoform protein demonstrated that PKCα, PKCβ, PKCε and PKCι were present (Figure 5.2.1). Antibodies to PKCγ, PKCθ, PKCδ, PKCη and PKCλ failed to locate any immunoreactive protein at the predicted molecular weight when used in immunoblots of whole chromaffin cell lysate (Figure 5.2.1, lane A).

The immunoreactivity of all PKC antibodies was verified using rat brain lysate (Figure 5.2.1, lane B) and bovine brain extract (Figure 5.2.1, lane C). Using a similar protein concentration of bovine and rat brain to that of the bovine chromaffin cell extract (30μg protein.lane^{-1}), antibodies to PKC-γ, -θ, -δ, -η and λ detected bands at the appropriate molecular weights, confirming the ability of the antibodies to detect these bovine isoforms.

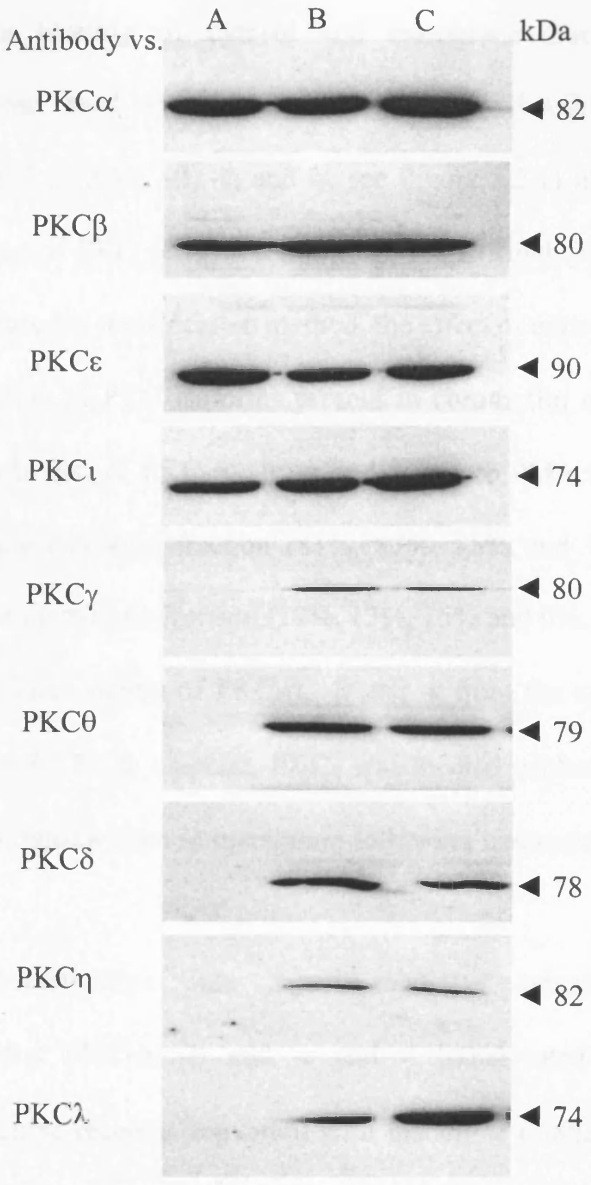


Figure 5.2.1. PKC isoform expression in bovine chromaffin cells. Lane A) chromaffin cell whole lysate (30μg protein.lane^{-1}) probed with a range of antibodies demonstrated the presence of PKC-α, -β, -ε and -ι isoforms. The immunoreactivity of all PKC antibodies was confirmed using rat brain lysate (30μg.lane^{-1}) (lane B) and bovine brain extract (30μg.lane^{-1}) (lane C) as positive controls. Immunoblots of chromaffin cell whole lysate are representative of five experiments (three different cell culture preparations) whereas immunoblots of either rat brain lysate or bovine brain extract are representative of three independent experiments (using the same frozen rat or bovine brain extracts).

Agonist-mediated translocation of PKC isoforms

Western blotting of cytosol and membrane fractions followed by densitometry was used to assess agonist-evoked redistribution of detectable isoforms of PKC (PKC-α, -β, -ε, and -ι, see Figure 5.2.1) in bovine chromaffin cells as an index of PKC activation (Newton, 1997; Mochly-Rosen and Gordon, 1998). To validate the translocation method, the effect of exposure to PMA (1μM) on the distribution of PKC isoforms present in chromaffin cells was examined. The basal distribution of PKC-α, -β, -ε and -ι before PMA treatment was more prominent in the cytosolic fraction (81%, 87%, 85% and 100%, respectively) compared to the membrane fraction (19%, 13%, 15% and 0%, respectively). PMA resulted in the translocation of PKC-α, -β and -ε from the cytosol to membrane fraction (Figure 5.2.2). In contrast, PKCι was located exclusively in the cytosol and showed no translocation to membrane following treatment with PMA (Figure 5.2.2).

This investigation into agonist-mediated translocation of PKC demonstrated that PKC-α, -β and -ε and -ι translocated to the membrane following histamine receptor activation with histamine (100μM). Histamine was used at 100μM as this concentration was previously shown to induce maximal Ca^{2+} responses in these cells (see Chapter 2). The rapid loss of PKCε from the cytosol to the membrane after 1min of histamine treatment was more profound than loss of PKCβ, PKCα and PKCι (Figure 5.2.3: panel A and B). Indeed, following 1 min of stimulation with histamine, PKCε was the only isoform to show a significant translocation (Figure 5.2.3). The pattern of loss of the PKC isoforms from the cytosol was mirrored by changes in the immunoreactivities within the membrane fraction (Figure 5.2.3: panel C and D). Stimulation with

another $G\alpha_{q/11}$-coupled receptor agonist, angiotensin II (100nM) (this concentration was also shown to evoke maximal Ca^{2+} responses in these cells, see Chapter 2) did not cause a significant loss of either PKCβ or PKCι from the cytosol. Furthermore, recruitment of PKCα to the membrane fraction was only significant after 10 min incubation with angiotensin II. In contrast, there was a complete loss of PKCε from the cytosol at 10 min (Figure 5.2.4: panel A and B). In addition, the membrane bound PKCε dramatically increased between 2 and 10 min of stimulation (Figure 5.2.4: panel C and D).

Activation of nicotinic receptors with nicotine (100μM) (a concentration chosen based on its ability to evoke maximal Ca^{2+} responses in these cells, see Chapter 2) caused a translocation of PKC-α, -β and -ε to the plasma membrane. The membrane association of PKCε was greater than that of PKCα and PKCβ (Figure 5.2.5: panel C and D). In contrast, PKCι showed no significant translocation from the cytosolic fraction over a 10 min stimulation period (Figure 5.2.5: panel A and B).

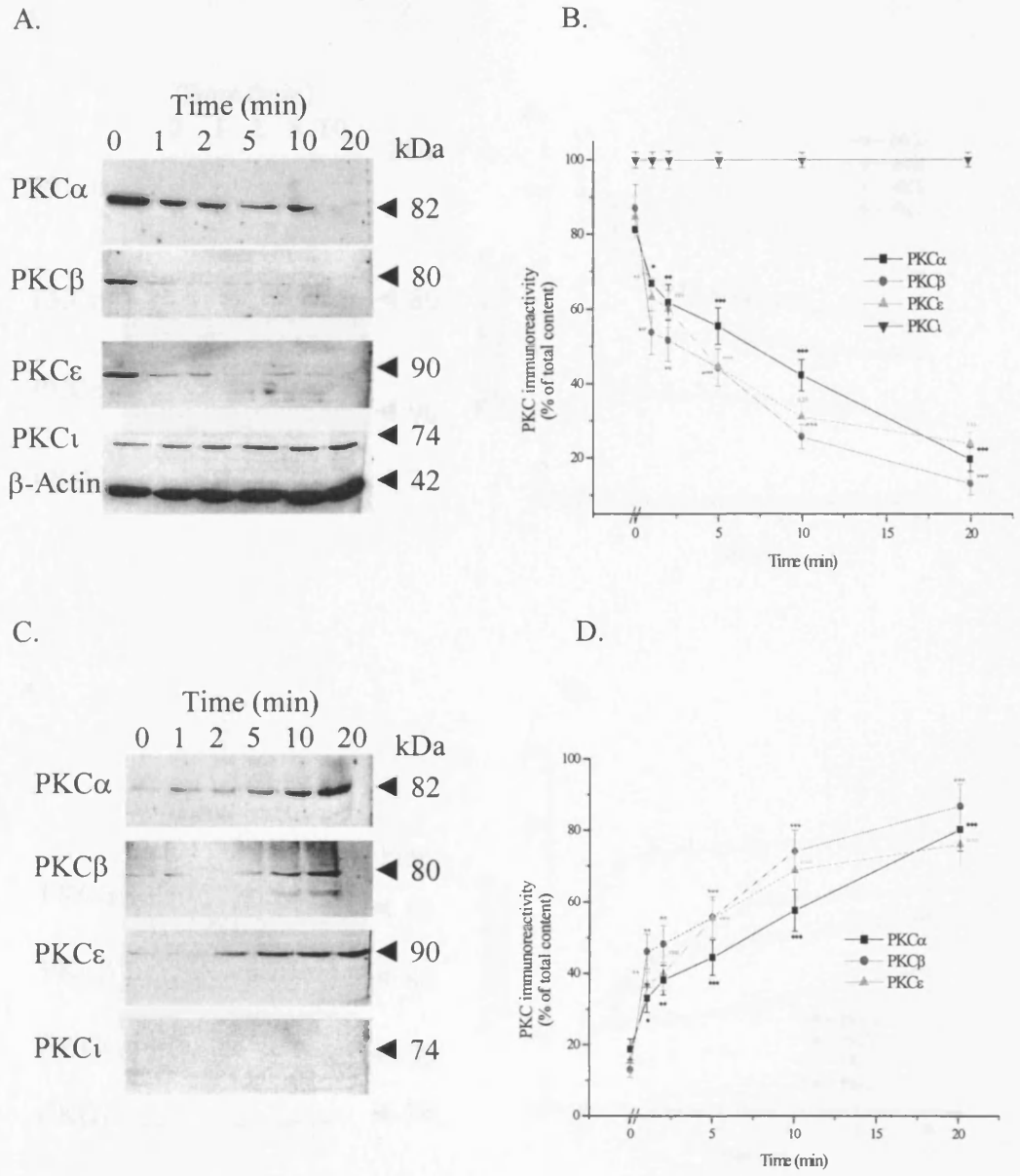


Figure 5.2.2. PMA-mediated translocation of PKC isoforms. A, C: immunoblots showing the effect of exposure to PMA (1μM) on PKC-α, -β, -ε, and -ι associated with either the cytosol (A) or membrane (C) fractions from cultured bovine adrenal chromaffin cells. Cells were treated for the indicated times after which membrane and cytosol fractions were prepared and immunoblotted for the PKC isoforms. The densities of the immunoblots in cytosolic (B) and membrane (D) fractions were quantified using Scion Image analysis software (v4.02 beta, Frederick, Maryland, USA). Cytosolic and membrane values were calculated as a percentage of the total content (i.e. membrane plus cytosolic). Immunoblots (A, B) representative of four experiments on different cell cultures. Mean data (C, D) are mean ± SEM, n=4. For * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by one-way ANOVA followed by Dunnett's test vs. zero time. The cytosolic fraction was probed with β-actin to confirm equal loading.

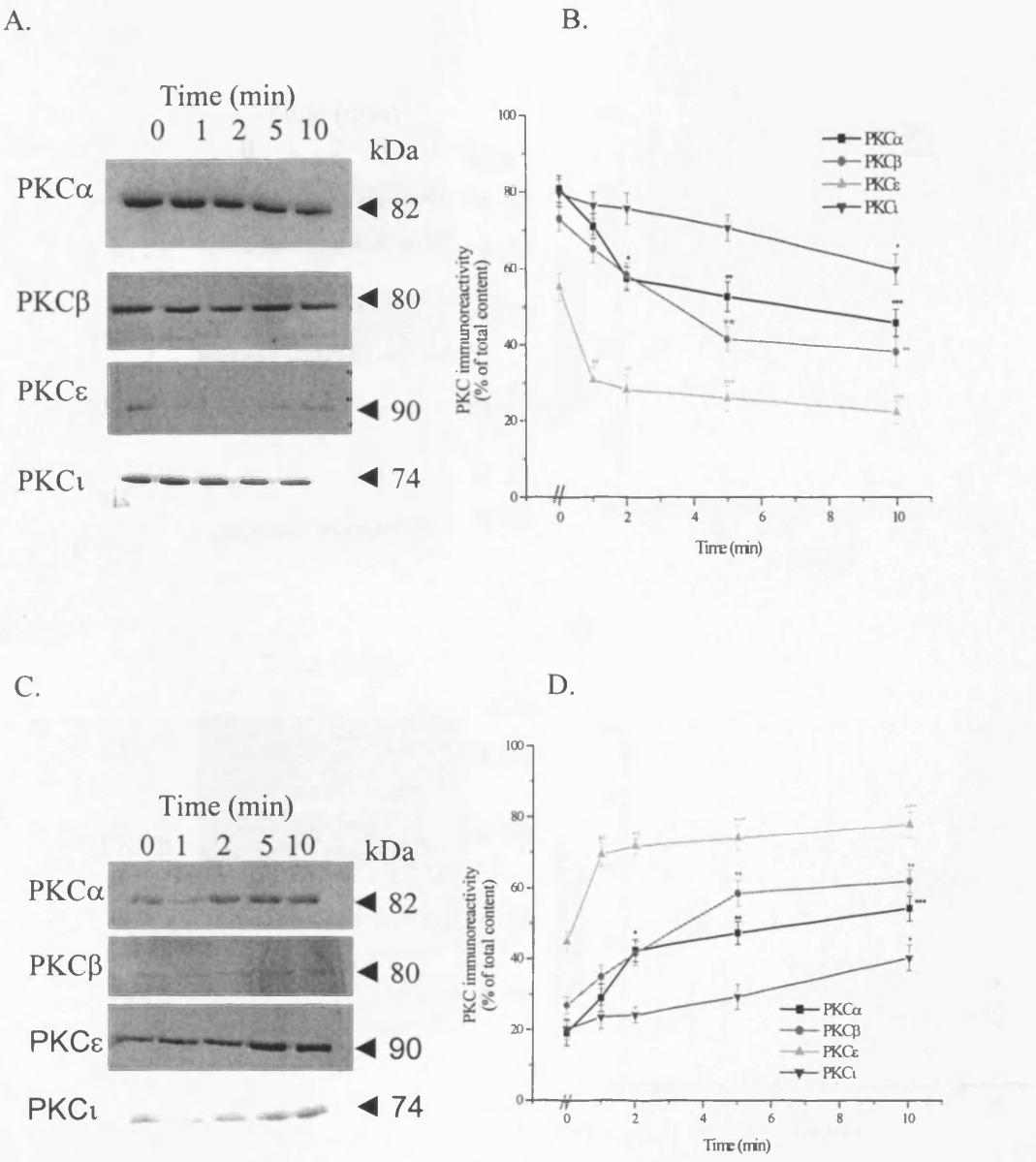


Figure 5.2.3. Histamine-mediated translocation of PKC isoforms. A, C: immunoblots showing the effect of exposure to histamine (100μM) on PKC-α, -β, -ε, and -ι associated with either the cytosol (A) or membrane (C) fractions from cultured bovine adrenal chromaffin cells. Cells were treated for the indicated times after which membrane and cytosol fractions were prepared and immunoblotted for PKC isoforms. The densities of the immunoblots in cytosolic (B) and membrane (D) fractions were quantified using Scion Image analysis software (v4.02 beta, Frederick, Maryland, USA). Cytosolic and membrane values were calculated as a percentage of the total content (i.e. membrane plus cytosolic). Immunoblots (A, B) representative of four experiments on different cell cultures. Mean data (C, D) are mean ± SEM, n=4. For * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by one-way ANOVA followed by Dunnett's test vs. zero time.

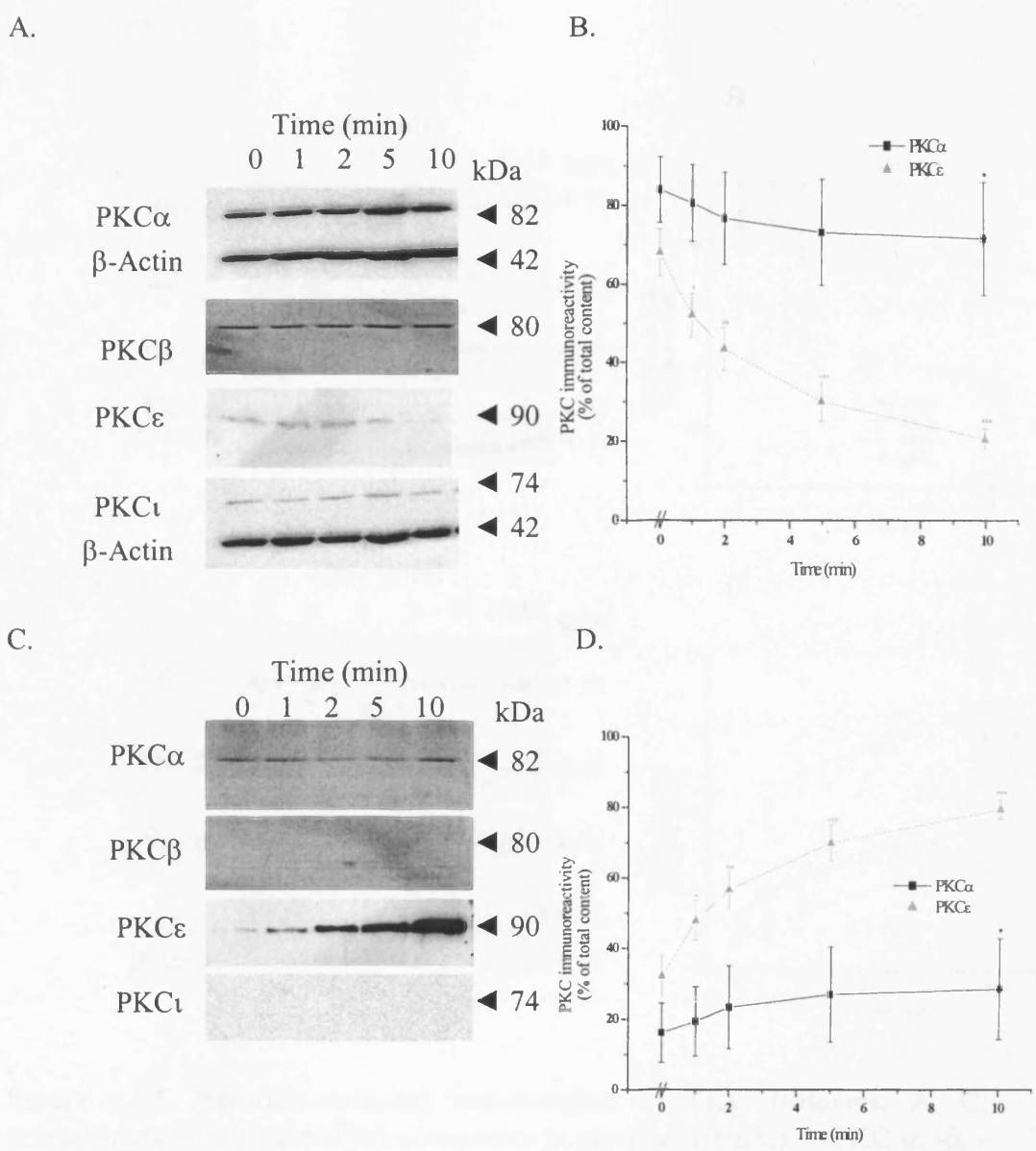


Figure 5.2.4. Angiotensin II-mediated translocation of PKC isoforms. A, C: immunoblots showing the effect of exposure to angiotensin II (100nM) on PKC-α, -β, -ε and -ι associated with either the cytosolic (A) or membrane (C) fractions from cultured bovine adrenal chromaffin cells. Cells were treated for the indicated times after which membrane and cytosol fractions were prepared and immunoblotted for PKC isoforms. The densities of the immunoblots in cytosolic (B) and membrane (D) fractions were quantified using Scion Image analysis software (v4.02 beta, Frederick, Maryland, USA). Cytosolic and membrane values were calculated as a percentage of the total content (i.e. membrane plus cytosolic). Immunoblots (A, B) representative of four experiments on different cell cultures. Mean data (C, D) are mean ± SEM, n=4. For * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by one-way ANOVA followed by Dunnett's test vs. zero time. Note that the distribution of PKC-β and -ι was exclusively associated with the cytosolic fraction throughout and have therefore been omitted from the graphs for clarity. The cytosolic fraction was probed with β-actin to confirm equal loading.

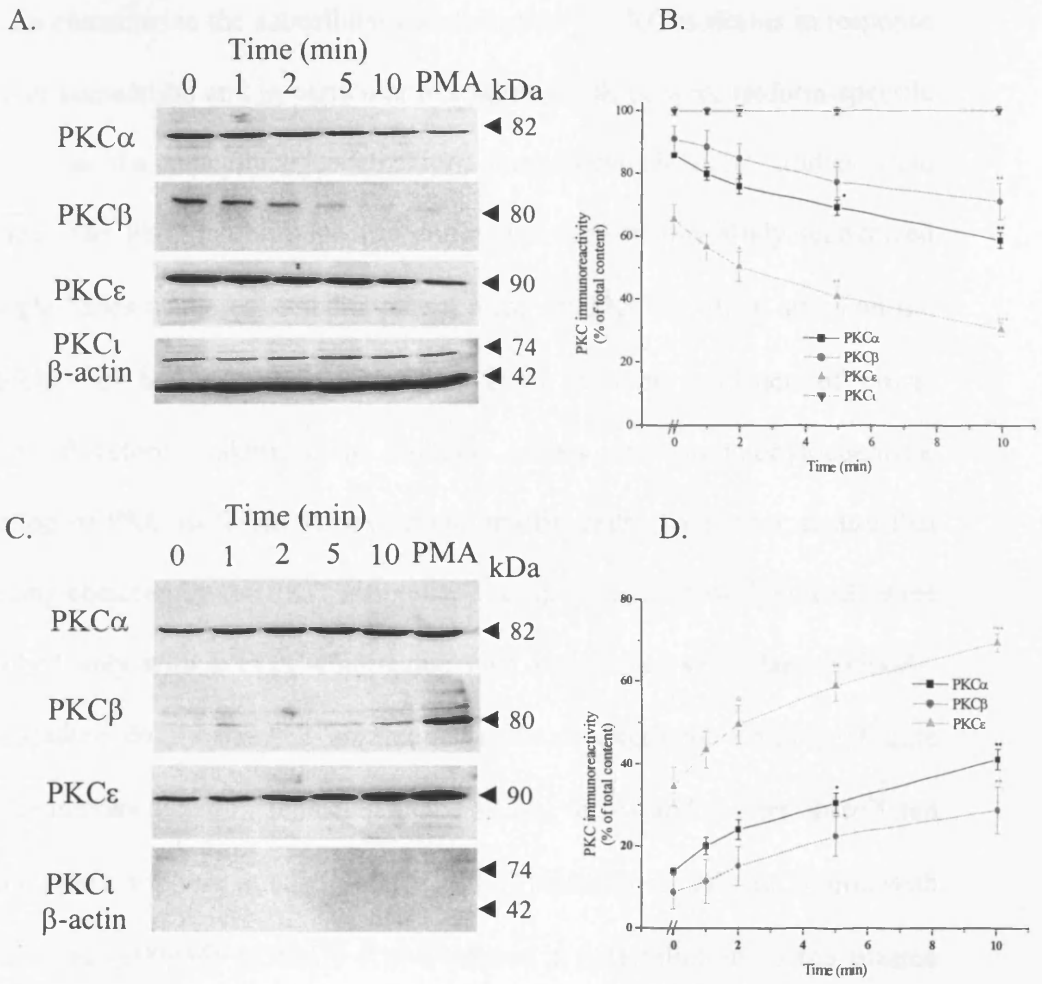


Figure 5.2.5. Nicotine-mediated translocation of PKC isoforms. A, C: immunoblots showing the effect of exposure to nicotine (100μM) on PKC-α, -β, -ε and -ι associated with either the cytosolic (A) or membrane (C) fractions from cultured bovine adrenal chromaffin cells. Cells were treated for the indicated times after which membrane and cytosol fractions were prepared and immunoblotted for PKC isoforms. The densities of the immunoblots in cytosolic (B) and membrane (D) fractions were quantified using Scion Image analysis software (v4.02 beta, Frederick, Maryland, USA). Cytosolic and membrane values were calculated as a percentage of the total content (i.e. membrane plus cytosolic). Immunoblots (A, B) representative of four experiments on different cell cultures. Mean data (C, D) are mean ± SEM, n=4. For * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by one-way ANOVA followed by Dunnett's test vs. zero time. The cytosolic and membrane fractions were probed with β-actin antibody to confirm equal loading and exclude contamination of membrane fraction with cytosol during the cell fractionation process.

Agonist-mediated subcellular redistribution of PKC isoforms

To characterize the subcellular redistribution of PKC isoforms in response to receptor stimulation and in particular to examine if there were isoform-specific differences in the subcellular localization, immunocytochemical studies were performed. The PKC isoform-specific antibodies used in this study recognized only single bands at the expected apparent mass for PKC-α, -β, -ε and ι on the immunoblots of bovine chromaffin cells. There was no evidence of cross-reactivity, therefore making them suitable probes for immunocytochemical localization of PKC isoforms in bovine chromaffin cells. To further ensure that the staining obtained by the PKC antibodies was specific, control fixed cells were also probed only with a FITC-conjugated goat anti-mouse secondary antibody. This procedure did not reveal any non-specific, background staining (Figure 5.2.6). Immunolocalization indicated that PKC-α, -ε, -β and ι were distributed throughout the cytoplasm in unstimulated cells. Treatment of cells for 10 min with either nicotine (100μM) or PMA (1μM) caused a redistribution to the plasma membrane of PKC-α, -ε and -β but not PKCι. Stimulation of $G\alpha_{q/11}$-coupled histamine receptors with histamine (100μM) for 10 min resulted in the redistribution of all PKC isoforms (PKC-α, -ε, -β and -ι) in chromaffin cells to the plasma membrane. In contrast, activation of $G\alpha_{q/11}$-coupled angiotensin receptors with angiotensin II (100nM) for 10 min caused the redistribution of only PKC-α and -ε to the plasma membrane (Figure 5.2.6) (PKC expression and localization are summarized in Table 5.2.1).

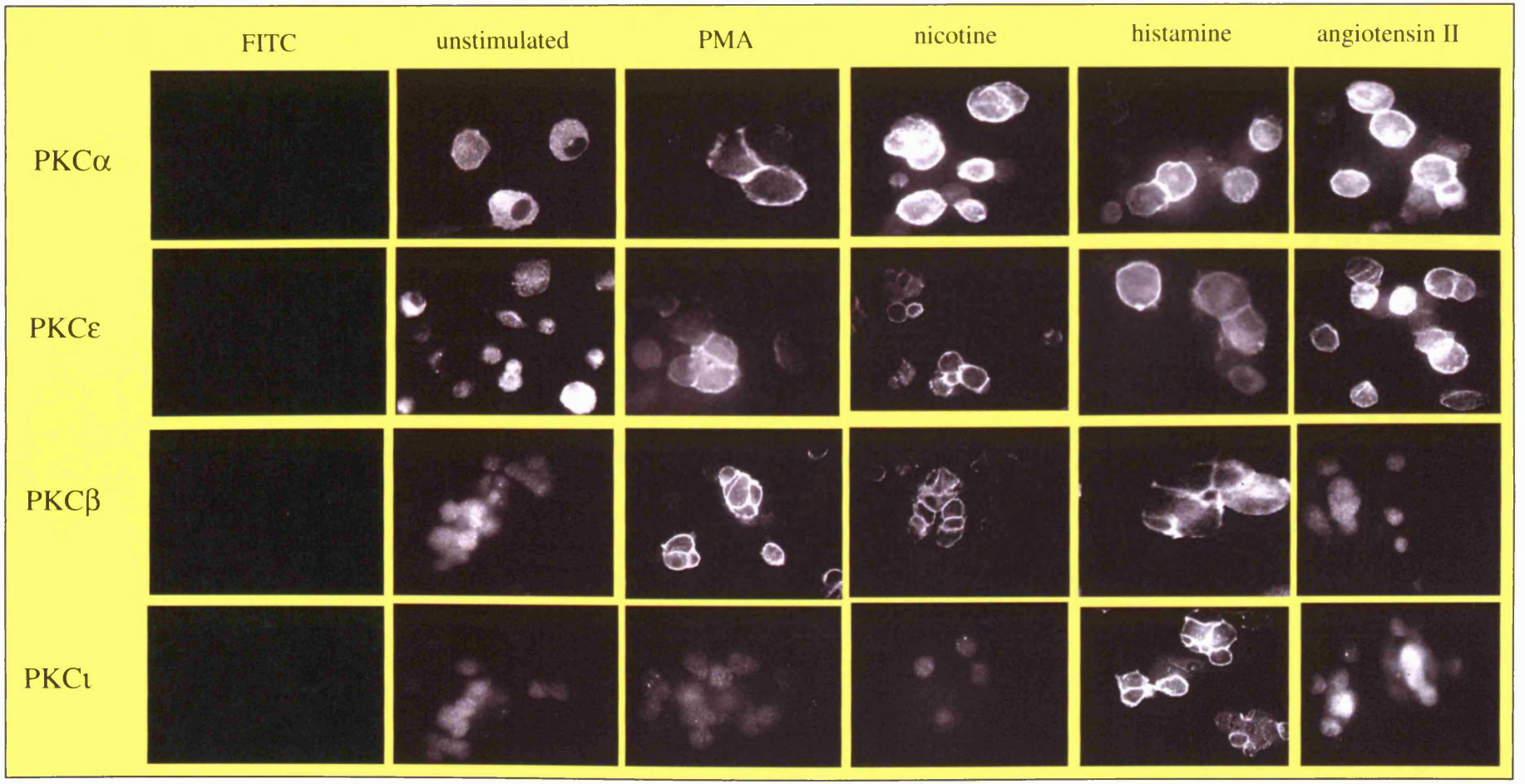


Figure 5.2.6. Immunolocalization of PKC-α, -ε, -β and ι in cultured bovine adrenal chromaffin cells. Cells cultured on coverslips, were fixed and processed for immunocytochemistry as described in "Materials and Methods". PKC -α, -ε, -β and ι were distributed throughout the cytoplasm before stimulation with agonists. Cells treated for 10 min with PMA (1μM), nicotine (100μM), or angiotensin II (100nM) caused a redistribution of PKC-α, -ε and -β to the plasma membrane while treatment of the cells with histamine (100μM) for 10 min caused a redistribution of PKC-α, -ε, -β and -ι to the plasma membrane. Unstimulated cells were also probed only with a FITC-conjugated goat anti-mouse secondary antibody to ensure specificity of the immunochemical reactivity. Pictures are representative of four different cell culture preparations.

Table 5.2.1 Summary of the expression of PKC isoforms and their translocation to the plasma membrane in response to PMA or receptor activation in bovine adrenal chromaffin cells.

PKC isoform	Localisation in unstimulated cells (immunoblot)	Cytosol to membrane translocation			
		PMA (1μM)	Histamine (100μM)	ATII (100nM)	Nicotine (100μM)
α	cytosol: 83% membrane: 17%	+	+	+	+
β	cytosol: 88% membrane: 12%	+	+	-	+
ε	cytosol: 68% membrane: 37%	+	+	+	+
ι	cytosol: 100% membrane: 0%	-	+	-	-

Data are compiled from Figures 5.2.2, 5.2.3, 5.2.4, 5.2.5 and 5.2.6. Note that these data are compiled from both immunoblots and immunocytochemistry.
+ indicates translocation, - indicates no translocation.
Based on 10 min stimulation.

Agonist-mediated translocation of eGFP-PKCβII and eGFP-PKCδ

Whilst the immunoblotting and immunocytochemical approaches give valuable information on the translocation and activation of PKC isoforms, these techniques have limited temporal resolution. To overcome this, fusion constructs between eGFP (Tsien, 1998) and either PKCβII (Feng and Hannun, 1998) or PKCδ (Chiesa *et al.*, 2001) were used to monitor the dynamics of membrane translocation of PKC isoforms in real time in living cells. These isoforms represent the conventional and novel classes of PKC isoforms, respectively. Even though PKCδ is not present in bovine adrenal chromaffin cells, its availability was used to enable monitoring of the activation of novel PKC isoforms. To examine whether activation of either $G\alpha_{q/11}$-coupled receptors or nicotinic receptors recruits either eGFP-PKCβII or eGFP-PKCδ to the plasma membrane, cells were transfected with either eGFP-tagged PKCβII or PKCδ. In unstimulated cells eGFP-PKCβII and eGFP-PKCδ were distributed uniformly throughout the cytoplasm and exclude from the nucleus. Application of PMA (1μM) caused a slow (50s) and permanent fluorescence movement towards the edges of the cell reflecting translocation of either eGFP-PKCβII (Figure 5.2.7) or eGFP-PKCδ (Figure 5.2.11) to the plasma membrane.

Stimulation with histamine (100μM) induced a rapid but transient translocation of either eGFP-PKCβII (Figure 5.2.8) or eGFP-PKCδ (Figure 5.2.12) from the cytosol to the plasma membrane. Similarly, angiotensin II (100nM) induced recruitment of either eGFP-PKCβII (Figure 5.2.9) or eGFP-PKCδ (Figure 5.2.13) to the plasma membrane. Following the initial translocation to the plasma membrane there was some reversal in the continued presence of

angiotensin II, although there was clearly a sustained movement (Figures 5.2.9 and 5.2.13). Nicotinic receptor stimulation with nicotine (100μM) mediated a rapid and sustained recruitment of either eGFP-PKCβII (Figure 5.2.10) or eGFP-PKCδ (Figure 5.2.14) to the plasma membrane. The mean data reflecting the reduction in cytosolic fluorescence mediated by these agonists are summarized in Table 5.2.2.

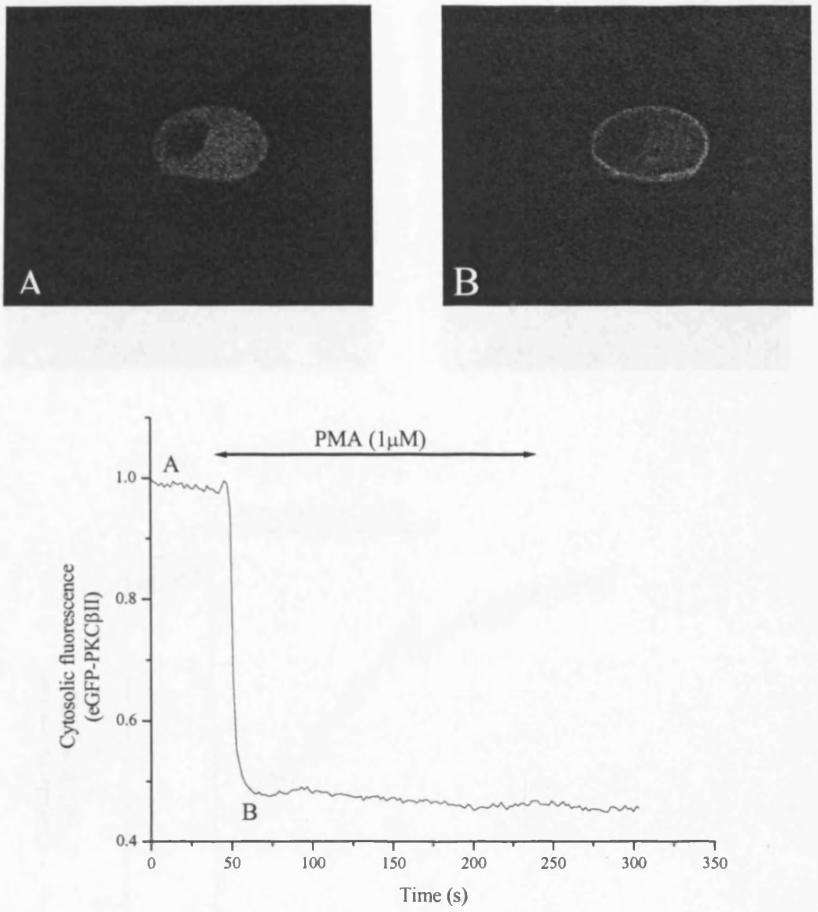


Figure 5.2.7. Single cell imaging of PMA-induced translocation of eGFP-PKCβII. Chromaffin cells transiently transfected with eGFP-PKCβII were perfused with 1μM PMA and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 25 cells).

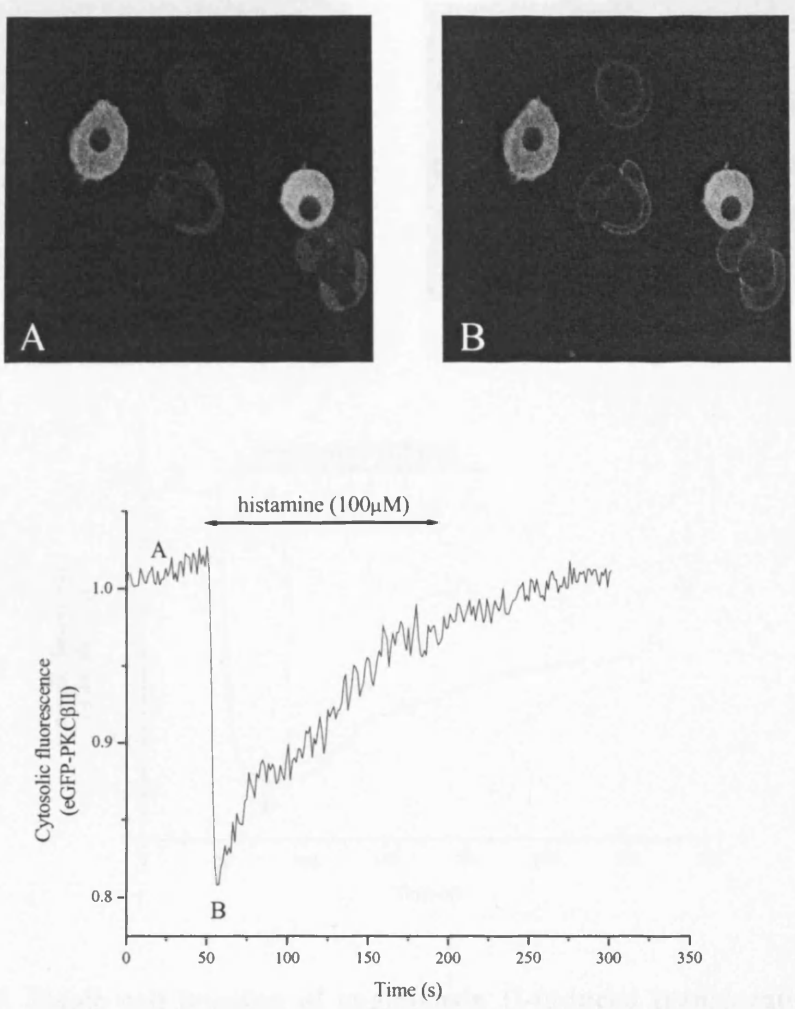


Figure 5.2.8. Single cell imaging of histamine-induced translocation of eGFP-PKCβII. Chromaffin cells transiently transfected with eGFP-PKCβII were perfused with 100μM histamine and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 30 cells).

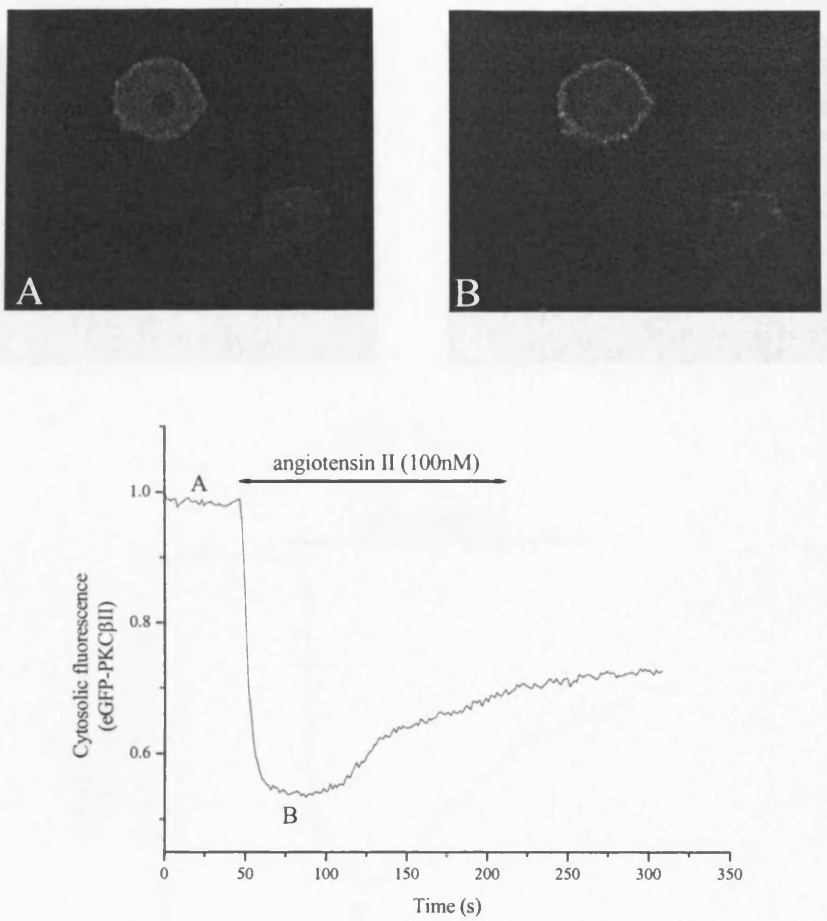


Figure 5.2.9. Single cell imaging of angiotensin II-induced translocation of eGFP-PKCβII. Chromaffin cells transiently transfected with eGFP-PKCβII were perfused with 100nM angiotensin II and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 35 cells).

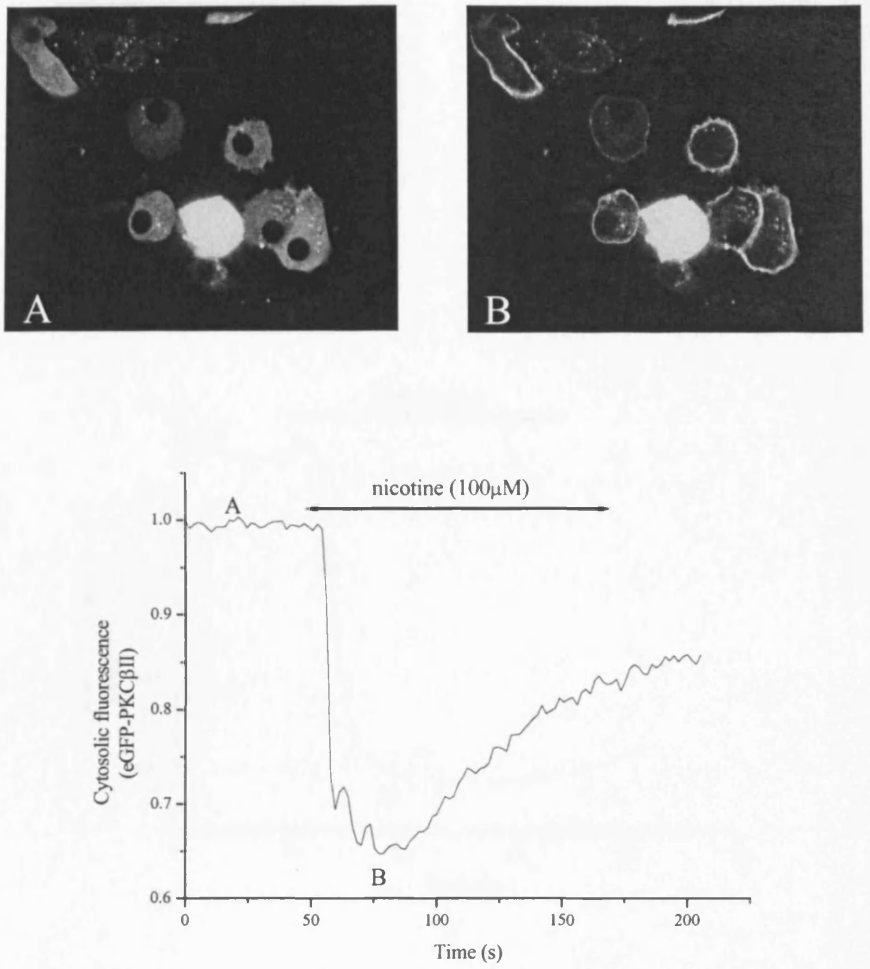


Figure 5.2.10. Single cell imaging of nicotine-induced translocation of eGFP-PKCβII. Chromaffin cells transiently transfected with eGFP-PKCβII were perfused with 100μM nicotine and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 32 cells).

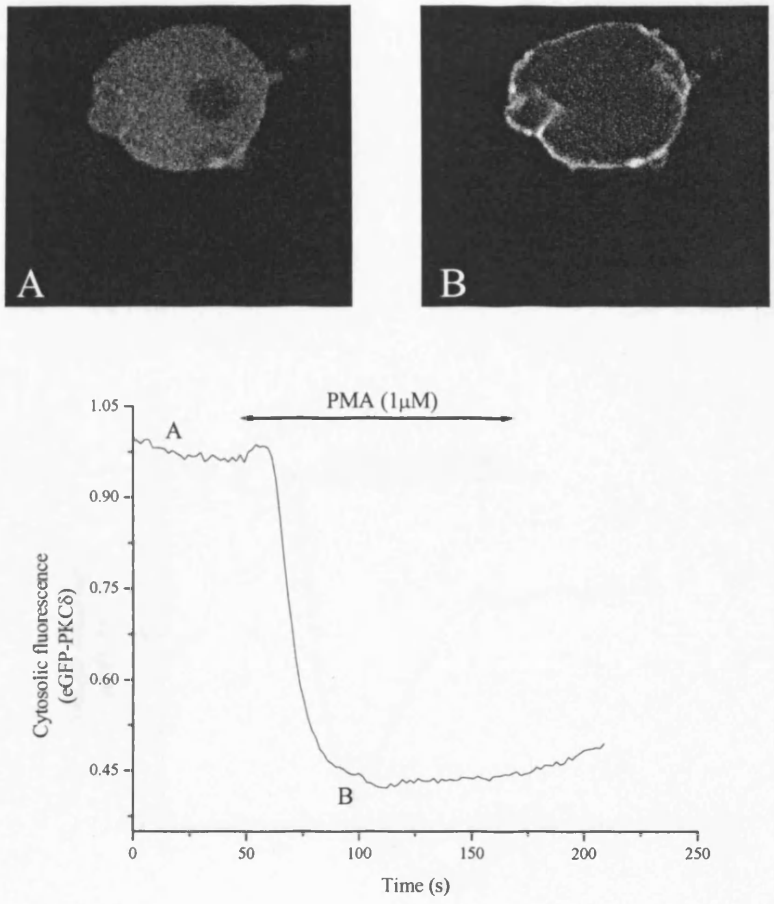


Figure 5.2.11. Single cell imaging of PMA-induced translocation of eGFP-PKCδ. Chromaffin cells transiently transfected with eGFP-PKCδ were perfused with 1μM PMA and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 27 cells).

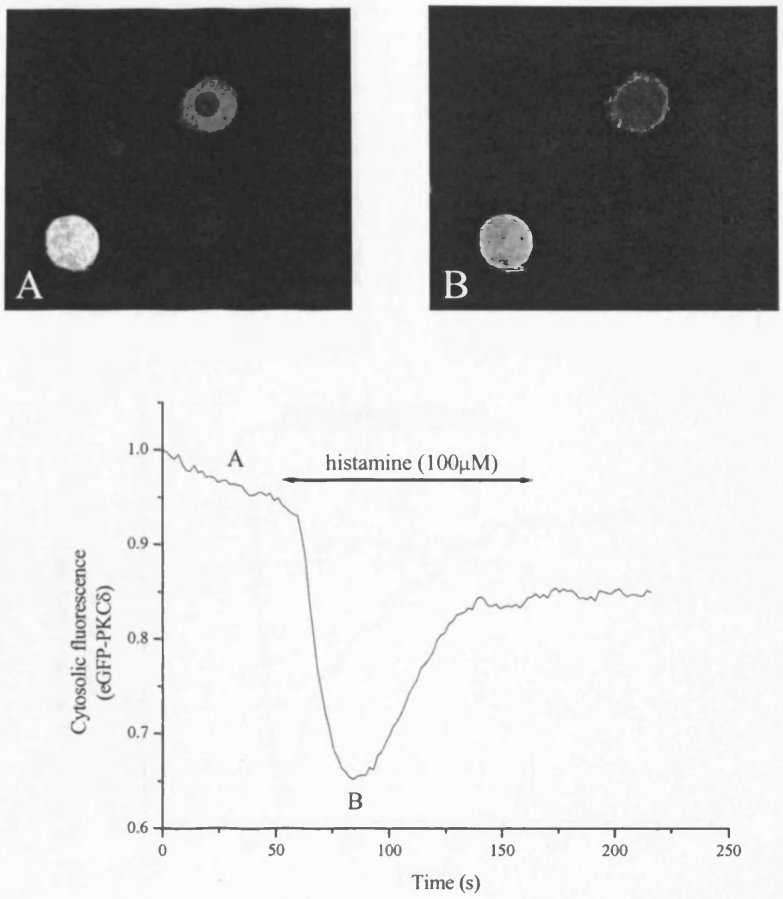


Figure 5.2.12. Single cell imaging of histamine-induced translocation of eGFP-PKCδ. Chromaffin cells transiently transfected with eGFP-PKCδ were perfused with 100μM histamine and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 25 cells).

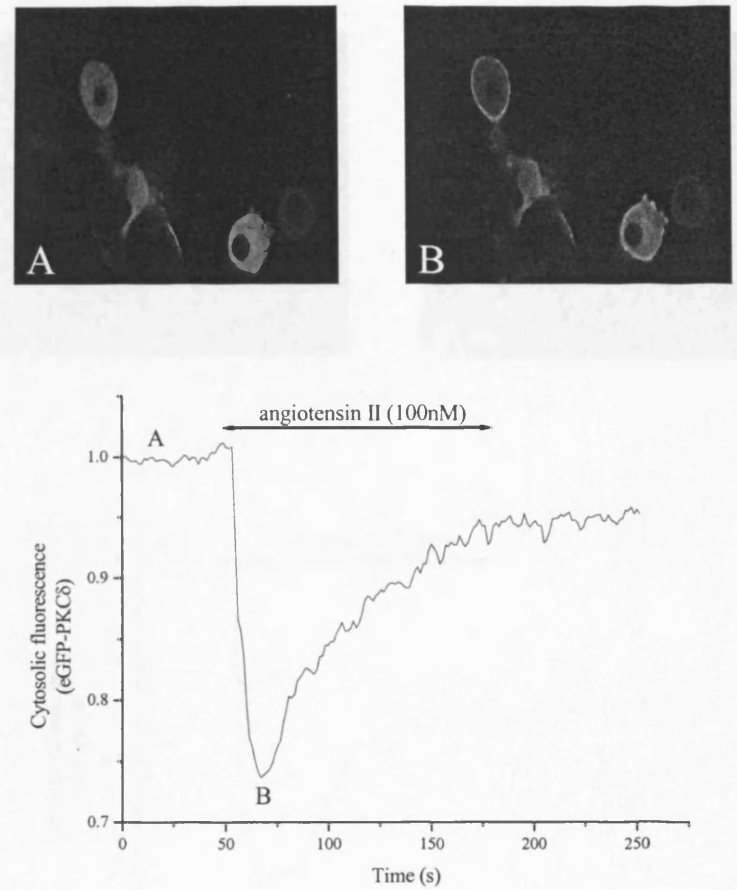


Figure 5.2.13. Single cell imaging of angiotensin II-induced translocation of eGFP-PKCδ. Chromaffin cells transiently transfected with eGFP-PKCδ were perfused with 100nM angiotensin II and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 25 cells).

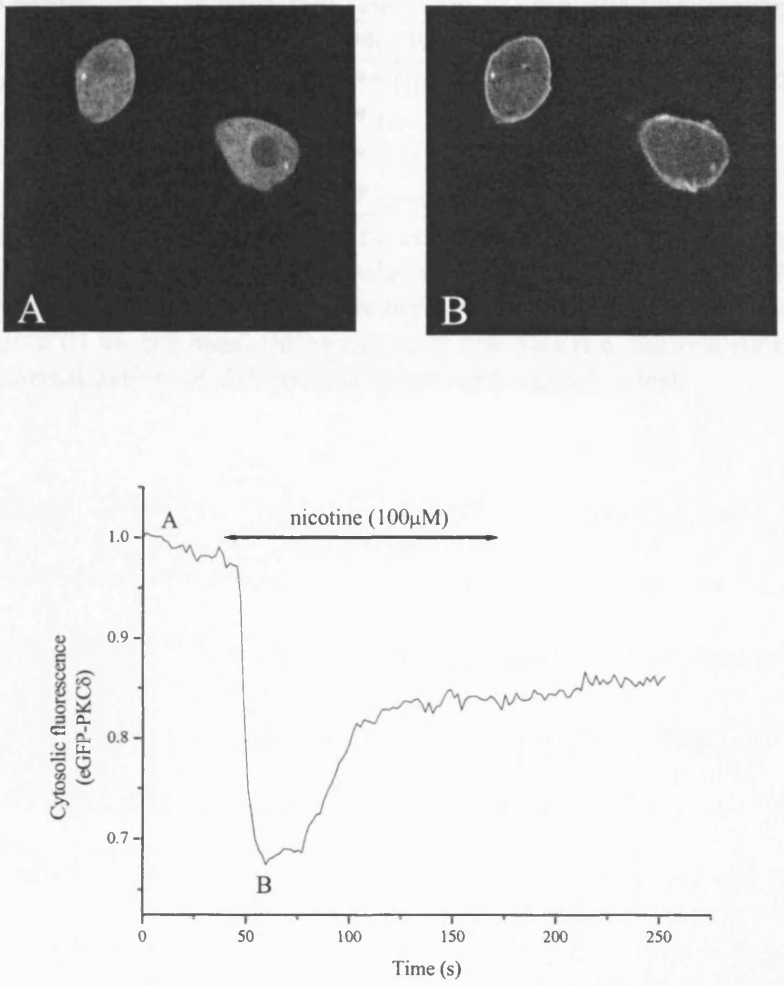


Figure 5.2.14. Single cell imaging of nicotine-induced translocation of eGFP-PKCδ. Chromaffin cells transiently transfected with eGFP-PKCδ were perfused with 100μM nicotine and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 35 cells).

Table 5.2.2 Summary of agonist-induced translocation of eGFP-tagged PKC constructs

Agonist	Maximal reduction of cytosolic fluorescence	
	eGFP-PKCβII	eGFP-PKCδ
PMA (1μM)	0.45 ± 0.02** (n=25)	0.49 ± 0.03** (n=27)
Histamine (100μM)	0.85 ± 0.09* (n=30)	0.64 ± 0.05* (n=25)
Angiotensin II (100nM)	0.53 ± 0.03* (n=35)	0.73 ± 0.06* (n=25)
Nicotine (100μM)	0.65 ± 0.04* (n=32)	0.67 ± 0.04* (n=35)

Data are mean ± SEM. n= the number of cells examined. Data are expressed as fold change in cytosolic fluorescence relative to basal fluorescence (=1.0) and a smaller number therefore indicates a greater change in cytosolic fluorescence. * $P<0.05$, ** $P<0.01$ vs. the basal fluorescence of raw data (i.e. fluorescence units) before normalization, as determined by paired Student's *t*-test.

Role of PKC in secretion of catecholamines evoked by nicotinic receptors and $G\alpha_{q/11}$-coupled receptors

The role of PKC in either the facilitation of nicotinic receptor mediated catecholamine release or in the regulation of $G\alpha_{q/11}$-coupled receptor-evoked secretion of catecholamines was examined by treating cells with isoforms-selective PKC inhibitors. A chemical oxidation method was used to measure the released and cellular levels of catecholamines as described (Materials and Methods).

PMA (1μM, 10 min) evoked a small secretion of catecholamines, which although not significantly different from basal release was abolished by inhibition of PKC using either Ro31-8220 (10μM, 15 min preincubation) or myristoylated pseudosubstrate peptide inhibitor (myr-ΨPKC inhibitor, 10μM, 30 min preincubation). These two non-selective PKC inhibitors were selected because of their different structures and modes of action. Thus, Ro31-8220 is a membrane-permeant bisindolylmaleimide IX derivative and acts as a highly selective, competitive inhibitor at the ATP binding site of PKC (Davis *et al.*, 1992). In contrast, myr-ΨPKC inhibitor appears not to interfere with ATP binding and acts by interacting with the substrate binding site in the catalytic domain, thereby keeping the enzyme in the inactive state.

Preincubation of cells with Ro31-8220 significantly attenuated secretion of catecholamines evoked by either submaximal (3μM, 10 min) or maximal (100μM, 10 min) concentrations of nicotine either alone or in combination with PMA (1μM) (Figure 5.2.15). This combination of PMA and nicotine was used to examine whether PMA modulates catecholamine secretion evoked by nicotinic receptor activation. PMA also potentiated catecholamine secretion evoked by

either submaximal or maximal concentration of nicotine. Similarly, the myr-ΨPKC inhibitor markedly reduced secretion of catecholamines mediated by either nicotine alone or in combination with PMA (Figure 5.2.15).

Stimulation of $G\alpha_{q/11}$-coupled receptors with either histamine (10μM, 10 min) or angiotensin II (10nM, 10 min) evoked a lower secretion of catecholamines than that evoked by nicotine. This secretion was reduced in the presence of PMA (1μM) (Figures 5.2.16 and 5.2.17). Inhibition of PKC with Ro31-8220 markedly potentiated secretion of catecholamines induced by either histamine or angiotensin II alone or in combination with PMA (Figure 5.2.16 and Figure 5.2.17). Note that lower concentrations of either histamine (10μM) or angiotensin II (10nM) were used here to avoid full saturation of receptors that obscure any potentiation effect due to the maximal concentrations of either histamine or angiotensin II.

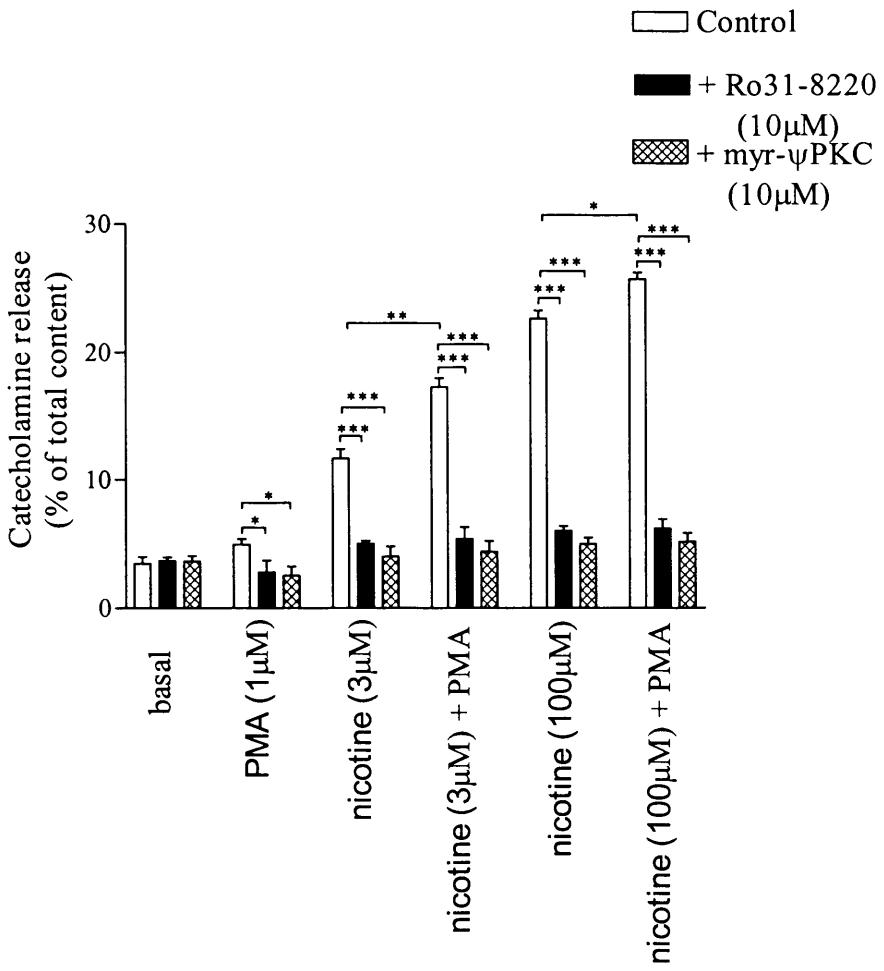


Figure 5.2.15. Effect of inhibiting PKC on catecholamine secretion evoked by nicotine and PMA. Chromaffin cells were incubated with the non-selective PKC inhibitors Ro31-8220 (10μM, 15 min) or myr-ψPKC (10μM, 30 min) at 37°C before stimulation with PMA (1μM) or either submaximal (3μM) or maximal (100μM) concentrations of nicotine for 10 min. Catecholamine release was measured as described in "Materials and Methods" and is expressed as a percentage of the total content. Data are mean + SEM from three different cell preparations. For * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by one-way ANOVA followed by post ANOVA test (Bonferroni's test). Note that not all comparsions are shown on the graph.

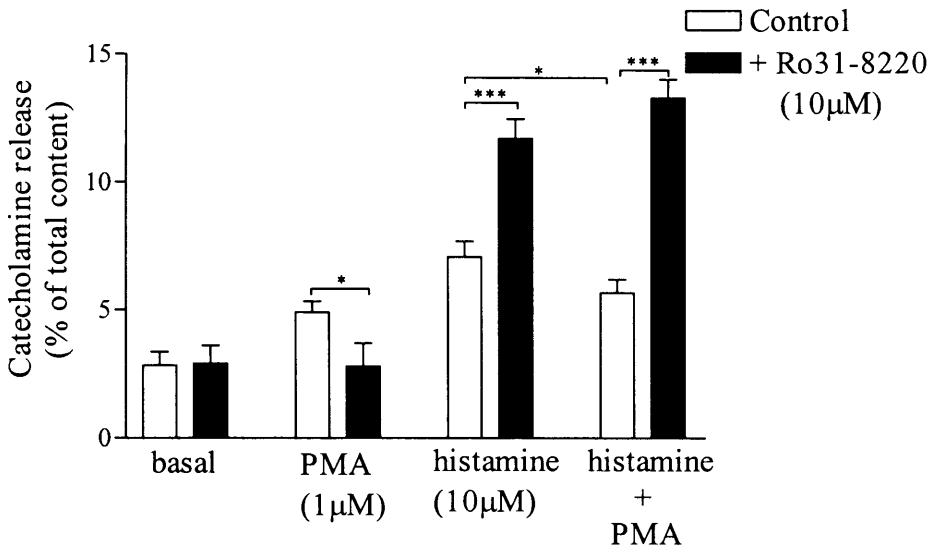


Figure 5.2.16. Effect of inhibiting PKC on catecholamine secretion evoked by histamine and PMA. Chromaffin cells were incubated with the non-selective PKC inhibitor Ro31-8220 (10μM, 15 min) at 37°C before stimulation with either PMA (1μM) or histamine (10μM) for 10 min. Catecholamine release was measured as described in "Materials and Methods" and is expressed as a percentage of the total cellular content. Data are mean + SEM from three different cell preparations. For * $P<0.05$, *** $P<0.001$ by one-way ANOVA followed by post ANOVA test (Bonferroni's test). Note that not all comparsions are shown on the graph.

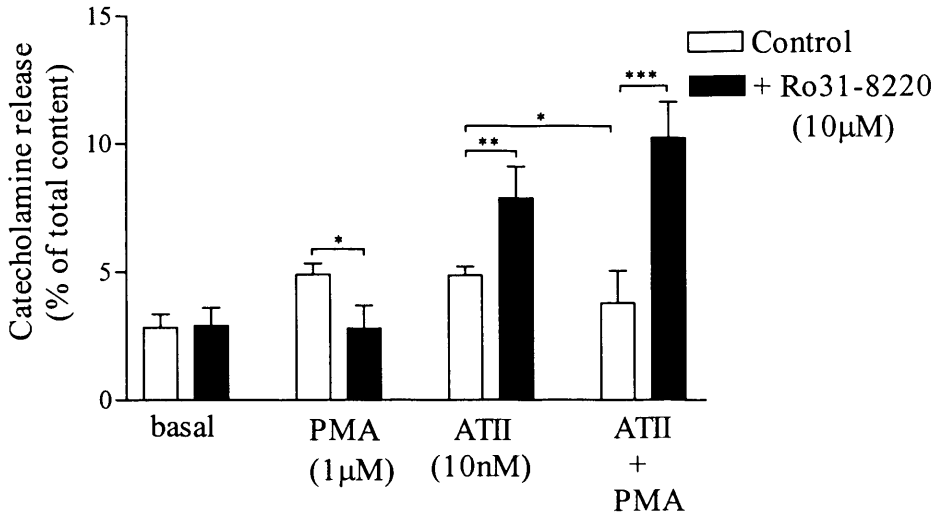


Figure 5.2.17. Effect of inhibiting PKC on catecholamine secretion evoked by angiotensin II and PMA. Chromaffin cells were incubated with the non-selective PKC inhibitor Ro31-8220 (10μM, 15 min) at 37°C before stimulation with either PMA (1μM) or angiotensin II (10nM) for 10 min. Catecholamine release was measured as described in "Materials and Methods" and is expressed as a percentage of the total content. Data are mean + SEM from three different cell preparations. For * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by one-way ANOVA followed by post ANOVA test (Bonferroni's test). Note that not all comparsions are shown on the graph.

Regulation of phospholipase C activity by PKC

Measurement of PLC activity using the accumulation of ^{3}H-inositol phosphates ([^{3}H]-InsP_x) under Li^+-block (Willars *et al.*, 1998b; 1999; Tovey and Willars, 2004) was used to determine if the reason that PKC inhibition potentiated catecholamine secretion was due to a negative feedback loop in which PKC inhibits GPCR-mediated signalling. Removal of such feedback by inhibition of PKC would be expected to enhance GPCR-mediated signalling and consequently enhance catecholamine release.

In ^{3}H-inositol-labeled cells, in which inositol monophosphatase activity had been blocked with Li^+, activation of $G\alpha_{q/11}$-coupled receptors with either histamine (100μM) or angiotensin II (100nM) mediated a significant increase in [^{3}H]-InsP_x accumulation. These accumulation of [^{3}H]-InsP_x were significantly potentiated by inhibition of PKC using Ro31-8220 (Figure 5.2.18).

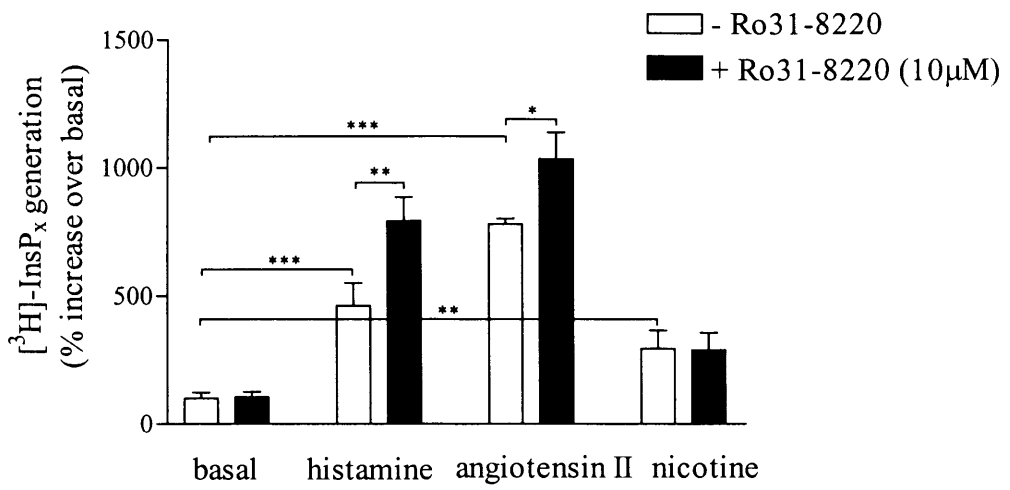


Figure 5.2.18. Effect of PKC inhibition on stimulation of PLC by either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors. Cells were prelabelled with [^{3}H]-inositol for 48 h before stimulation for 30 min in the presence of Li^+, with either PMA (1μM), nicotine (100μM), histamine (100μM) or angiotensin II (ATII, 100nM). The accumulation of [^{3}H]-$InsP_x$ was measured as described in "Materials and Methods". Data are presented as percentage increase over basal (the basal value was 250 ± 18 dpm/well). Data are mean + SEM from three different cell preparations (each in triplicate). For * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by one-way ANOVA followed by post ANOVA test (Bonferroni's test). Note that not all comparsions are shown on the graph.

Role of specific PKC isoforms in regulating the secretion of catecholamines evoked by nicotinic receptors and $G\alpha_{q/11}$-coupled receptors

A number of commercially available isoform-selective PKC inhibitors were used to probe the involvement of specific PKC isoforms in the secretion of catecholamines evoked by either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors.

The selection of inhibitors was made based on the isoforms expressed and the availability of inhibitors. The first isoform-specific PKC inhibitor used was HBDDE, which is a derivative of ellagic acid that is reported to act as an isoform-selective inhibitor of PKCα and γ with IC_{50} values of 43 and 50nM, respectively, determined using an *in vitro* assay (Kashiwada *et al.*, 1994) (note that as PKCγ is not expressed in bovine adrenal chromaffin cells HBDDE is here considered as a PKCα selective inhibitor). However, it acts in a competitive manner at the ATP-binding site in the catalytic domain and its IC_{50} values determined in intact cells lays within the 5-10μM range (Mathur and Vallano, 2000; Rong *et al.*, 2002). Preincubation of cells with HBDDE (100μM, 30 min) inhibited the release of catecholamines induced by PMA (1μM, 10 min) (Figure 5.2.19). HBDDE also significantly reduced catecholamine release in response to both submaximal (3μM) and maximal (100μM) concentrations of nicotine (Figure 5.2.19).

In contrast, preincubation of cells with HBDDE had no significant effect on release evoked by histamine (100μM) (Figure 5.2.22) whereas, secretion of catecholamines induced by angiotensin II (100nM) was potentiated following pre-treatment of cells with HBDDE (Figure 5.2.23).

The role of PKCβ in regulating catecholamine release in response to activation of either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors was examined by treating cells with either LY333531 (10μM, 30 min) or PKCβC2-4 (10μM, 30

min) before application of agonists. These two inhibitors were also selected because of their different modes of action and selectivity toward PKCβ isoforms. The bisindolymaleimide compound LY333531 (Way *et al.*, 2000), is a highly specific inhibitor of PKCβI and PKCβII (Ishii *et al.*, 1996) with IC_{50} values of 4.7nM and 5.9nM, respectively (Jirousek *et al.*, 1996). These concentrations are approximately 50 fold lower than the concentration required to inhibit other isoforms (Ishii *et al.*, 1996). This compound acts as a direct competitive inhibitor with ATP for binding to PKCβI with a K_i of 2nM (Jirousek *et al.*, 1996; Kowluru *et al.*, 1998).

PKCβC2-4 is a nonopeptide derived from the C2 domain of PKCβ and inhibits translocation of C2-containing PKC isoforms. This peptide fragment is a highly selective inhibitor of PKCβ translocation (Ron *et al.*, 1994).

Pre-treatment of cells with either LY333531 (10μM, 30 min) (Efendiev *et al.*, 1999) or PKCβC2-4 (10μM, 30 min) (Yedovitzky *et al.*, 1997; Ron *et al.*, 1995) reduced PMA-mediated release of catecholamines (Figure 5.2.20). Similarly, release of catecholamines evoked by submaximal (3μM) or maximal (100μM) concentrations of nicotine was significantly attenuated by either LY333531 (Figure 5.2.20: panel A) or PKCβC2-4 (Figure 5.2.20: panel B).

In contrast, LY333531 failed to influence catecholamine secretion induced by either histamine (10μM) (Figure 5.2.22) or angiotensin II (10nM) (Figure 5.2.23). Although, the peptide translocation inhibitor of PKCβ (PKCβC2-4 inhibitor) did not influence angiotensin II-mediated release it did potentiate secretion of catecholamines evoked by histamine (Figures 5.2.22 and 5.2.23).

PKCεV1-2, is a short peptide derived from the V1 region of PKCε which inhibits translocation of PKCε (Johnson *et al.*, 1996). Inhibition of PKCε by preincubating cells with PKCεV1-2 (10μM, 30 min) (Yedovitzky *et al.*, 1997; Mayne and Murray, 1998) reduced catecholamine secretion evoked by PMA (1μM). Furthermore, PKCεV1-2 significantly decreased secretion of catecholamines mediated by either submaximal (3μM) or maximal (100μM) concentrations of nicotine (Figure 5.2.21). In contrast, PKCεV1-2 potentiated histamine (10μM) induced catecholamine secretion (Figure 5.2.22) although it failed to affect angiotensin II (10nM) evoked secretion of catecholamines (Figure 5.2.23).

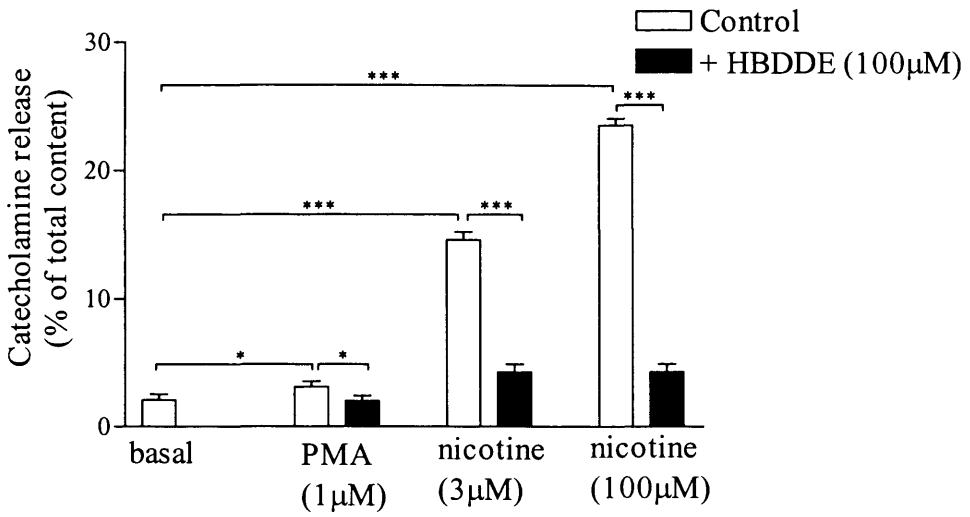
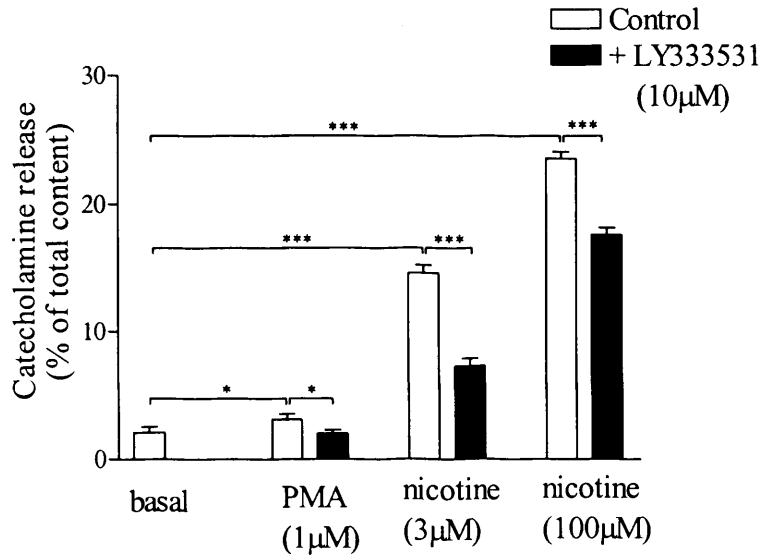


Figure 5.2.19. Effect of a PKCα/γ-selective inhibitor on catecholamine secretion evoked by nicotine or PMA. Chromaffin cells were incubated with the selective PKCα/γ inhibitor HBDDE (100μM, 30 min) at 37°C before stimulation with either PMA (1μM) or submaximal (3μM) or maximal (100μM) concentrations of nicotine for 10 min. Catecholamine release was measured as described in "Materials and Methods" and is expressed as a percentage of total cellular content. Data are mean + SEM for three different cell preparations. For * $P<0.05$, *** $P<0.001$ by one-way ANOVA followed by post ANOVA test (Bonferroni' test). Note that not all comparisons are shown on the graph.

A.



B.

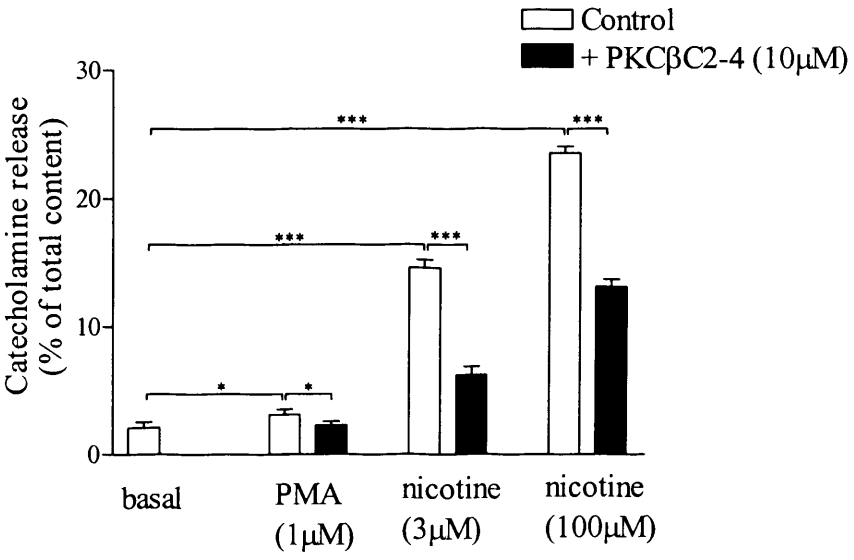


Figure 5.2.20. Effect of PKCβ-selective inhibitors on catecholamine secretion evoked by nicotine or PMA. Chromaffin cells were incubated with the selective PKCβ inhibitors LY333531 (10μM, 30 min, panel A) or PKCβC2-4 inhibitor (10μM, 30 min, panel B) at 37°C before stimulation with either PMA (1μM) or submaximal (3μM) or maximal (100μM) concentrations of nicotine for 10 min. Catecholamine release was measured as described in "Materials and Methods" and is expressed as a percentage of total cellular content. Data are mean + SEM for three different cell preparations. For * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by one-way ANOVA followed by post ANOVA test (Bonferroni's test). Note that not all comparisons are shown on the graph.

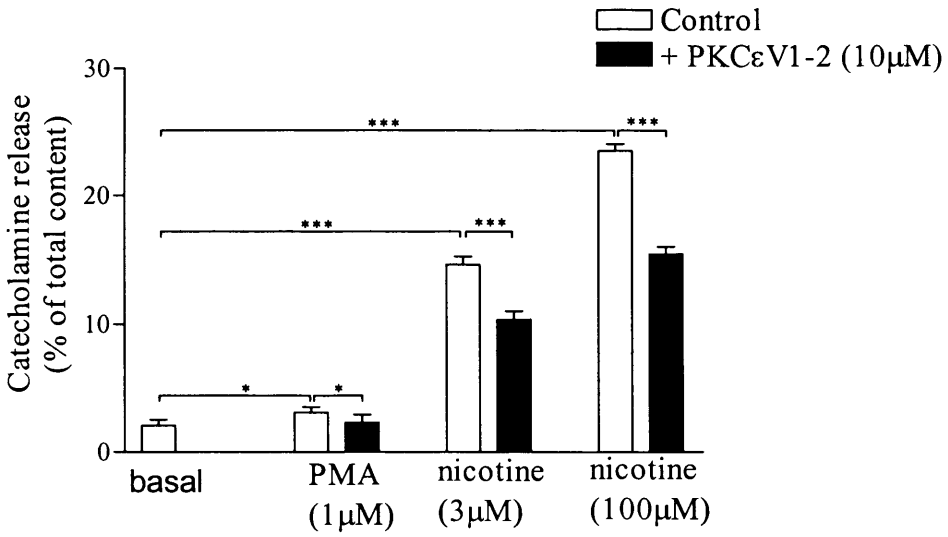
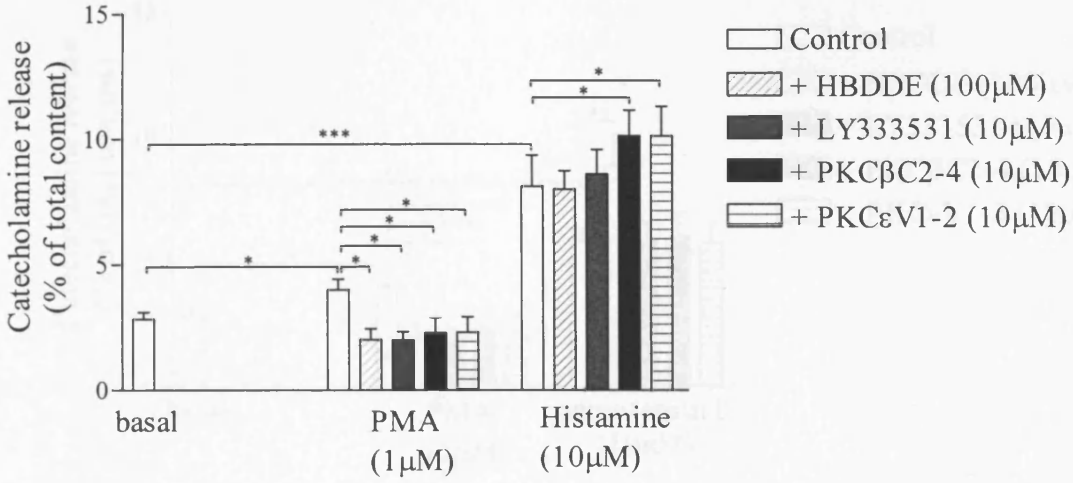


Figure 5.2.21. Effect of a PKCε-selective inhibitor on catecholamine secretion evoked by nicotine or PMA. Chromaffin cells were incubated with the selective PKCε inhibitor PKCεV1-2 (10μM, 30 min) at 37°C before stimulation with either PMA (1μM) or submaximal (3μM) or maximal (100μM) concentrations of nicotine for 10 min. Catecholamine release was measured as described in "Materials and Methods" and is expressed as a percentage of total cellular content. Data are mean + SEM from three different cell preparations. For * $P<0.05$, *** $P<0.001$ by one-way ANOVA followed by post ANOVA test (Bonferroni's test). Note that not all comparisons are shown on the graph.

Figure 5.2.22. Effect of selective PKC inhibitors on catecholamine secretion



evoked by histamine and PMA. Chromaffin cells were incubated with HBDDE (100μM, 30 min), LY333531 (10μM, 30 min), PKCβC2-4 (10μM, 30 min), or PKCεV1-2 (10μM, 30 min) at 37°C before stimulation with either histamine (100μM) or PMA (1μM). Catecholamine release was measured as described in "Materials and Methods" and is expressed as a percentage of total cellular content. Data are mean + SEM from three different cell preparations. For * $P<0.05$, *** $P<0.001$ by one-way ANOVA followed by post ANOVA test (Bonferroni's test). Note that not all comparisons are shown on the graph.

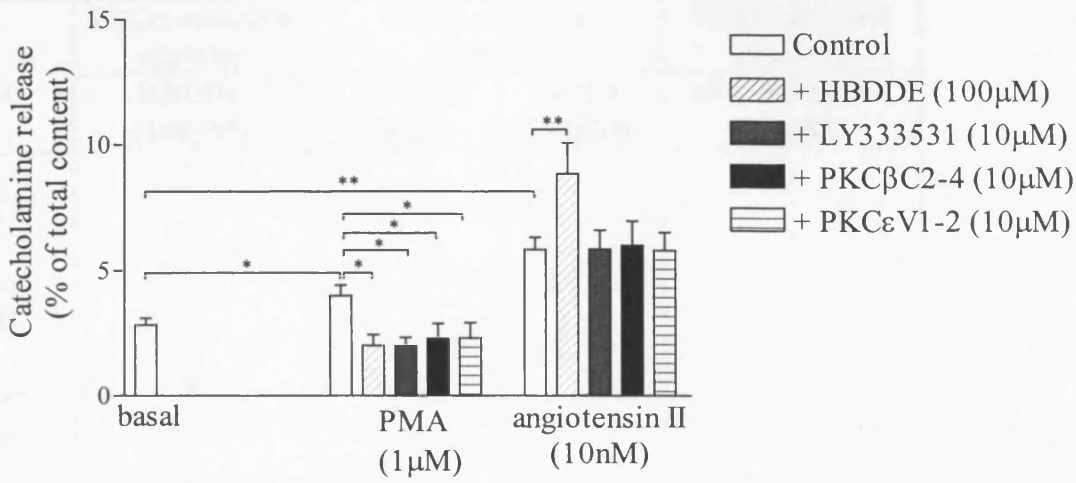


Figure 5.2.23. Effect of selective PKC inhibitors on catecholamine secretion evoked by angiotensin II and PMA. Chromaffin cells were incubated with HBDDE (100μM, 30 min), LY333531 (10μM, 30 min), PKCβC2-4 (10μM, 30 min), or PKCεV1-2 (10μM, 30 min) at 37°C before stimulation with either angiotensin II (10nM) or PMA (1μM). Catecholamine release was measured as described in "Materials and Methods" and is expressed as a percentage of total content. Data are mean + SEM from three different cell preparations. For * $P<0.05$ by *** $P<0.001$,one-way ANOVA followed by post ANOVA test (Bonferroni's test). Note that not all comparisons are shown on the graph.

Table 5.2.3 Summary of the effects of isoform-selective PKC inhibitors on the evoked secretion of catecholamines.

Agonist	PKCα-selective inhibitor	PKCβ-selective inhibitors		PKCε-selective inhibitor
	HBDDE (100μM)	**LY333531 (10μM)**	**βC2-4 (10μM)**	**εV1-2 inhbitor (10μM)**
PMA (1μM)	- - -	- - -	- -	-
Nicotine (100μM)	- - - -	- -	- - -	-
Histamine (10μM)	X	X	+	+
Angiotensin II (10nM)	++	X	X	X

- Sign shows degree of inhibition (- weak, ---- strong) (- =10-20% inhibition, -- =25-35% inhibition, --- =35-45% inhibition, and ---- =above 60% inhibition).
+ Sign shows degree of potentiation (+ weak, ++ strong) (+ = 10-20% potentiation, ++ =25-35% potentiation).
X Sign shows no effect.

The role of PKC in agonist-mediated F-actin disassembly

The role of specific PKC isoforms in mediating the disassembly of cortical F-actin in response to activation of either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors was investigated using confocal microscopy of fixed chromaffin cells stained with rhodamine-phalloidin (Vitale *et al.*, 1991).

The confocal image for control cells (unstimulated cells) inserted in the box at the left upper corner of the image shows an intact bright fluorescent ring and weak cytosolic staining (Figure 5.2.24a). The confocal image was analysed to produce a three dimensional plot in which the image intensity is reflected in the z-axis and set to a maximum of 4096 arbitrary units (main box) (Figure 5.2.24a).

Upon stimulation of cells with cytochalasin D (2μM, 20 min), a known F-actin destabilizer, there was a breakdown (fragmentation) of the cortical fluorescent ring as seen in the confocal image (Figure 5.2.24a). This fragmented fluorescent ring was reflected in the image analysis (main box) (Figure 5.2.24a).

Stimulation of chromaffin cells with either PMA (1μM, 10 min), nicotine (100μM, 40s), histamine (100μM, 10 min), or angiotensin II (100nM, 10 min) also caused disruption of the cortical F-actin (Figure 5.2.24a). Whilst it was difficult to quantitate the integrity of the cortical F-actin ring for individual cells, cells could be scored as having either a continuous (intact) or discontinuous ring. Using a single-blind trial design and a random selection of cells, the proportion of cells with continuous and discontinuous F-actin rings were quantified for each condition (see Materials and Methods). Based upon this analysis the levels of cortical F-actin disassembly mediated by cytochalasin D > PMA > nicotine > histamine > angiotensin II (Figure 5.2.24b).

Cortical F-actin disassembly mediated by PMA was significantly reduced by either the selective PKCα inhibitor HBDDE (100μM, 30 min) or non-selective myristoylated PKC inhibitor myr-ψPKC (10μM, 30 min). Similarly, nicotine caused fragmentation of the cortical F-actin ring and this was significantly attenuated by either HBDDE or myr-ψPKC (Figure 5.2.24b). However, these inhibitors failed to prevent disruption of cortical F-actin caused by either cytochalasin D, histamine or angiotensin II (Figure 5.2.24b). HBDDE was selected in particular for this study since out of the PKC inhibitors tested it caused the greatest reduction of catecholamine secretion evoked by nicotinic receptor stimulation.

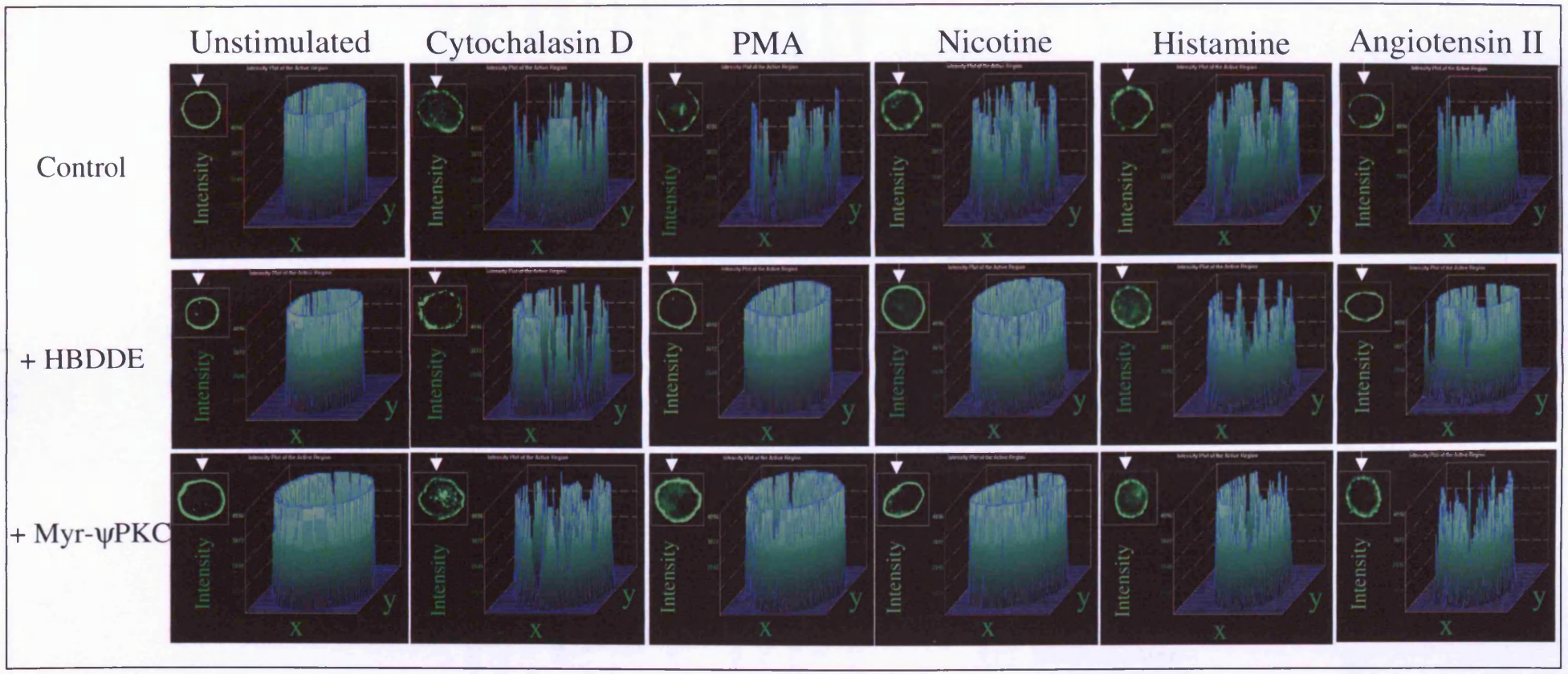


Figure 5.2.24a. Effects of PKC inhibitors on cortical F-actin disassembly mediated by cytochalasin D, PMA, nicotine, histamine, or angiotensin II. Chromaffin cells were incubated with either cytochalasin D (2μM, 30 min), PMA (1μM, 10 min), nicotine (100μM, 40s), histamine (100μM, 10 min), or angiotensin II (100nM, 10 min) following a 30 min preincubation with either vehicle (control), HBDDE (100μM) or myr-ψPKC inhibitor (10μM). Cells were then fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under " Materials and Methods" . The arrows in the left hand corner of the panels indicate the confocal image of the cell. Three-dimensional image analysis was performed using Ultra*View* confocal software (Perkin-Elmer Life Science, Cambridge, UK) where the maximum intensity of the fluorescence was set to 4096 arbitrary units (main box). Images are representative of a total of six hundred cells for each condition from three different cell culture preparations.

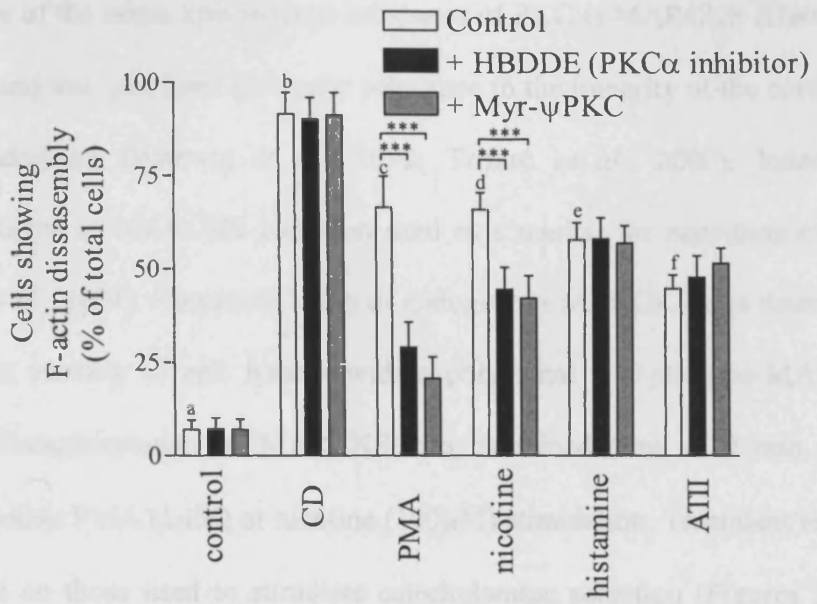
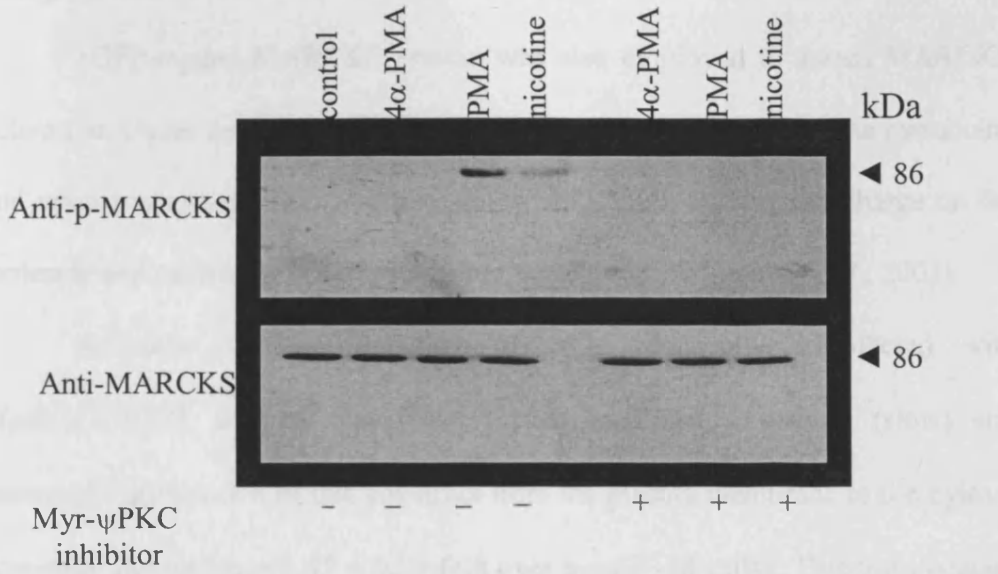


Figure 5.2.24b. Effects of HBDDE and myr-ψPKC on cortical F-actin disassembly mediated by cytochalasin D, PMA, nicotine, histamine or angiotensin II. Chromaffin cells were incubated with cytochalasin D (2μM, 30 min), PMA (1μM, 10 min), nicotine (100μM, 40s), histamine (100μM, 10 min), and angiotensin II (ATII) (100nM, 10 min) following a 30 min preincubation with either vehicle (control), HBDDE (PKCα inhibitor, 100μM) or myr-ψPKC (10μM). Cells were then fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under "Materials and Methods". The rhodamine cortical staining of chromaffin cells was analysed and classified as being either continuous or discontinous and the percentage of cells displaying cortical F-actin disassembly (disrupted cortical rhodamine staining) was calculated. A total of six hundred cells for each condition were examined. Data shown are mean + SEM. For *** $P<0.001$ for a vs. b, c, d, e and f, *** $P<0.001$ vs. control, one-way ANOVA followed by post ANOVA test (Bonferroni's test). Note that not all comparisons are shown on the graph.

Nicotinic receptor-mediated phosphorylation of MARCKS and its prevention by inhibition of PKC

One of the major downstream substrates of PKC is MARCKS (Hartwig *et al.*, 1992) and this may have particular relevance to the integrity of the cortical F-actin cytoskeleton (Hartwig *et al.*, 1992; Trifaró *et al.*, 2000). Indeed the phosphorylation of MARCKS has been used as a marker for activation of PKC (Bar-Am *et al.*, 2004). Phosphorylation of endogenous MARCKS was determined by Western blotting of cell lysates with a polyclonal anti-phospho-MARCKS antibody. Phosphorylation of MARCKS was examined over a 10 min period following either PMA (1μM) or nicotine (100μM) stimulation. Treatment regimes were based on those used to stimulate catecholamine secretion (Figures 5.2.15, 5.2.19 and 5.2.20). Phospho-MARCKS was detected following these treatments as a single band with an apparent molecular mass of 86 kDa (Figure 5.2.25: panel A). The inactive phorbol ester, 4α-PMA (1μM, 10 min) did not mediate phosphorylation of MARCKS. To ensure levels of MARCKS were equivalent, lysates were also immunoblotted for total-MARCKS using a mouse monoclonal antibody specific for bovine MARCKS (Figure 5.2.25: panel A). Pre-incubation of cells with the PKC inhibitor, myr-ψPKC (10μM, 30 min) abolished the phosphorylation of MARCKS in response to challenge of cells with either PMA or nicotine (Figure 5.2.25: panel B).



B.

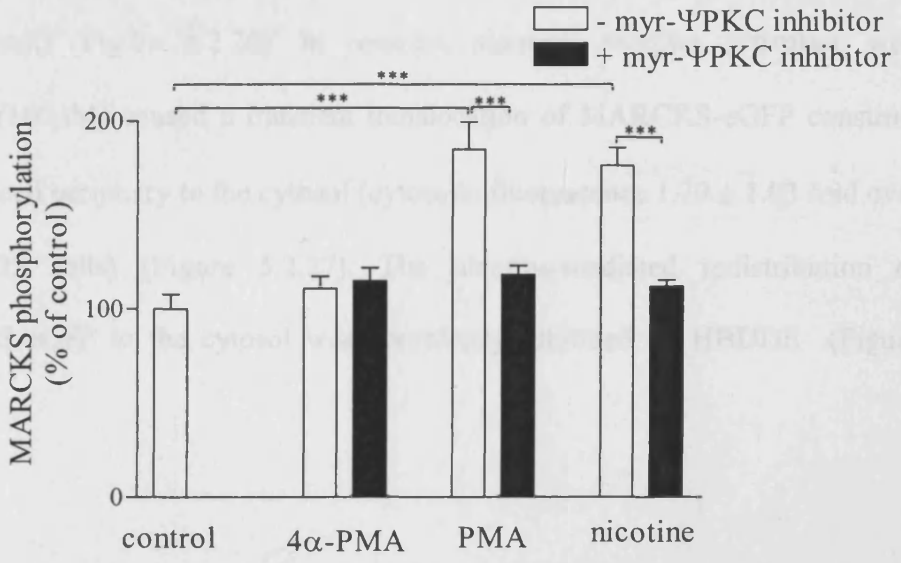


Figure 5.2.25. Role of PKC in PMA- and nicotine-mediated MARCKS phosphorylation. Chromaffin cells were incubated with vehicle control (buffer) or myr-ψPKC (10μM, 30 min) at 37°C before stimulation with either nicotine (100μM), PMA (1μM), or 4α-PMA for 20 min. Western blots of the whole cell lysate were prepared as described in "Materials and Methods". Panel A) shows the immunoblot of phospho-MARCKS. The amount of phospho-MARCKS (p.MARCKS) was quantified using Scion Image analysis software and expressed as a percentage of control (unstimulated cells). Panel B) Cumulative data on MARCKS phosphorylation obtained from experiments carried out on four different cell culture preparations. Data are mean + SEM. For *** $P<0.001$, one-way ANOVA followed by Bonferroni's test.

Using MARCKS-eGFP to assess the activation of MARCKS in real-time

eGFP-tagged MARCKS protein was also employed to assess MARCKS activation. Under resting conditions, MARCKS localizes to the plasma membrane and upon stimulation PKC phosphorylates MARCKS, altering the charge on the molecule and causing a loss of membrane association (Arbuzova *et al.*, 2002).

Real-time confocal imaging of cells transiently transfected with MARCKS-eGFP showed that PMA (1μM) mediated a gradual (slow) and sustained translocation of this construct from the plasma membrane to the cytosol (cytosolic fluorescence 1.45 ± 0.09 fold over basal (~18 cells). This translocation was inhibited by the PKCα-selective inhibitor HBDDE (10μM, 30 min pretreatment) (Figure 5.2.26). In contrast, nicotinic receptor activation with nicotine (100μM) caused a transient translocation of MARCKS-eGFP construct from the cell periphery to the cytosol (cytosolic fluorescence 1.79 ± 1.03 fold over basal, ~25 cells) (Figure 5.2.27). The nicotine-mediated redistribution of MARCKS-eGFP to the cytosol was completely inhibited by HBDDE (Figure 5.2.26).

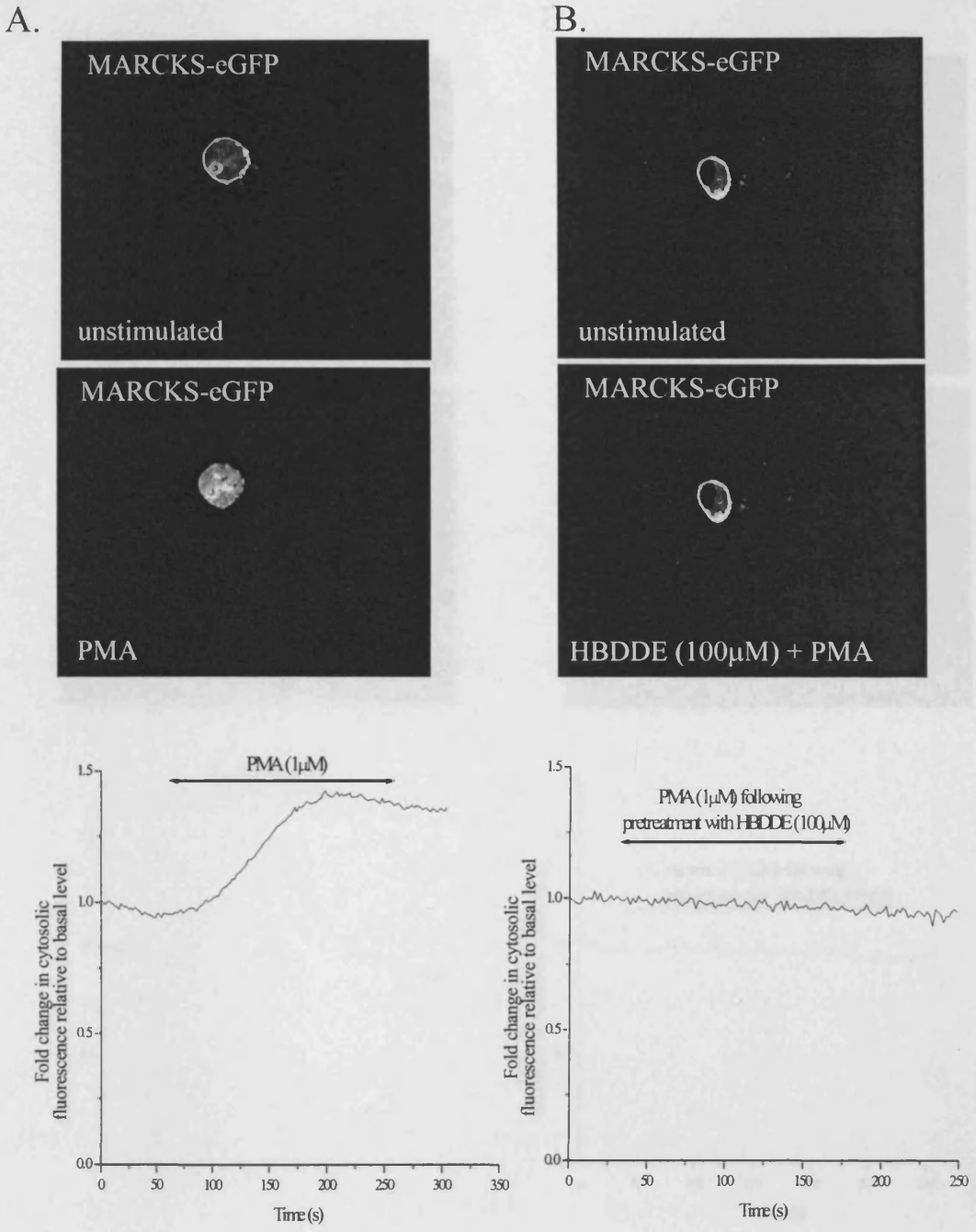


Figure 5.2.26. Single cell imaging of MARCKS-eGFP. Chromaffin cells transiently transfected with MARCKS-eGFP were perfused with PMA (1μM) in the absence of HBDDE (A). Cells were preincubated with HBDDE (100μM, 30 min) at 37°C then challenged with PMA (1μM) (B). The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n=18).

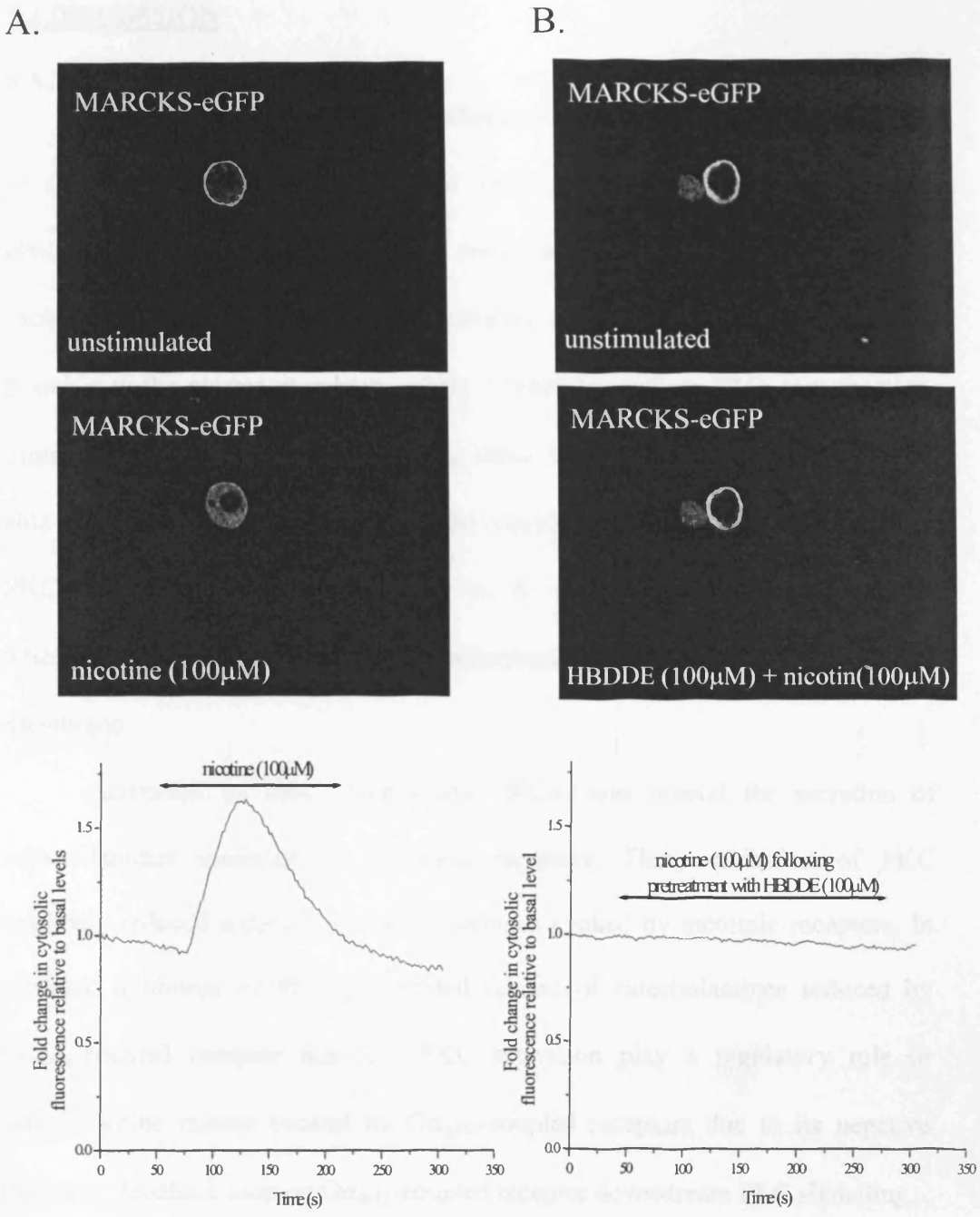


Figure 5.2.27. Single cell imaging of MARCKS-eGFP. Chromaffin cells transiently transfected with MARCKS-eGFP were perfused with nicotine (100μM) in the absence of HBDDE (A). Cells were preincubated with HBDDE (100μM, 30 min) at 37°C then challenged with nicotine (100μM) (B). The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n=25).

5.3 DISCUSSION

5.3.1 Summary of data

The work described within this chapter identified the PKC isoforms -α, -β, -ε and -ι representing each class of PKC isoforms within bovine adrenal chromaffin cells. Different techniques were used to assess PKC activation and each indicated that nicotinic receptor activation mediated recruitment of PKC-α, -β and -ε to the plasma membrane while it failed to mediate PKCι translocation. Similarly, $G\alpha_{q/11}$-coupled receptors for either histamine or angiotensin II were able to activate PKC isoforms. Histamine caused translocation of all the expressed PKC isoforms in chromaffin cells (PKC-α, -β, -ε and -ι) to the plasma membrane whereas angiotensin II only induced redistribution of PKCα and ε to the plasma membrane.

Activation of PKC, particularly PKCα, was crucial for secretion of catecholamines mediated by nicotinic receptors. Thus, inhibition of PKC markedly reduced secretion of catecholamines evoked by nicotinic receptors. In contrast, inhibition of PKC potentiated release of catecholamines induced by $G\alpha_{q/11}$-coupled receptor therefore PKC activation play a regulatory role in catecholamine release evoked by $G\alpha_{q/11}$-coupled receptors due to its negative inhibitory feedback loop on $G\alpha_{q/11}$-coupled receptor downstream PLC signalling.

In addition, investigations in this study highlight the importance of cortical F-actin disassembly in secretory process. Since stimulation of nicotinic receptor activates PKC which subsequently phosphorylates MARCKS thereby reducing its ability to cross-link F-actin filaments. The disruption of cortical F-actin would result in an increase the size of readily releasable pool of secretory granules (Vitale *et al.*, 1995) allowing enhanced exocytosis of catecholamines.

5.3.2 PKC isoform expression in chromaffin cells

The present immunoblot analysis revealed that among the 12 PKC isoforms, bovine chromaffin cells possess only PKC-α, -β, -ε and -ι at levels detectable by immunoblotting. Interestingly, these detectable PKC isoforms in bovine chromaffin cells are representative of the three PKC subfamily classes, PKC-α and -β belong to the conventional class while PKCε represents the novel class and PKCι represents the atypical PKC class. The presence of PKC-α, -β, -ε and -ι isoforms in chromaffin cells is in full agreement with previous studies (Pavlović-Šurjančev *et al.*, 1993; Penberthy and Dahmer, 1994; Sena *et al.*, 1996; 2001, Cox and Parsons, 1997; Vainio *et al.*, 1998; Yanagita *et al.*, 2000).

The ability of each PKC antibody to detect the PKC isoform was confirmed by the parallel study in rat brain and bovine brain extracts, which used to check antibodies. In this study all antibodies were able to detect proteins at the expected molecular weight in both tissues giving confidence that they were not expressed or expressed at very low levels in bovine adrenal chromaffin cells. It has been proposed that the absence of PKC-γ, -δ, -η, -θ and -λ may reflect either an inability of the antisera raised against the carboxyterminal peptides of rat PKC (Wetsel *et al.*, 1992) to interact with the bovine PKC-γ, -δ, -η, -θ and -λ or a lack of expression of these isoforms in bovine chromaffin cells (Pavlović-Šurjančev et al., 1993). The present data show that each of the antibodies could detect these PKC isoforms of bovine origin supporting the view that they are not expressed or expressed at very low levels in bovine chromaffin cells.

5.3.3 PKC translocation

The translocation of PKC isoforms from cytosol to the plasma membrane is assumed to be a hallmark of PKC activation (Newton, 1997; Mochly-Rosen and

Gordon, 1998). In this study two techniques namely Western blotting and immunocytochemistry were used to study the translocation of identified PKC isoforms (PKC-α, -β, -ε and -ι) in bovine adrenal chromaffin cells.

The results clearly demonstrate that stimulation of chromaffin cells with PMA, resulted in a rapid and intense redistribution of PKC-α, -β and -ε from a predominantly cytosolic location in resting cells to membrane-associated sites. The complete loss of PKCβ from the cytosol at 10 min treatment with PMA was more profound than the loss of PKCε and PKCα. This is inconsistent with a previous study that indicated translocation of PKCε was more profound than translocation of PKCα in bovine adrenal chromaffin cells (Sena *et al.*, 1996). Translocation of each of these isoforms in response to PMA is in agreement with other reports in bovine adrenal chromaffin cells that have showed translocation of cPKC and nPKC isoforms in response to several minutes of PMA treatment. The current study is also in agreement with these studies showing an inability of PMA to induce the redistribution of aPKC isoforms (Olivier and Parker, 1992; Ways *et al.*, 1992; Sena *et al.*, 1996; Sena *et al.*, 2001; Yanagita *et al.*, 2000).

The C1 domain constitutes the membrane-targeting regulatory domain in cPKC and nPKC and this domain is required for their interaction with PMA. However, PKCι is insensitive to PMA because the aPKC family possess only one cysteine-rich zinc finger instead of two incorporated in C1 region (Way *et al.*, 2000).

In this study, there is clear evidence of translocation of PKC-α, -β, -ε and -ι in response to histamine receptor activation detected by both Western blotting and immunocytochemical studies. Although over a ten minutes period the degree of translocation was approximately equivalent for each isoform, the movement of

PKCε was the fastest, occurring predominantly in the first one minute of stimulation. Chromaffin cells have been shown to express histamine H_1 receptors, which are linked by $G\alpha_{q/11}$ proteins to PLC (Plevin and Boarder, 1988; Burgoyne, 1991; Challiss *et al.*, 1991; Bunn *et al.*, 1992) (see Chapter 3). The activation of PLC results in the generation of $Ins(1,4,5)P_3$ and DAG in chromaffin cells. Thus, histamine might activate PKC via elevation of intracellular Ca^{2+} and/or DAG generation resulting in the activation of DAG- and Ca^{2+}-dependent isoforms of PKC (i.e., cPKC and nPKC) (Alvarez *et al.*, 1997; Marley, 2003). The translocation of PKC isoforms to the plasma membrane mediated by histamine was inconsistent with other studies that indicate failure of histamine to induce any significant sustained activation (translocation) of any of the PKC isoforms expressed in bovine chromaffin cells (PKC-α, -ε and -ξ) (Sena *et al.*, 1996; Donald *et al.*, 2002), although a tendency to reduce cytosolic and increase particulate PKCε was noted in (Sena *et al.*, 1996). Interestingly, in the present study, histamine did cause the translocation of PKCι detected by both Western blotting and immunocytochemistry. The mechanisms underlying translocation of PKCι are unclear. Indeed, it is unclear whether translocation is required for activation. However, the current data suggest that histamine, probably in a Ca^{2+}- and DAG-independent manner does activate PKCι. The functional consequences of this are not clear.

Activation of angiotensin receptors by angiotensin II only recruited PKC-α and -ε to the plasma membrane with the translocation of PKCε being more prominent. However, the translocation of PKCε was slower than that following histamine receptor stimulation suggesting a different signal or strength of signal following activation of histamine and angiotensin II receptors. It has been

suggested that the angiotensin II type 1 receptor (AT_1) in chromaffin cells are coupled to a plethora of different types of G-protein (Richards *et al.*, 1999; Teschemacher and Seward, 2000). Thus, the inhibitory effects of $G_{i/o}$ activation receptor on VOCCs and hence Ca^{2+} signalling (Takahashi *et al.*, 1996; Powell *et al.*, 2000) could counteract the stimulatory effect of $G\alpha_{q/11}$-proteins on Ca^{2+} signalling. Under these circumstances, PKCα would be expected to be affected more than PKCε, since PKCα requires both Ca^{2+} and DAG for activation (Newton and Johnson, 1998) whereas PKCε is DAG-sensitive and Ca^{2+}-insensitive. Indeed, the Ca^{2+} signal in response to angiotensin II was much shorter in duration than that in response to histamine. Whether this is due to differences in G-protein coupling or the rate of receptor desensitisation, this could account for the inability of angiotensin II to activate the Ca^{2+}/DAG-sensitive PKCβ. Similarly, this could account for inability of angiotensin II to activate PKCι.

Nicotine caused a dramatic loss of PKCε from the cytosolic fraction that was much more marked than the loss of either PKC-α or -β. Activation of PKC-α or -β following nicotinic receptor activation could be secondary to a rise of $[Ca^{2+}]_i$ (Wolf *et al.*, 1985; Gopalakrishna *et al.*, 1986; Akers and Routtenberg, 1987). However, in the case of Ca^{2+}-independent PKCε activation could occur following Ca^{2+}-triggered PLC activity and an increased formation of DAG, a necessary activator of PKCε. This will be explored further in Chapter 6. The ability of nicotine to increase membrane-bound PKCα and ε is largely in agreement with previous studies (Messing *et al.*, 1989; Sontag *et al.*, 1990; Rodríguez Del Castillo *et al.*, 1992; Cox and Parsons, 1997; Vainio *et al.*, 1998; Izrael *et al.*, 2004).

It has been demonstrated that translocation mediated by tumor-promoting phorbol esters (in this case PMA) cause a dramatic decrease in cytosolic PKC

activity accompanied by a large increase in membrane-bound activity in many cell types (Kraft and Anderson, 1982; Nishizuka, 1995; Mochly-Rosen, 1995). This is in agreement with the present study in which PKC-α and -β translocation mediated by PMA was more prominent than translocation mediated by activation of $G\alpha_{q/11}$-coupled receptors. Interestingly the activation of $G\alpha_{q/11}$-coupled receptors increased membrane-association of PKCε more than PMA suggesting that factors other than PMA/DAG binding are crucial in the regulation of PKCε.

The problem that may emerge from using Western blotting to study the translocation of PKC isoforms in chromaffin cells is that it only allows study of changes of PKC distribution in populations of cells and not single cells. This presents a particular problem when a heterogenous population of cells like chromaffin cells are used (Tischler, 2002). Thus, chromaffin cell preparations might be contaminated by other cell types such as fibroblasts, cortical cells, and adrenal medulla endothelial cells (Bossu *et al.*, 1992; Vinet and Vargas, 1999; Benavides *et al.*, 2004). Furthermore, Western blotting fails to show the pattern of subcellular localization. To characterize the subcellular distribution of PKC isoforms and their potential redistribution, immunocytochemical studies were performed. Immunocytochemical studies using isoform-specific antibody confirmed the cytosolic localization of all expressed PKC isoforms in unstimulated cells and demonstrated the membrane localization of PKC-α, -β, -ε but not -ι with either PMA or nicotinic receptor activation. Histamine also caused the plasma membrane localization of all expressed PKC isoforms whereas AT_{1A} receptor activation caused the redistribution of PKC-α and -ε to the plasma membrane.

These data are consistent with those obtained by Western blotting and demonstrate that a substantial proportion of cytosolically located PKC translocates to the plasma membrane rather than other intracellular membranes.

Whilst the immunoblotting and immunocytochemical methods give valuable information on the translocation and activation of PKC isoforms these techniques have limited temporal resolution. To overcome this, fusion constructs between enhanced green fluorescent (eGFP) (Tsien, 1998) and PKCβII (Feng and Hannun, 1998) or PKCδ (Chiesa *et al.*, 2001) were used to monitor the dynamics of membrane translocation of PKC isoforms in real time in living cells. These eGFP-tagged constructs represent the conventional and novel classes of PKC respectively. In the present study eGFP-PKCδ was used as positive control although immunoblotting analysis indicated that PKCδ was not expressed in chromaffin cells.

As expected, PMA caused robust and sustained translocation of either eGFP-PKCβII or eGFP-PKCδ. Activation of histamine receptors caused a rapid but transient translocation of eGFP-PKCβII or eGFP-PKCδ from the cytosol to the plasma membrane. The transient nature of these translocations were inconsistent with the activation profiles that were observed using immunoblotting. It is possible that this is a reflection of high expression levels of the constructs. However, activation of either angiotensin II receptors or nicotinic receptors did cause a rapid and sustained translocation of eGFP-PKCβII and eGFP-PKCδ from the cytosol to the plasma membrane. Thus, the activation profiles of eGFP-PKCβII or eGFP-PKCδ translocation mediated by either angiotensin II or nicotine were consistent with the activation profiles observed using immunoblotting.

5.3.4 Role of PKC in secretion of catecholamines

The main function of adrenal chromaffin cells is secretion of catecholamines. As discussed elsewhere (see main introduction) PKC has been reported to regulate secretion in both chromaffin cells and other secretory cells. Given that there are structural differences in the regulatory domains of PKC isoforms expressed in chromaffin cells, these isoforms may have distinct roles in the facilitation and regulation of secretion of catecholamines from adrenal chromaffin cells (Terbush and Holz, 1986; 1990; Terbush *et al.*, 1988; Graham *et al.*, 2000; Taylor *et al.*, 2000). Although, the role of PKC in secretion of catecholamines is well documented, there is debate about the underlying mechanisms (Waters and Smith, 2000). Several groups have suggested that PKC has an obligatory role in exocytosis based on the results obtained from a variety of relatively non-specific PKC inhibitors (Knight *et al.*, 1988). In this study non-selective PKC inhibitors were initially used to confirm the role of PKC in release of catecholamines mediated either by nicotinic receptors or $G\alpha_{q/11}$-coupled receptors. Isoform-selective PKC inhibitors were then used to probe the role of specific PKC isoforms. The non-selective inhibitors used were Ro31-8220 and myristoylated pseudosubstrate peptide (myr-ψPKC). The selection of these two inhibitors was based on the fact that they have different chemical structures and modes of action. Thus, Ro31-8220, a membrane-permeant bisindolylmaleimide IX derivative acts by competing with ATP for binding to PKC (Davis *et al.*, 1992; Bit *et al.*, 1993). Ro31-8220, which possess a straight chain alkyl side group with a cationic substituent shows little PKC isoform selectivity and inhibits most, if not all PKC isoforms known to date, including the isoforms expressed in chromaffin cells (Wilkinson *et al.*, 1993; Shen and Glazer, 1998; Sena *et al.*, 2001).

Furthermore, at nanomolar concentration (IC_{50} =10nM), Ro31-8220 fully inhibits PKC while still retaining specificity (Davis *et al.*, 1992). This IC_{50} value (10nM) was determined by *in vitro* kinase assays in which the ATP concentration is generally around 100μM. However, cellular ATP concentrations are of the order of 2mM and much higher concentrations of inhibitor are required. Ro31-8220 was used at 10μM as reported using a variety of cell types including chromaffin cells (Turner *et al.*, 1996; Willars *et al.*, 1999; Soliakov and Wonnacott, 2001). In contrast, myr-ψPKC is a specific, cell permeable a myristoylated peptide inhibitor and expectedly not interferes with ATP binding (Eichholtz *et al.*, 1993). This peptide inhibitor is related to the pseudosubstrate site of PKC and competes with substrate binding site in the catalytic domain and keeps the enzyme in the inactive state (House and Kemp, 1987). This peptide is competent to inhibit all PKC isoforms as all contain a pseudosubstrate domain. Structurally, its sequence resembles a PKC phosphorylation site and it features a set of basic amino acids. However, the phosphate acceptor Ser or Thr residues are substituted by Ala (Gschwendt *et al.*, 1991). This compound has been shown to inhibit PKC when used at 10μM in several cell types (eg. human fibroblast, jurkat cells, islets, human T lymphocytes and bovine chromaffin cells) (Ioannides *et al.*, 1990; Eichholtz *et al.*, 1993; Harris *et al.*, 1996; Alexander *et al.*, 1989; Terbush and Holz, 1990).

The current study demonstrated that inhibition of PKC using either Ro31-8220 or myr-ψPKC markedly reduced secretion of catecholamines evoked by either PMA or nicotine suggesting a crucial role for PKC in catecholamine release mediated by nicotinic receptor activation. This is consistent with previous studies in chromaffin cells in which there was a correlation between nicotinic receptor

stimulation, PKC activation and increased exocytosis (Brocklehurst *et al.*, 1985; Wakade *et al.*, 1986; Terbush *et al.*, 1988; Terbush and Holz, 1990; Cox and Parsons, 1997).

PMA might be expected to enhance nicotinic receptor mediated catecholamine release when they are used concurrently based on previous studies (Wakade *et al.*, 1986; Terbush *et al.*, 1988; Vitale *et al.*, 1992; Cox and Parsons, 1997). Indeed, this study demonstrated that PMA potentiated catecholamine secretion evoked by nicotinic receptor activation. To examine the influence of nicotinic receptor-mediated PKC activation on nicotinic receptor-evoked secretion, the influence of the general PKC inhibitors Ro31-8220 and myr-ψPKC was examined. The data revealed that inhibition of PKC significantly antagonized secretion mediated by nicotine, providing strong evidence of the crucial role of PKC in facilitating secretion evoked by nicotinic receptor activation.

It has been previously demonstrated that Ca^{2+} influx due to nicotinic receptor activation induced translocation of PKC from cytosol to the membrane and that this correlates well with the secretory response (Terbush *et al.*, 1988). In addition, it has been suggested that nicotinic receptor-mediated binding of PKC to membranes involves two components, one mediated by Ca^{2+} alone and the other by Ca^{2+} and DAG. The source of Ca^{2+} and DAG for nicotinic receptor mediated catecholamine secretion will be explored elsewhere (see Chapter 6).

Surprisingly, secretion of catecholamines evoked by the $G\alpha_{q/11}$-coupled receptors either histamine or angiotensin II was reduced by direct activation of PKC with PMA whilst inhibition of PKC potentiated release. $G\alpha_{q/11}$-coupled receptor-evoked catecholamine release from bovine adrenal chromaffin cells requires PLC activation (Plevin and Boarder, 1988; Sasakawa *et al.*, 1989; Bunn

et al., 1990). The activation of PLC results in the hydrolysis of membrane PtdIns(4,5)P_2. The generation of DAG and Ins(1,4,5)P_3 with subsequent Ca^{2+} release from intracellular stores evokes catecholamine secretion. In addition, depletion of Ca^{2+} from internal stores results in store-operated Ca^{2+} entry (capacitative Ca^{2+} entry) (Putney, 1990) and capacitative Ca^{2+} entry has been reported to evoke secretion from both PC12 cells and chromaffin cells (Powis *et al.*, 1996a; Fomina and Nowycky, 1999; Taylor and Peers, 1999). The PLC-dependent generation of DAG, and in some instances Ca^{2+} results in the activation of PKC following $G\alpha_{q/11}$-coupled receptor stimulation. Despite this, PKC does not appear to potentiate catecholamine release under these conditions as inhibition of PKC potentiates release. The reason for this is likely to be a reflection of a short inhibitory feedback loop from PKC perhaps onto PLC (Boarder and Challis, 1992; Bunn, 1993; Alvarez *et al.*, 1997; Sena *et al.*, 1996; Teschemacher and Seward, 2000; Marley, 2003). There is also evidence that PKC-dependent phosphorylation of GPCRs may be responsible for a negative feedback loop (Willars *et al.*, 1996; Diviani *et al.*, 1997; Liang *et al.*, 1998). Thus, removal of this loop by inhibition of PKC may potentiate receptor-mediated signalling. This negative feedback loop from PKC onto PLC was further confirmed by measurement of PLC activity using the accumulation of ^{3}H-inositol phosphates ([^{3}H]-InsP_x) under Li^+-block (Willars *et al.*, 1998a; 1999; Tovey and Willars, 2004). This study indicated that [^{3}H]-InsP_x generation mediated by $G\alpha_{q/11}$-coupled receptors was potentiated by inhibiting PKC using Ro31-8220. These data provide additional evidence of the negative regulatory role of PKC on catecholamine secretion evoked by $G\alpha_{q/11}$-coupled receptors.

Although, Ro31-8220 and myr-ψPKC demonstrated the contribution of PKC to catecholamine secretion evoked by either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors, these general PKC inhibitors did not provide information on the role of individual PKC subtypes in exocytosis. Therefore, isoform-specific PKC inhibitors were used to dissect the contribution of specific PKC isoforms to nicotinic receptor stimulated catecholamine release. It was also hoped that it may be possible to determine the isoforms responsible for the negative effects of PKC on $G\alpha_{q/11}$-coupled receptor-mediating signalling, potentially enabling facilitating effects of PKC on catecholamine release to be uncovered.

The present study suggests that PKCα is the prominent isoform involved in potentiating the catecholamine release in response to nicotinic receptor activation since inhibition of this isoform using HBDDE completely abolished secretion of catecholamines evoked by nicotinic receptors. This bisindolymaleimide compound acts in a competitive manner at the ATP-binding site in the catalytic domain (Mathur and Vallano, 2000). This compound has been used previously at the concentration used here (100μM) to selectively inhibit PKCα in chromaffin cells and other cell types (Rong *et al.*, 2002; Markos *et al.*, 2005). Two structurally and functionally different PKCβ inhibitors also inhibited nicotinic receptor-mediated catecholamine release. The bisindolymaleimide compound LY333531 (Way *et al.*, 2000) inhibits both PKCβI and PKCβII (Ishii *et al.*, 1996) with IC_{50} values of 4.7nM and 5.9nM, respectively (Jirousek *et al.*, 1996). It has been to act as a direct competitive inhibitor with ATP for binding to PKCβI with a K_i of 2nM (Jirousek *et al.*, 1996; Kowluru *et al.*, 1998). In contrast, PKCβC2-4 is derived from the C2 domain of PKC and this peptide fragment is a highly selective inhibitor of PKCβ translocation. The inhibitory effect of

PKCβC2-4 on nicotinic receptor-evoked secretion of catecholamines was slightly greater than that achieved with LY333531. In contrast, both PKCβC2-4 and LY333531 were equally effective in inhibition of PMA mediated secretion.

The PKCε inhibitor, PKCεV1-2 also had less much inhibitory effect than PKCβ inhibitors on nicotinic receptor-mediated catecholamine release. This was used at 10μM as previously reported (Ron *et al.*, 1995; Yedovitzky *et al.*, 1997). This peptide is derived from the V1 region of PKCε lead to inhibiting translocation of this isoforms (Johnson *et al.*, 1996). Despite the fact that PKCε translocation to the plasma membrane mediated by nicotinic receptors was more profound than the translocation of the other expressed isoforms; PKCε contributes the least to catecholamine secretion evoked by nicotinic receptors.

From the present results it can be suggested that PKCα plays the most important role in potentiating the release of catecholamines from chromaffin cells triggered by nicotine, consistent with previously reported studies (Terbush *et al.*, 1988; Cox and Parsons, 1997; Smith, 1999; Sena *et al.*, 2001; Fontainhas *et al.*, 2005). Based on the use of selective PKCβ inhibitors, this isoform would also appear to play role in catecholamine secretion mediated by nicotinic receptor activation. However, PKCα was selected as a target throughout this study since inhibition of PKCα caused the greatest attenuation of nicotinic receptor-mediated catecholamine secretion and this may require further confirmation.

In contrast, it is difficult to dissect the roles of the PKC isoforms to the regulation of catecholamine secretion evoked by $G\alpha_{q/11}$-coupled receptors. Since the degree of potentiations caused by the wide range of isoforms-selective inhibitors were approximately comparable.

There is range of sites of PKC action including ion channels (Wakade *et al.*, 1986; Majewski and Iannazo, 1998; Maeno-Hikichi *et al.*, 2003), the actin cytoskeleton (Vitale *et al.*, 1995) and exocytotic machinery proteins that are involved in regulated exocytosis (Rink *et al.*, 1983; Nagy *et al.*, 2002; Barclay *et al.*, 2003) (see Chapter 1). Although, these sites of PKC action have been extensively investigated, one PKC substrate, MARCKS and its link to F-actin and secretion has received little attention particularly in response to nicotinic receptor activation.

5.3.5 The role of PKC in agonist-mediated F-actin disassembly

It has been suggested that the actin microfilament network localized underneath the plasma membrane of chromaffin cells (Lee and Trifaró; 1981; Trifaró *et al.*, 1984; Cheek and Burgoyne, 1986) acts as barrier (negative clamp) to the movement of secretory granules, blocking their access to exocytotic sites at the plasma membrane (Trifaró *et al.*, 1982; Burgoyne *et al.*, 1989; Burgoyne, 1991). In chromaffin cells, F-actin forms a cortical network that excludes the large majority of secretory vesicles from plasma membrane docking and maintains them in reserve pool. Disassembly of cortical F-actin in response to stimulation is thought to allow the movement of vesicles from this reserve compartment into the release-ready pool (Vitale *et al.*, 1991; 1995). It is evident that stimulation of chromaffin cells produces disassembly of actin networks and removal of a barrier to secretory vesicle mobility (Trifaró *et al.*, 1982; 1984; Cheek and Burgoyne, 1986; Burgoyne *et al.*, 1989; Trifaró and Vitale, 1993; Zhang *et al.*, 1995b; Trifaró *et al*; 2000; 2002). F-actin network disassembly has also been observed in mast cells on stimulation (Koffer *et al.*, 1990), in depolarised synaptosomes

(Bernstein and Bamburg, 1985; Trifaró and Vitale, 1993; Walaas and Sefland, 2000) and PC12 cells (Geeraert *et al.*, 2003).

Two pathways are known to be involved in the control of the chromaffin cell cortical F-actin network during secretion (Trifaró *et al.*, 2000). These are the Ca^{2+}-scinderin and PKC-MARCKS pathways. The first pathway constitutes three Ca^{2+}-dependent actin-binding proteins that control actin filament length: gelsolin (Yin and Stossel, 1979), fodrin (Perrin and Aunis, 1985) and scinderin (Rodríguez Del Castillo *et al.*, 1990). Gelsolin is an actin-servering protein expressed in many cells including chromaffin cells (Yin and Stossel, 1979; Trifaró *et al.*, 1985; Bader *et al.*, 1986). Fodrin, is also an actin-binding protein present in the cortical region of chromaffin cells (Perrin and Aunis, 1985). Scinderin is an 80 kDa cytosolic protein that shortens actin filaments (Rodríguez Del Castillo *et al.*, 1990). It is evident that during cell stimulation, subplasmalemal scinderin, but not gelsolin, is redistributed in chromaffin cells and this redistribution precedes exocytosis (Vitale *et al.*, 1991). The second pathway constitutes the PKC substrate protein, which MARCKS. MARCKS is a member of a family of PKC substrates that potentially interact with F-actin and calmodulin. MARCKS is widely distributed in the nervous system (Hartwig *et al.*, 1992; Ouimet *et al.*, 1990) and is also expressed in chromaffin cells (Zhang *et al.*, 1995b; Powis *et al.*, 1996b).

MARCKS binds actin and cross-links the actin filaments (Hartwig *et al.*, 1992) which remain intact unless MARCKS is phosphorylated by PKC as phospho-MARCKS is unable to cross-link actin filaments (Hartwig *et al.*, 1992). Furthermore, it has been suggested that in chromaffin cells, MARCKS mediates the effects of PKC activation by controlling the F-actin network dynamics during secretion in response to specific stimuli (Vitale *et al.*, 1995; Trifaró *et al.*, 2000).

The cytochemical experiments with rhodamine-labelled phalloidin (as a probe for F-actin) clearly demonstrated that the positive control, cytochalasin D depolymerises F-actin and disrupts actin filaments as previously reported for this fungal toxin (Schliwa, 1982; Flanagan and Lin, 1980; Vitale *et al.*, 1995; Cuchillo-Ibáñez *et al.*, 2004). The fragmentation of cortical F-actin induced by cytochalasin D was not prevented by inhibition of PKC, giving a clear indication as expected that PKC was not involved in cytochalasin D-mediated F-actin disassembly. Activation of PKC with PMA caused disassembly of the cortical F-actin network. Such PMA-induced disassembly of cortical F-actin has previously been demonstrated to facilitate the translocation to the plasma membrane (0-50 nm area) of those vesicles that are in the proximity of the plasma membrane but separated from it by the actin filament barrier (Vitale *et al.*, 1995). The PMA result confirmed the contribution of PKC particularly α isoform to cortical F-actin disruption evoked by PMA since inhibition of PKC with selective PKCα inhibitor, HBDDE dramatically attenuated this disruption. This is consistent with previous studies that used less specific PKC inhibitors such as sphingosine, staurosporine and calphostin that blocked in a concentration dependent manner PMA-induced cortical F-actin network disassembly (Vitale *et al.*, 1992; Rodríguez Del Castillo *et al.*, 1992).

Previous results (see Chapter 4) demonstrated that jasplakinolide, F-actin stabilizer prevents F-actin disassembly mediated by nicotinic receptor activation and as consequence prevented release of catecholamines mediated by nicotinic receptors. This indicates that disassembly of the cortical F-actin plays a crucial role in nicotinic receptor evoked catecholamine secretion as previously suggested (Trifaró, 1984; Cheek and Burgoyne, 1986; Trifaró *et al.*, 1989; Vitale *et al.*,

1991; Vitale *et al.*, 2000; Tchakarov *et al.*, 1998). The inability of the PKC inhibitors to fully prevent nicotinic receptor-mediated disassembly suggests that either PKC inhibition was incomplete or that a mechanism other than PKC might be involved in controlling F-actin network dynamics. This is highlighted by a previous study in chromaffin cells that revealed a Ca^{2+}-dependent scinderin mechanism to be responsible for at least 80% of the cortical F-actin disassembly and catecholamine release in response to nicotinic receptor stimulation since PKC inhibitors only produced 20% inhibition of F-actin disassembly and catecholamine release (Trifaró *et al.*, 2002).

An immunoblot approach was used to study the phosphorylation of endogenous MARCKS. Data revealed that MARCKS phosphorylation increased upon stimulation with PMA and this seems to be due to PKC since this was completely abolished by inhibition of PKC. In addition, MARCKS-eGFP was used to assess the activation of MARCKS in real-time (Ohmori *et al.*, 2000). MARCKS localizes to the plasma membrane and upon stimulation PKC phosphorylates MARCKS leading to its translocation to the cytosol (Arbuzova *et al.*, 2002). In this study, activation of PKC by PMA treatment mediated translocation of MARCKS to the cytosol. This translocation was markedly prevented by inhibition of PKCα using HBDDE suggesting a role for PKCα in the activation of MARCKS and the subsequent F-actin disassembly.

Although, the effects of PMA on MARCKS phosphorylation and translocation have been studied in chromaffin cells (Vitale *et al.*, 1992; 1995; Rosē *et al.*, 2001; Trifaró *et al.*, 2000; Trifaró *et al.*, 2002), no study has previously shown a role for PKC in nicotinic receptor-mediated MARCKS phosphorylation and translocation. Clarification of the role of PKCα and

MARCKS in vesicular trafficking in chromaffin cells might be achieved by studying the effect of antisense oligonucleotides or siRNAs specific for MARCKS and PKCα.

Activation of $G\alpha_{q/11}$-coupled receptors also facilitated F-actin network disassembly supporting previously published work (Trifaró *et al.*, 2000; 2002; Zhang *et al.*, 1995b; Samain *et al.*, 1999). The non-selective PKC inhibitor (myr-ψPKC) and selective PKCα inhibitor (HBDDE) failed to block F-actin filament disruption. Thus, F-actin disassembly in response to activation of $G\alpha_{q/11}$-coupled receptors would seem to be independent of the PKC-MARCKS pathway. It is possible that Ca^{2+}-scinderin pathway could therefore play a major role in controlling cortical F-actin dynamics under these conditions.

Data in this chapter demonstrated that it is difficult to dissect which PKC isoforms are involved in the regulation of catecholamine secretion evoked by $G\alpha_{q/11}$-coupled receptors and this aspect was not pursued further and the focus was diverted to the role of PKC in catecholamine secretion evoked by nicotinic receptors as shown in the next chapter (Chapter 6).

6: MECHANISMS UNDERLYING PKC ACTIVATION MEDIATED BY NICOTINIC RECEPTOR STIMULATION

6.1 INTRODUCTION

Catecholamine secretion from bovine adrenal chromaffin cells is physiologically stimulated by nicotinic receptor-mediated influx of extracellular Ca^{2+} and a subsequent rise in the $[Ca^{2+}]_i$ (Wilson and Kirshner, 1977; Kao and Schneider, 1986). Although there is strong evidence as seen in this (see Chapter 5) and previous studies (Brocklehurst *et al.*, 1985; Vainio *et al.*, 1998; Soliakov and Wonnacott, 2001; Mahata *et al.*, 2002) indicating that nicotinic receptor-mediated activation of PKC enhances secretion of catecholamines, the mechanism(s) by which PKC is activated are not fully understood. Thus, work described within this chapter sought to investigate how nicotinic receptor activation results in the activation of PKC specifically. PKC-α, -β and -ε (see Chapter 5) that are either activated by DAG or Ca^{2+} and DAG in combination. Earlier experiments (Chapter 3) demonstrated elevation of $[Ca^{2+}]_i$ in response to nicotinic receptor activation. Many isoforms of PLC are Ca^{2+}-sensitive and it is possible therefore that PLC is activated in response to nicotinic receptor activation. Enhanced PLC activity would generate DAG (and potentially additional Ca^{2+} from intracellular stores via $Ins(1,4,5)P_3$) and this DAG, either alone or in combination with Ca^{2+}, could activate PKC. Indeed, under equilibrium conditions, biological membranes contain very little DAG (Hodgkin *et al.*, 1998) and DAG generation may, therefore, be required to support PKC activation. Previous data have suggested that Ca^{2+} influx following nicotinic- or high K^+-mediated depolarisation in chromaffin cells can increase activity of PLC (Eberhard and Holz, 1988; Nakaki *et al.*, 1988; Sasakawa *et al.*, 1989; Roberts-Thomson *et al.*, 2000).

Furthermore, previous studies have shown that activity of the key signal transduction enzyme PLC is enhanced by secretory stimuli is chromaffin cells (Bunn *et al.*, 1990; Roberts-Thomson *et al.*, 2000; Sasakawa *et al.*, 1989). Furthermore, the PLC substrate, PtdIns(4,5)P_2 is essential for secretion (Hay *et al.*, 1995). These observations suggest that PLC activity could play an important role in the exocytotic response (O'Connel *et al.*, 2003).

Advances in imaging techniques and the advent of GFP-tagged proteins with high selectivity for intracellular messengers make it possible to image the cellular level of Ins(1,4,5)P_3 in a real time in single cells (Stauffer *et al.*, 1998; Hirose *et al.*, 1999). The pleckstrin homology domain of PLCδ1 ($PH_{PLC\delta1}$) binds specifically and with high affinity to PtdIns(4,5)P_2 (Kavran *et al.*, 1998) and a fusion construct of $PH_{PLC\delta1}$ and GFP (eGFP-$PH_{PLC\delta1}$) enriches over the plasma membrane. Therefore, agonist-stimulated Ins(1,4,5)P_3 production is predominantly responsible for translocation of eGFP-$PH_{PLC\delta1}$ with little contribution of PtdIns(4,5)P_2 depletion as result of PLC activity (Nash *et al.*, 2001; Nahorski *et al.*, 2003; Bartlett *et al.*, 2005). Similarly, DAG levels can be monitored in single cells using the DAG binding domain of PKCγ (eGFP-Cl_2PKCγ) (Oancea and Meyer, 1998; Mogami *et al.*, 2003), which translocates to the plasma membrane in response to agonist-mediated DAG generation. In addition to these fluorescent biosensors, PLC activity can also be assessed by measurement of total [^{3}H]-inositol phosphates ([^{3}H]-InsPs) in Li^+-blocked cells (Willars and Nahorski, 1995b).

The PLC family comprises 11 isoforms classified into four groups (four PLCβ, two PLCγ, four PLCδ and one PLCε) (Rhee and Bae, 1997; Rhee, 2001). Recently, a novel sperm-specific PLC, PLCζ has been identified (Saunders *et al.*,

2002). Activation of PLCβ isozymes is initiated by ligand binding to G protein-coupled receptor. Apart from $G_{q/11}$ type α subunits coupled to these receptors, Gβγ subunits can also activate PLCβ. PLCγ activation involves phosphorylation by growth-factor-activated receptor protein tyrosine kinases. Activation of PLCδ isozymes is triggered by binding of Ca^{2+} to EF-hand and C2 domains of PLCδ. Ca^{2+} activates PLCδ 50-100 fold more than β and γ isoforms. Thus, PLCδ would be expected to be responsible for activity observed following an initial elevation of $[Ca^{2+}]_i$. PLCε is widely expressed in human tissues and most abundantly in heart. PLCε is activated by Ras and receptors for thrombin and lysophosphatidic acid that couple to $G\alpha_{12}$ (Rhee, 2001). Chromaffin cells express β, γ and δ isoforms of PLC (Choi *et al.*, 1993) but whether PLCε is expressed in chromaffin cells is not yet known.

Apart from the PI-PLC pathway, DAG can also be produced by two sequential reactions in which phosphatidylcholine is broken down by phosphatidylcholine-specific phospholipase D (PC-PLD) to yield phosphatidic acid, which is then converted to DAG by phosphatidic acid phosphohydrolase (Nishizuka, 1992; Hodgkin *et al.*, 1998; Wakelam, 1998). Indeed, PKC can also be activated by DAG generated PC-PLD pathways. Furthermore, PKC can phosphorylate and activate PLD and this may be a process that ensures continued production of DAG for sustained PKC activation (see reviews Newton, 1997; 2001) Metabolism of DAG either by DAG lipase or DAG kinase terminates its action (Hodgkin *et al.*, 1998).

DAG is able to bind to the C1 domains of a large number of proteins with diverse function (see Chapter 1). The most prominent DAG targets belong to the PKC family of serine/threonine kinases. Binding of DAG often is synergy with

Ca^{2+} leads to membrane translocation and activation of PKC isoforms (Nishizuka, 1992; Newton, 1995; 1997; 2001) (see Chapter 1 for details of role of Ca^{2+} and DAG in PKC activation and recruitment). The modulation of cellular processes by DAG and by the functionally analogous phorbol esters has been attributed primarily to activation of PKCs (Brose and Rosenmund, 2002). However, it has been shown that DAG, as well as phorbol esters can bind to an alternative non-kinase targets including Munc13, PKD kinases, chimaerin, RacGTPase-activating proteins (GAPs) and RasGRP (Ras guanyl nucleotide-releasing protein) (Yang and Kazanietz, 2003). In this study, attention was focused on Munc13 since there is large body of evidence indicating that activation of Munc13 facilitates neurotransmitter release (Betz *et al.*, 1998; Ron and Kazanietz, 1999; Kazanietz, 2002).

Munc13 proteins constitute a family of four mammalian homologues of *Caenorhabditis elegans* Unc13 including Munc13-1, Munc13-2, and Munc13-3 (Brose *et al.*, 1995) and recently identified novel Munc13-like protein (Munc13-4) (Koch *et al.*, 2000). Munc13 is specifically expressed in neuronal cells and Munc13-1 is localized at the presynaptic plasma membrane (Brose *et al.*, 1995; Augustin, 1999b; Betz *et al.*, 1998) whereas Munc13-4 is predominantly expressed in lung (Koch *et al.*, 2000). Chromaffin cells express low levels of Munc13-1 and Munc13-3 while Munc13-2 is not detectable (Ashery *et al.*, 2000). Munc13 isoforms are very proteins (195-225kDa) (Betz *et al.*, 1997) with phorbol ester and DAG binding C1 domain and two C2 domains that are Ca^{2+}/phospholipid binding domains (Brose *et al.*, 1995; Augustin *et al.*, 1999a; 1999b). All Munc13 isoforms bind phorbol esters and DAG with high affinity and in common with PKCs translocate to the plasma membrane (Betz *et al.*, 1998;

Ashery *et al.*, 2000). In *C. elegans*, Unc13 is essential for coordinated movement (Brenner, 1974). It has been shown that Unc13 and Munc13 are important for synaptic vesicle priming (Augustin *et al.*, 1999b; 2001; Richmond *et al.*, 1999; Aravamudan *et al.*, 1999; Varoqueaux *et al.*, 2002). Unc13 and Munc13s act by unfolding and activating the SNARE protein syntaxin and thereby promoting SNARE complex formation (Betz *et al.*, 1997; Brose *et al.*, 2000; Richmond *et al.*, 2001). In addition, it has been demonstrated that Munc13-1 interacts with the synaptic core complex by binding directly to syntaxin. Therefore, Munc13-1 has a central role in synaptic vesicle priming (Betz *et al.*, 1997).

The work described in this chapter aimed to determine whether the activation of PKC in response to nicotinic receptor activation (which was previously shown to enhance catecholamine release (see Chapter 5)) was mediated by DAG generated by activation of PLC.

In addition, this work sought to determine whether Munc13 was activated by nicotinic receptor stimulation and whether this was also dependent on PLC activation.

6.2 RESULTS

Single cell imaging of phospholipase C activity using fluorescent biosensors

Transient transfection of chromaffin cells with eGFP-$PH_{PLC\delta1}$ resulted in accumulation of fluorescence over the plasma membrane representing the association of the fusion protein with PtdIns(4,5)P_2. Challenge of cells with either nicotine (100μM), histamine (100μM) or angiotensin II (100nM) resulted in loss of membrane association and enrichment of cytosolic fluorescence in chromaffin cells (approximately 40-60% of transfected cells showed translocation of eGFP-$PH_{PLC\delta1}$ in response to each agonist) (Figures 6.2.1, 6.2.2 and 6.2.3). Plotting the change in cytosolic fluorescence relative to basal level against time during prolonged agonist challenge indicated that both nicotine and angiotensin II caused a transient increase in cytosolic fluorescence (Figure 6.2.3 and Figure 6.2.2). In contrast, challenge of cells with histamine induced a bigger peak than that mediated by nicotine and angiotensin II followed by a lower sustained phase. These changes in fluorescence returned to basal levels after perfusion with Krebs'/HEPES buffer to remove the agonist (Figure 6.2.1).

The potential impact of nicotinic receptors or $G\alpha_{q/11}$-coupled receptors on the other limb of the signalling pathway resulting from PLC-mediated hydrolysis of plasma membrane bound PtdIns(4,5)P_2 was assessed by determing DAG production using the eGFP-PKC$\gamma C1_2$ biosensor.

In cells transiently transfected with eGFP-PKC$\gamma C1_2$, challenge with either angiotensin II (100nM) or histamine (100μM) resulted in rapid and robust loss of cytosolic fluorescence from the cytosol (Figure 6.2.4 and Figure 6.2.5) (mean data are shown in Figure 6.2.7: panel B). Nicotine (100μM) caused a rapid (40s) and transient reduction in cytosolic fluorescence and a corresponding increase at the

plasma membrane (Figure 6.2.6). The mean data for agonist-mediated changes in cytosolic fluorescence in cells expressing either eGFP-$PH_{PLC\delta 1}$ or eGFP-$PKC\gamma Cl_2$ are summarized in Figure 6.2.7: panels A and B.

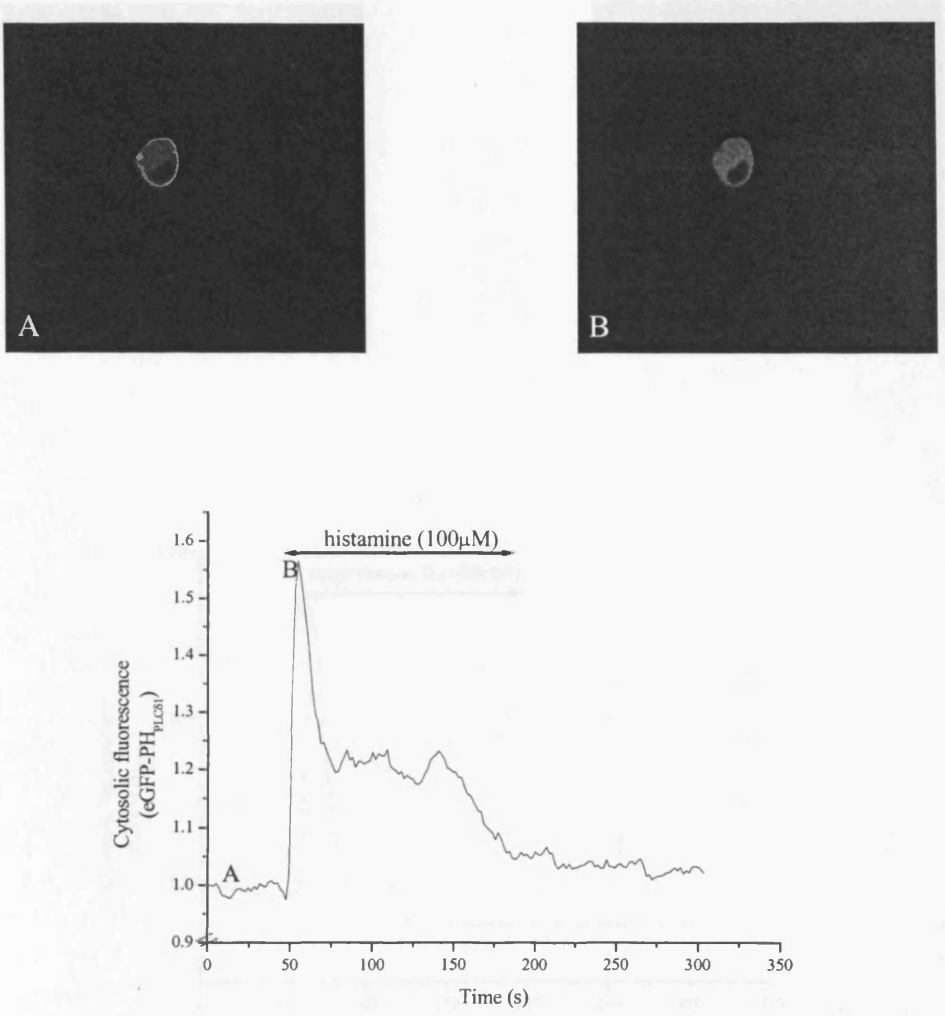
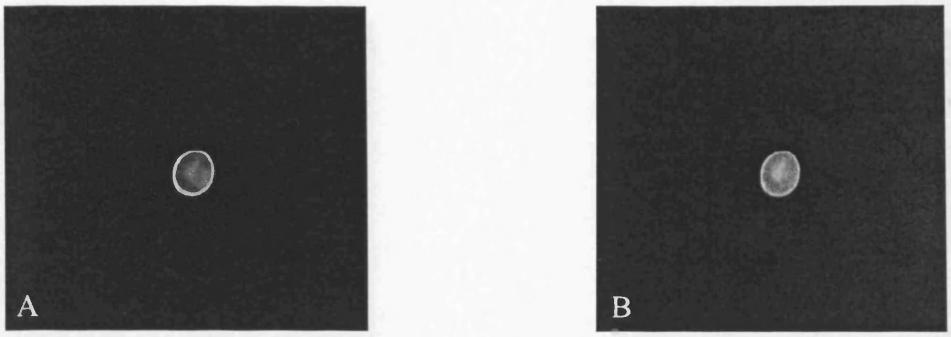


Figure 6.2.1. Single cell imaging of histamine-induced changes in the distribution of eGFP-PH$_{PLC\delta1}$ in cultured bovine adrenal chromaffin cells. Cells transiently transfected with eGFP-PH$_{PLC\delta1}$ were perfused with histamine (100μM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 30 cells in total (~ 60% of examined cells responded)).



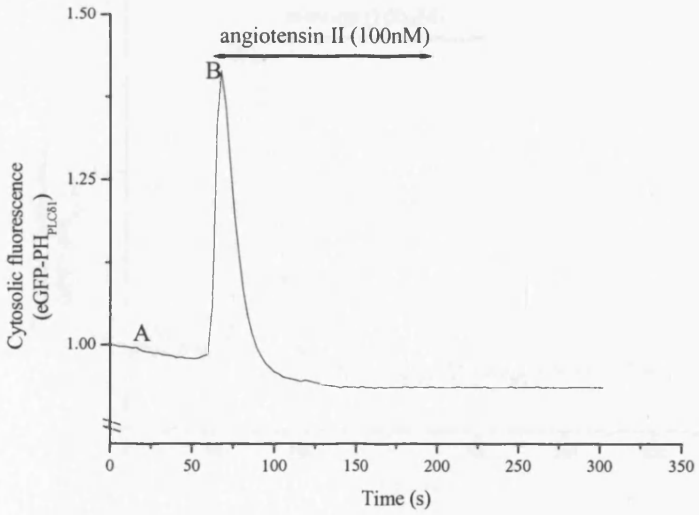


Figure 6.2.2. Single cell imaging of angiotensin II-induced changes in the distribution of eGFP-$PH_{PLC\delta1}$ in cultured bovine adrenal chromaffin cells. Cells transiently transfected with eGFP-$PH_{PLC\delta1}$ were perfused with angiotensin II (100nM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 20 cells in total (~ 40% of examined cells responded)).

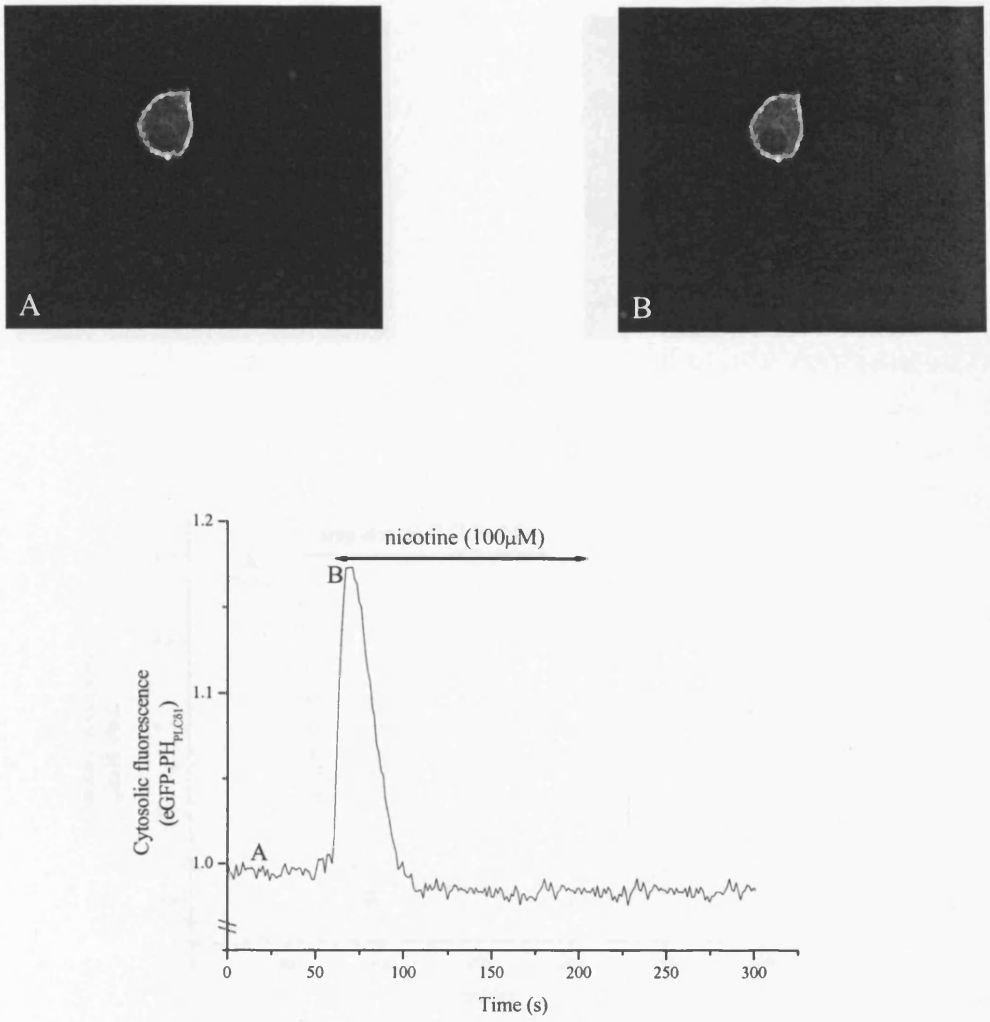


Figure 6.2.3. Single cell imaging of nicotine-induced changes in the distribution of eGFP-PH$_{PLC\delta1}$ in cultured bovine adrenal chromaffin cells. Chromaffin cells transiently transfected with eGFP-PH$_{PLC\delta1}$ were perfused with nicotine (100μM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 24 cells in total (~ 40% of examined cells responded)).

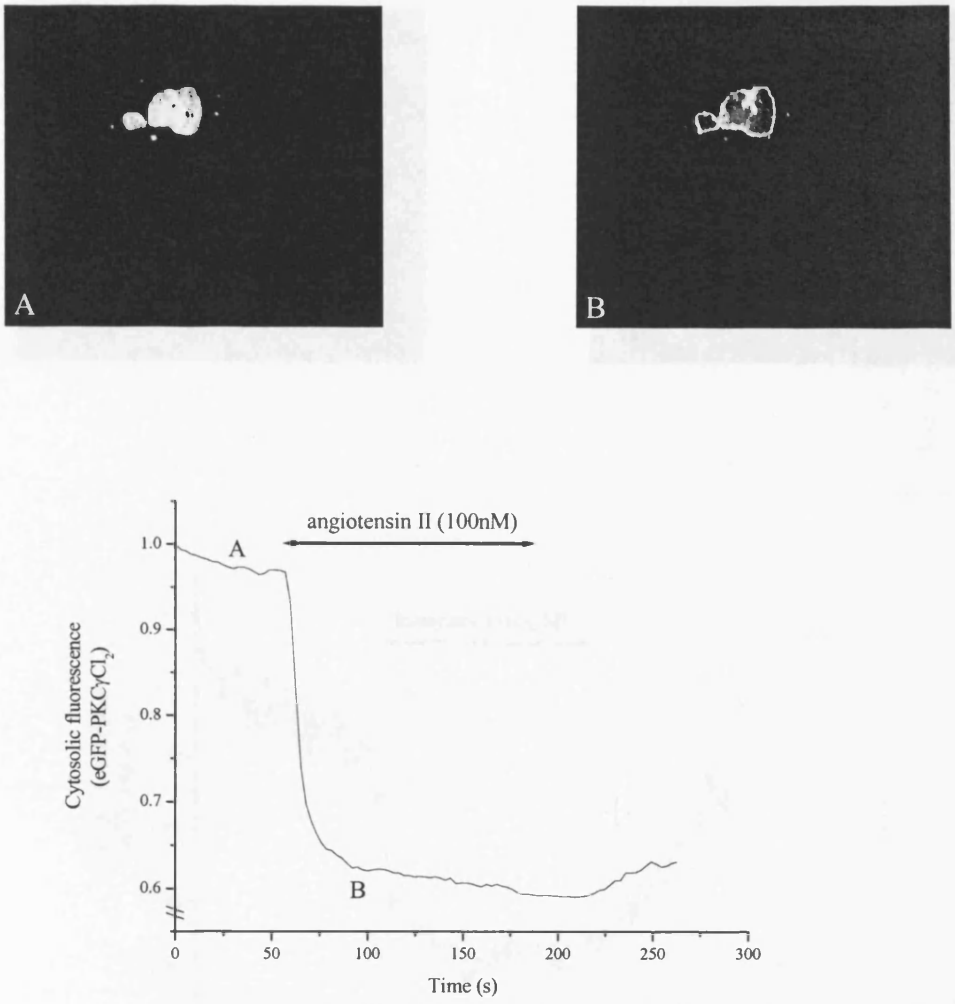


Figure 6.2.4. Single cell imaging of angiotensin II-induced changes in the distribution of eGFP-PKCγCl_2 in cultured bovine adrenal chromaffin cells. Cells transiently transfected with eGFP-PKCγCl_2 were perfused with angiotensin II (100nM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 18 cells in total (~ 42% of examined cells responded)).

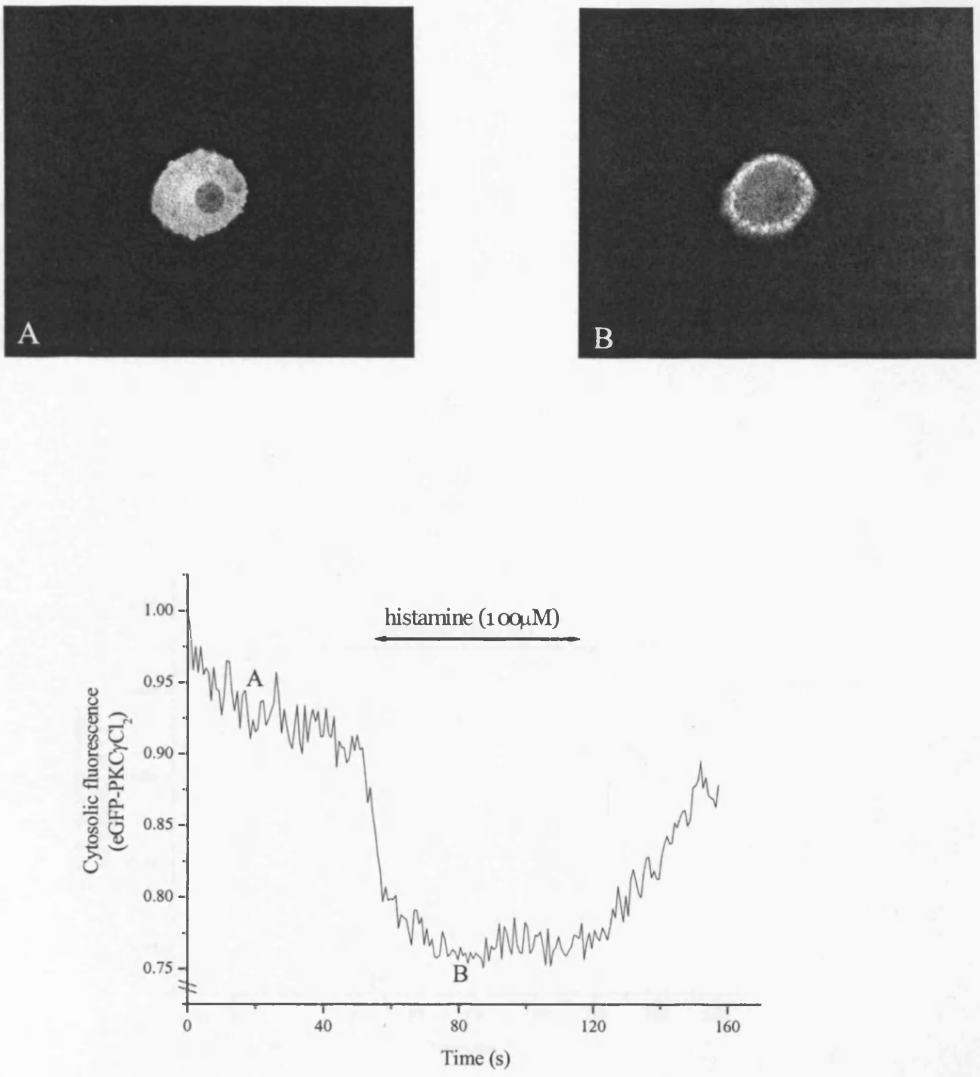


Figure 6.2.5. Single cell imaging of histamine-induced changes in the distribution of eGFP-PKCγCl_2 in cultured bovine adrenal chromaffin cells. Cells transiently transfected with eGFP-PKCγCl_2 were perfused with histamine (100μM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 29 cells in total (~ 37% of examined cells responded)).

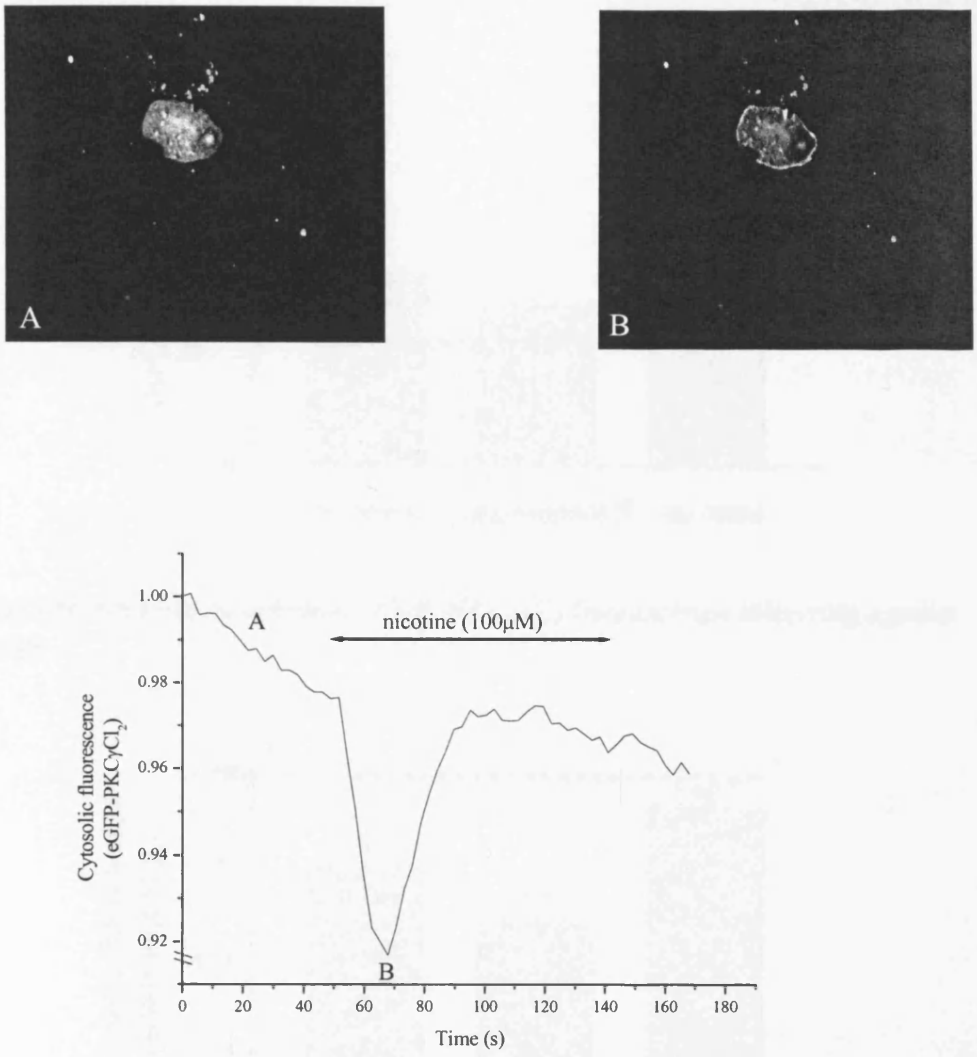
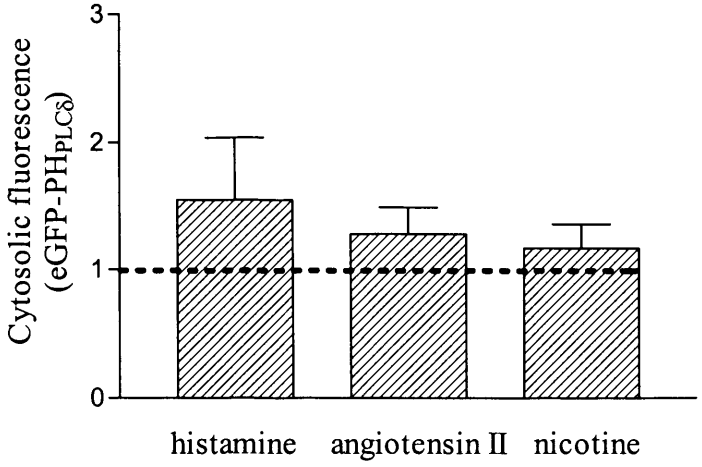


Figure 6.2.6. Single cell imaging of nicotine-induced changes in the distribution of eGFP-PKCγCl_2 in cultured bovine adrenal chromaffin cells. Chromaffin cells transiently transfected with eGFP-PKCγCl_2 were perfused with nicotine (100μM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 24 cells in total (~ 33% of examined cells responded)).

A. Peak changes in cytosolic eGFP-$PH_{PLC\delta1}$ fluorescence following agonist challenge



B. Maximal reduction in cytosolic eGFP-$PKC\gamma Cl_2$ fluorescence following agonist challenge

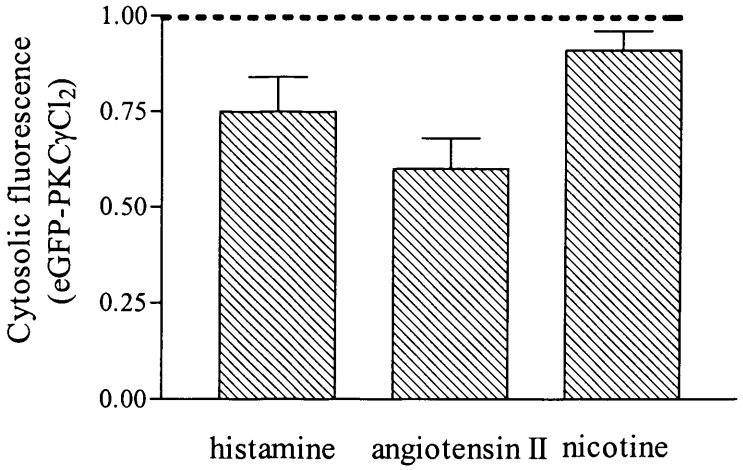


Figure 6.2.7. Summary of changes in cytosolic fluorescence in response to agonist stimulation in cultured bovine adrenal chromaffin cells. Cells transiently transfected with either eGFP-$PH_{PLC\delta1}$ or eGFP-$PKC\gamma Cl_2$ were perfused with agonists and confocal images were collected at a rate of 3-4 frames per second. Panel A: shows the mean maximal peak change in fluorescence for different agonists. Panel B: shows the mean maximal reduction in cytosolic fluorescence following agonist addition. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. The dotted lines indicate the basal fluorescence. Data are mean + SEM from three different cell preparations.

Temporal profiles of total inositol phosphate generation evoked by nicotinic receptor activation

In [^{3}H] inositol-labeled cells, in which inositol monophosphatase activity had been blocked with Li^+, nicotine (100μM) caused a time-dependent accumulation of [^{3}H]-$InsP_x$ (Figure 6.2.8). The greatest accumulation of [^{3}H]-$InsP_x$ evoked by nicotine was achieved at 30 min (203.90 ± 28.20% of basal levels). Similarly, either histamine (100μM) or angiotensin II (100nM) (used here as positive controls for PLC activation) were able to mediate time-related and robust [^{3}H]-$InsP_x$ accumulations (Figure 6.2.8).

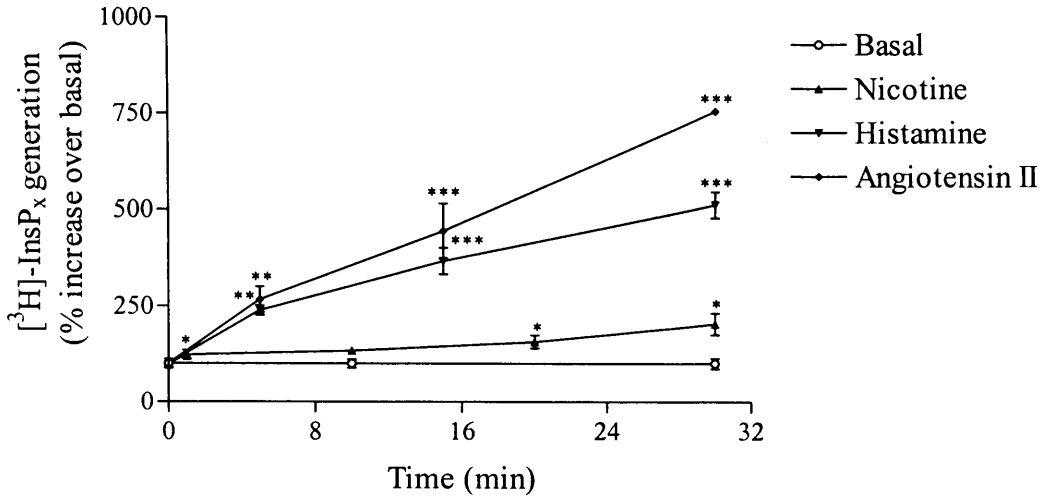


Figure 6.2.8. Temporal profiles of [^{3}H]-InsP$_x$ generation evoked by activation of either nicotinic receptors or G$\alpha_{q/11}$-coupled receptors. Chromaffin cells were prelabelled with [^{3}H]-inositol for 48h before stimulation in the presence of 10mM Li^+ with either nicotine (100μM), histamine (100μM) or angiotensin II (100nM). The accumulation of [^{3}H]-InsP$_x$ was measured as described in "Materials and Methods". Data are presented as percentage increase over basal. Data are mean ± SEM from three different cell preparations (each in triplicate). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. basal by one-way ANOVA followed by post-hoc Dunnett's test.

Effect of wortmannin on nicotine-mediated total [^{3}H]-InsP$_x$ accumulation

Although sterol-like wortmannin is well known for its ability to block phosphatidylinositol 3-kinase (PtdIns 3-kinase) (Okada *et al.*, 1994), at higher concentrations (μM) is also effective at blocking some isoforms of phosphatidylinositol 4-kinase (PtdIns 4-kinase) (Nakanishi *et al.*, 1995; Downing *et al.*, 1996; Willars *et al.*, 1998b), a crucial enzyme in maintaining the supply of the PLCβ substrate, PtdIns(4,5)P_2 by the phosphorylation of PtdIns. Thus, wortmannin was used to potentially inhibit PtdIns(4,5)P_2 generation and to examine the impact of this on nicotinic receptor mediated PLC activation and catecholamine release.

Cells prelabeled with [^{3}H]-inositol were preincubated for 20 min under Li^+ block with or without wortmannin (either 1μM or 10μM). Following this incubation, cells were stimulated for 30 min with either nicotine (100μM) or angiotensin II (100nM, used as a positive control for PLC activation). The [^{3}H]-InsP$_x$ generation evoked by either nicotine or angiotensin II was not affected by preincubation of cells with 1μM wortmannin (Figure 6.2.9). However, [^{3}H]-InsP$_x$ accumulation mediated by either nicotine or angiotensin II were markedly reduced by pre-treatment of cells with 10μM wortmannin (Figure 6.2.9). In this study, the need to use 10μM wortmannin to achieve inhibition of [^{3}H]-InsP$_x$ generation was consistent with Willars et al. (1998b). In addition, the inability of 1μM wortmannin to influence PLC activity shows that the effect was not via inhibition of PtdIns 3-kinase activity as this is inhibited at lower concentrations than PtdIns 4-kinase (Nakanishi *et al.*, 1995).

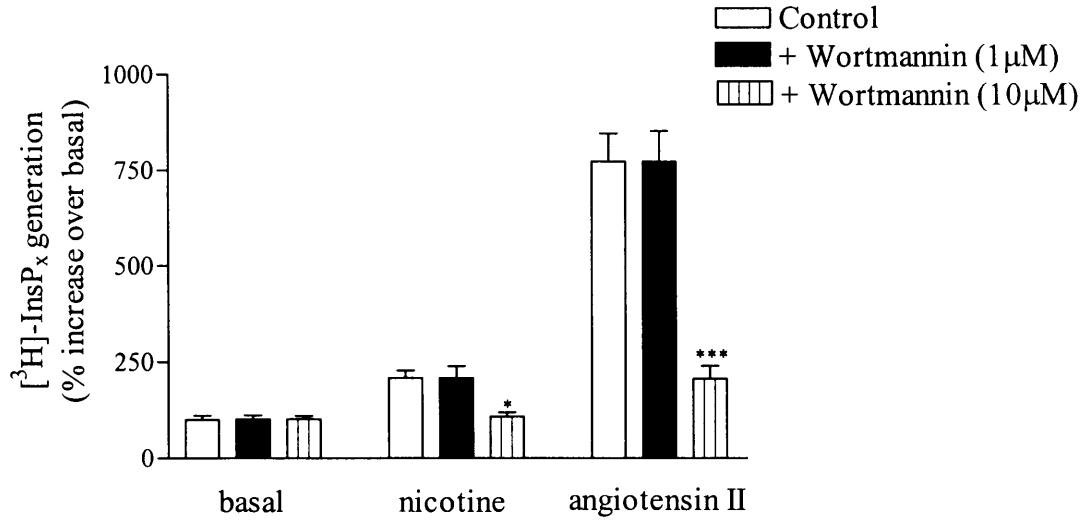


Figure 6.2.9. Effect of wortmannin on nicotinic receptor and $G\alpha_{q/11}$-coupled receptor mediated stimulation of PLC. Cells were prelabelled with [^{3}H]-inositol for 48h before treatment with wortmannin (1μM or 10μM) for 20 min at 37°C in the presence of 10mM Li^+. Cells were then stimulated with either nicotine (100μM) or angiotensin II (100nM) for a further 30 min. The accumulation of [^{3}H]-$InsP_x$ was measured as described in "Materials and Methods". Data are presented as percentage increase over basal (the basal values were 240 ± 16 dpm/well). Data are mean + SEM from three different cell preparations (each in triplicate). * $P<0.05$, *** $P<0.001$ vs. control by one-way ANOVA followed by post-hoc Dunnett's test.

Effect of wortmannin on catecholamine secretion evoked by nicotinic receptors activation

Wortmannin inhibits the generation of PtdIns(4,5)P_2 thereby inhibiting the activity of PLC. If either PtdIns(4,5)P_2 or PLC activity are required for catecholamine release, wortmannin would therefore be expected to inhibit nicotinic receptor-mediated release.

Cells were pre-incubated with or without wortmannin (10μM) for 20 min and then stimulated with nicotine (100μM) for a further 10 min. Catecholamine secretion was measured as described in "Materials and Methods". Inhibition of PtdIns 4-kinase by wortmannin had no effect on basal release while it markedly attenuated secretion of catecholamines evoked by nicotine (Figure 6.2.10).

Wortmannin also dramatically reduced the release of catecholamines mediated by either histamine (100μM) or angiotensin II (100nM) used as positive controls for the inhibition of PLC activity (Figure 6.2.10).

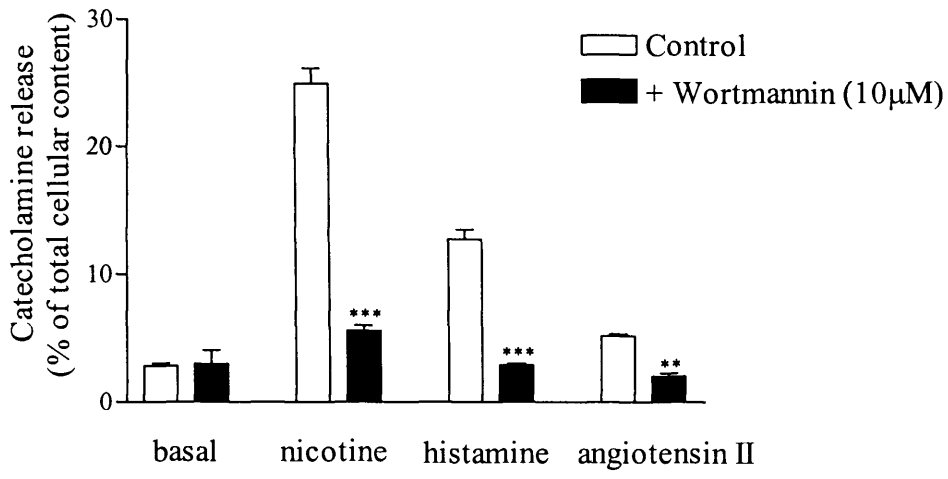


Figure 6.2.10. Effect of wortmannin on catecholamine secretion evoked by either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors. Chromaffin cells were incubated with wortmannin for 20 min at 37°C before stimulation with either nicotine (100μM), histamine (100μM) or angiotensin II (100nM). Catecholamine release over 10 min stimulation with agonist was measured as described in "Materials and Method" and is expressed as percentage of total cellular content. Data are mean + SEM from three different cell culture preparations. ** $P<0.01$, *** $P<0.001$ vs. appropriate control by unpaired Student's *t*-test.

Inhibition of PLC with U73122 reduces nicotine-mediated accumulation of total [^{3}H]-InsP$_x$ and catecholamine secretion

To assess the involvement of PLC in nicotinic receptor-mediated catecholamine secretion, the selective PLC inhibitor U-73122 or its inactive isomer U-73343 (Zhang *et al.*, 1995b) were used. Initially, the effect of PLC inhibition on total [^{3}H]-InsP$_x$ generation evoked by nicotine was examined and the impact of this inhibition on secretion of catecholamines was examined subsequently.

Pretreatment of cells with the PLC inhibitor U-73122 (either 1μM or 10μM, 30 min) significantly inhibited nicotine-mediated accumulation of [^{3}H]-InsP$_x$ (Figure 6.2.11) and catecholamine secretion (Figure 6.2.12).

As a positive control, U-73122 (either 1μM or 10μM, 30 min) was also shown to inhibit histamine-mediated [^{3}H]-InsP$_x$ accumulation (Figure 6.2.11) and release of catecholamines (Figure 6.2.12). In contrast, U-73343 (10μM), the inactive analogue of U-73122 did not influence either nicotine or histamine-evoked [^{3}H]-InsP$_x$ accumulation and secretion of catecholamines (Figure 6.2.11 and Figure 6.2.12).

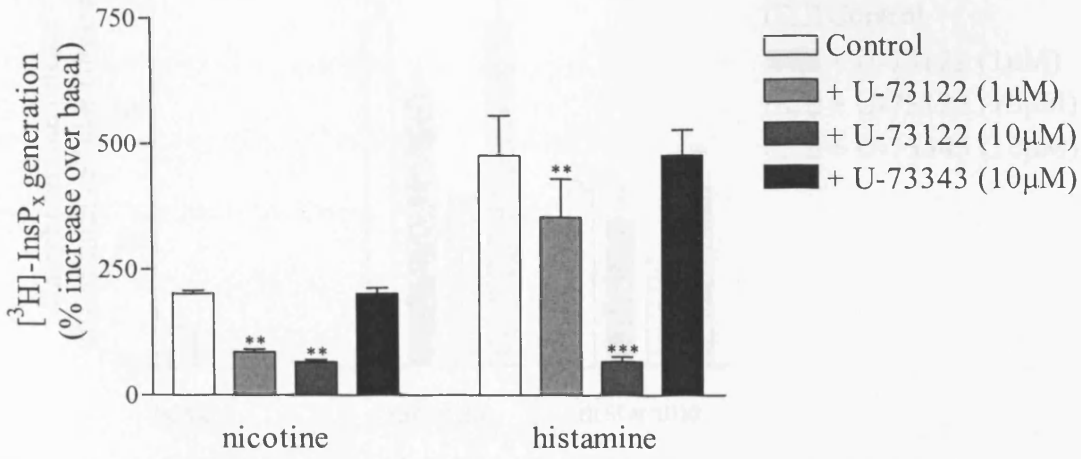


Figure 6.2.11. Effect of the PLC inhibitor U73122 or its negative control U73343 on nicotinic receptor and $G\alpha_{q/11}$-coupled receptor mediated stimulation of PLC. Chromaffin cells were prelabelled with [^{3}H]-inositol for 48h before treatment with either U73122 (1 or 10μM, 30 min) or U73343 (10μM, 30 min) at 37°C in the presence of 10mM Li^+ then stimulated with either nicotine (100μM) or histamine (100μM). The accumulation of [^{3}H]-$InsP_x$ was measured as described in "Materials and Methods". Data are presented as percentage increase over basal (the basal values are 242 ± 17 dpm/well). Data are mean + SEM from three different cell preparations (each in triplicate). ** $P<0.01$, *** $P<0.001$ vs. control by one-way ANOVA followed by post-hoc Dunnett's test.

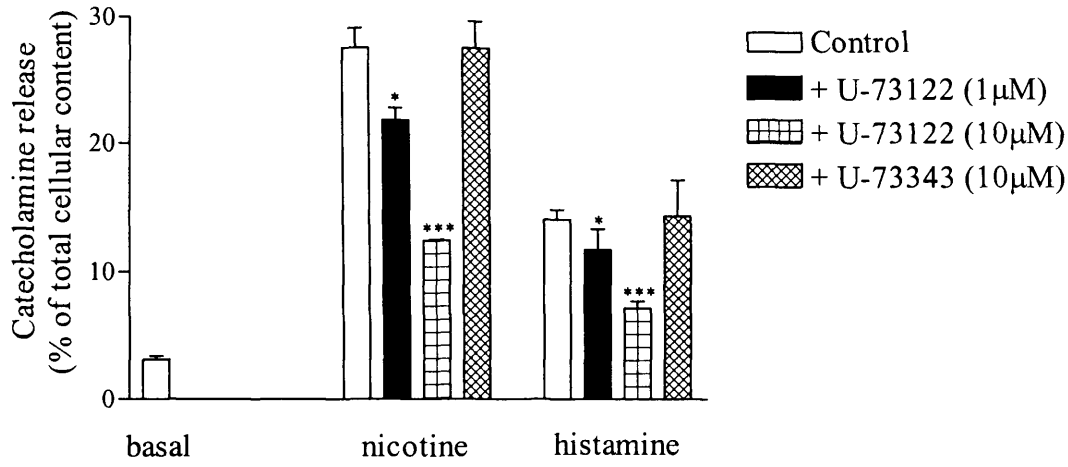


Figure 6.2.12. Effect of the PLC inhibitior U73122 or its negative control U73343 on catecholamine secretion evoked by nicotinic receptor and $G\alpha_{q/11}$-coupled receptor activation. Chromaffin cells were incubated with either U73122 (1μM or 10μM, 30 min) or U73343 (10μM, 30 min) at 37°C before stimulation with either nicotine (100μM) or histamine (100μM). Catecholamines released over 10 min stimulation with agonist were measured as described in "Materials and Methods" and are expressed as percentage of total cellular content. Data are mean + SEM from three different cell preparations. * $P<0.05$, *** $P<0.001$ vs. control by one-way ANOVA followed by post-hoc Dunnett's test.

The dependence on extracellular Ca^{2+} of PLC stimulation mediated by nicotinic receptors

Ca^{2+}-free buffer (i.e. no added Ca^{2+} to Krebs'/HEPES buffer) was used to test whether nicotinic receptor triggered [^{3}H]-$InsP_x$ generation (as an index of PLC activation) was dependent upon influx of extracellular Ca^{2+}.

Removal of extracellular Ca^{2+} essentially abolished nicotinic receptor-mediated accumulation of [^{3}H]-$InsP_x$ (Figure 6.2.13). In contrast, removal of extracellular Ca^{2+} did not abolish but did attenuate [^{3}H]-$InsP_x$ accumulation in response to either histamine or angiotensin II, particularly at later time points (i.e. 15 and 30 min) (Figure 6.2.14).

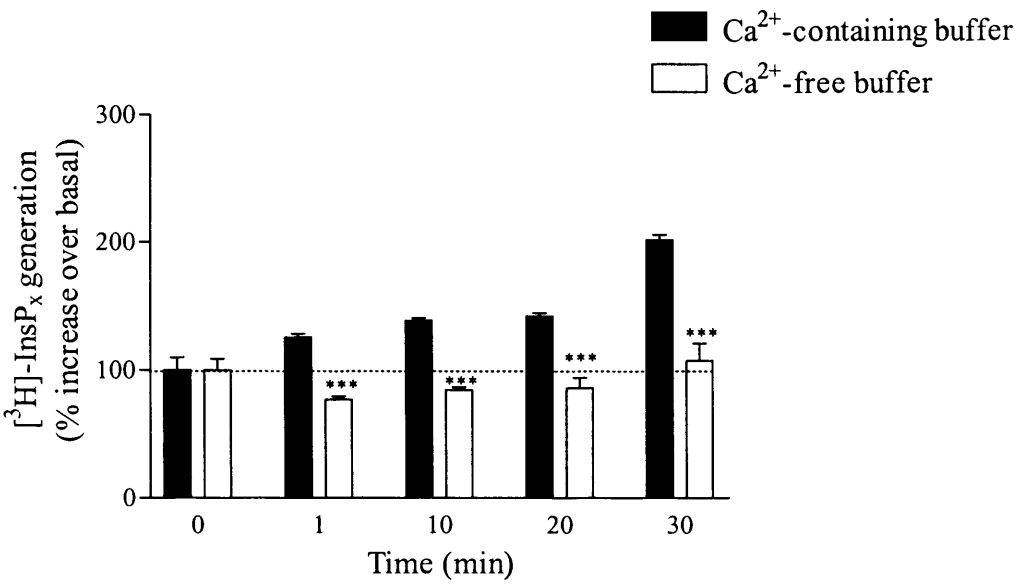
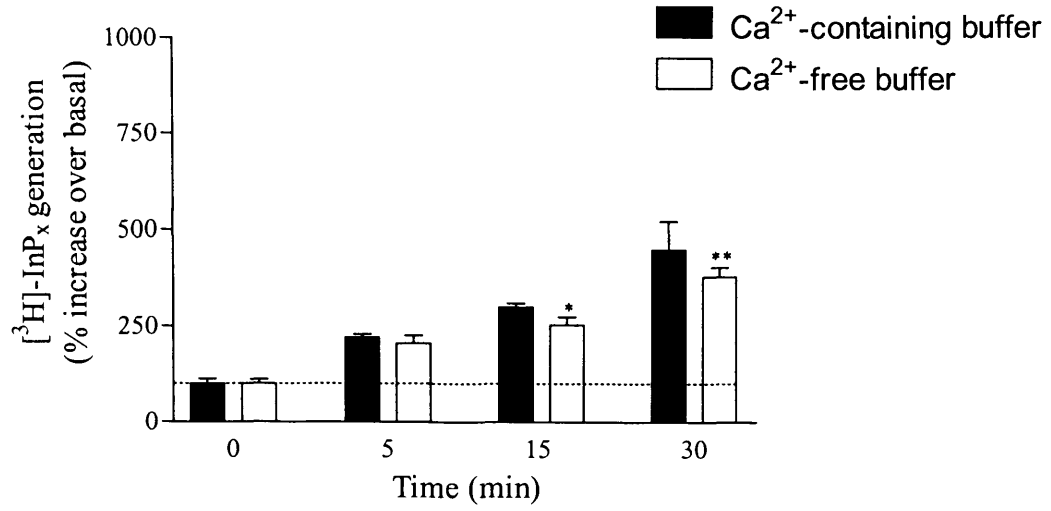


Figure 6.2.13. Time course of nicotinic receptor-mediated accumulation of total inositol phosphates in the presence or absence of extracellular Ca^{2+}. Cells were prelabelled with [^{3}H]-inositol for 48h before stimulation with nicotine (100μM) in the presence of 1.3mM $[Ca^{2+}]_e$ or its absence (i.e., no added Ca^{2+}). The accumulation of [^{3}H]-$InsP_x$ was measured as described in "Materials and Methods". Data are presented as percentage increase over basal. Dotted line indicates the basal [^{3}H]-$InsP_x$ (223 ± 15 dpm/well). Data are mean + SEM from three different cell preparations (each in triplicate). *** $P<0.001$, vs. control in the presence of extracellular Ca^{2+} by unpaired Student's *t*-test.

A.



B.

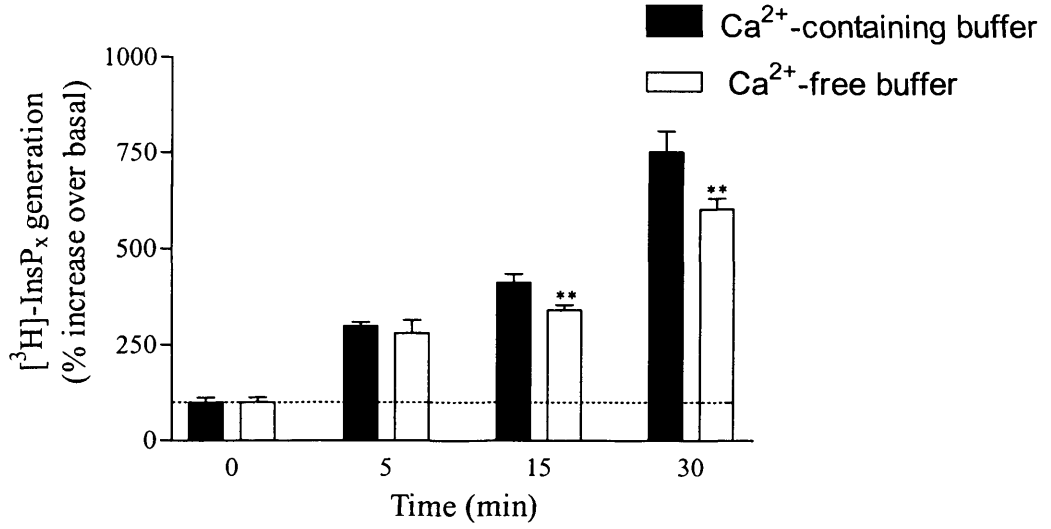


Figure 6.2.14. Time course of $G\alpha_{q/11}$-coupled receptor-mediated accumulation of total inositol phosphates in the presence or absence of extracellular Ca^{2+}. Cells were prelabelled with [^{3}H]-inositol for 48h before stimulation with either histamine (100μM) (panel A) or angiotensin II (100nM) (panel B) in the presence of 1.3mM $[Ca^{2+}]_e$ or its absence (i.e. no added Ca^{2+}). The accumulation of [^{3}H]-$InsP_x$ was measured as described in "Materials and Methods". Data are presented as percentage increase over basal. Dotted lines indicate the basal [^{3}H]-$InsP_x$ (239 ± 13 dpm/well). Data are mean + SEM from three different cell preparations (each in triplicate). * $P<0.05$, ** $P<0.01$ vs. control in the presence of extracellular Ca^{2+} by unpaired Student's t-test.

Effect of cholinergic receptor antagonists on nicotine and acetylcholine mediated activation of PLC

Since acetylcholine is the physiological secretagogue for chromaffin cells (Douglas and Rubin, 1961) and is able to activate both types of cholinergic receptors (nicotinic and muscarinic) (Wilson and Kirshner, 1977), it was used here along with selective nicotinic and muscarinic receptor antagonists to further explore the role of these receptors in the activation of PLC.

As seen in Figure 6.2.15 both nicotine (100μM) and acetylcholine (100μM) elicited [^{3}H]-InsP$_x$ accumulation although that evoked by acetylcholine was significantly greater. Preincubation of cells with atropine (10μM), a selective muscarinic receptor (mAChR) antagonist significantly reduced acetylcholine-induced total [^{3}H]-InsP$_x$ generation but had no effect on nicotine-evoked [^{3}H]-InsP$_x$ production.

In contrast, preincubation with either d-tubocurarine (100μM) or hexamethonium (1mM), selective nicotinic receptor (nAChR) blockers, essentially abolished nicotine-mediated [^{3}H]-InsP$_x$ accumulation and partially inhibited the response to acetylcholine.

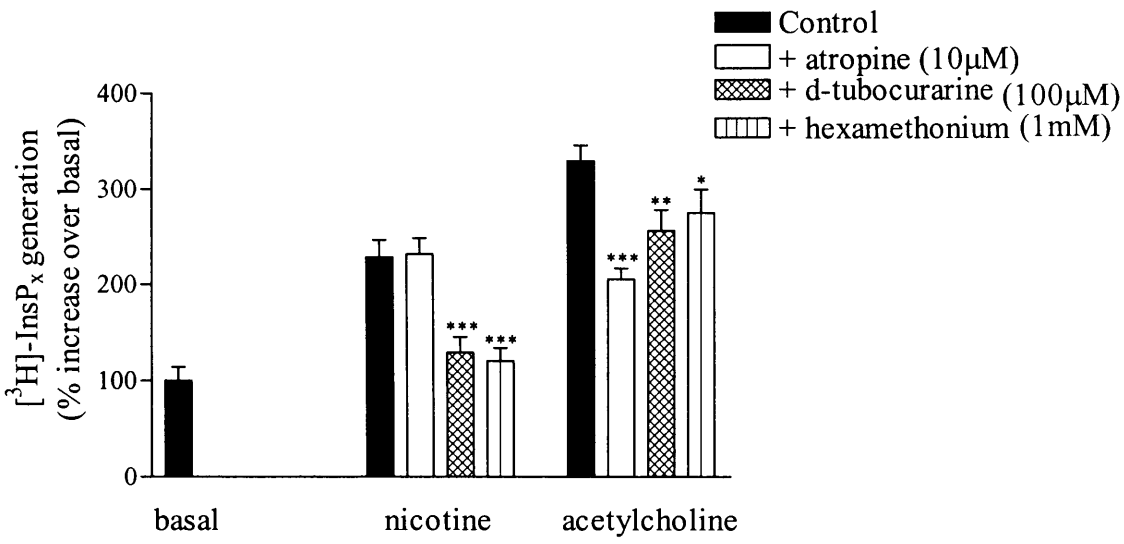


Figure 6.2.15. Effect of cholinergic receptor antagonists on nicotine- and acetylcholine-mediated stimulation of PLC. Cells were prelabelled with [^{3}H]-inositol for 48h before treatment with either vehicle control, atropine (10μM), d-tubocurarine (100μM), or hexamethonium (1mM) for 10 min at 37°C in the presence of 10mM Li^+. Cells were then stimulated with either nicotine (100μM) or acetylcholine (100μM). The accumulation of [^{3}H]-$InsP_x$ was measured as described in "Materials and Methods". Data are presented as percentage increase over basal (210 ± 11 dpm/well). Data are mean + SEM from three different cell culture preparations (each in triplicate). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control by one-way ANOVA followed by post-hoc Dunnett's test.

Effect of apyrase on total [^{3}H]-InsP$_x$ accumulation mediated by nicotinic receptors

Chromaffin cells of the adrenal medulla store catecholamines, nucleotides and peptides in membrane-bound organelles: the chromaffin vesicles (Trifaró, 1977; Winkler and Westhead, 1980). Upon stimulation chromaffin vesicles release their content to the cell exterior by exocytosis. Thus, ATP is co-released with catecholamines and could act via P_2Y purinoceptors that are coupled to PLC. Indeed, chromaffin cells express P2Y receptors (Currie and Fox, 1996) and thus ATP could activate PLC following nicotinic receptor stimulation.

To test the contribution of ATP to nicotinic receptor-mediated PLC activation, the effect of apyrase (ATP diphosphohydrolase, which converts nucleotide polyphosphates to nucleoside monophosphates (Curdova *et al.*, 1982; Tim *et al.*, 2002) was examined as the ATP hydrolysis products adenosine and AMP do not increase $[Ca^{2+}]_i$ or stimulate inositol lipid metabolism (Gosink and Forsberg, 1993).

Preincubation of cells with apyrase (10 u.ml^{-1}, grade III, 10 min) under Li^+-block of inositol monophosphatase did not elicite any significant inhibition of total [^{3}H]-InsP$_x$ accumulation triggered by nicotinic receptor stimulation. However, apyrase significantly reduced total inositol [^{3}H]-InsP$_x$ generation evoked by UTP (300μM), a P_2Y_2 selective agonist that was used here as a positive control (Figure 6.2.16).

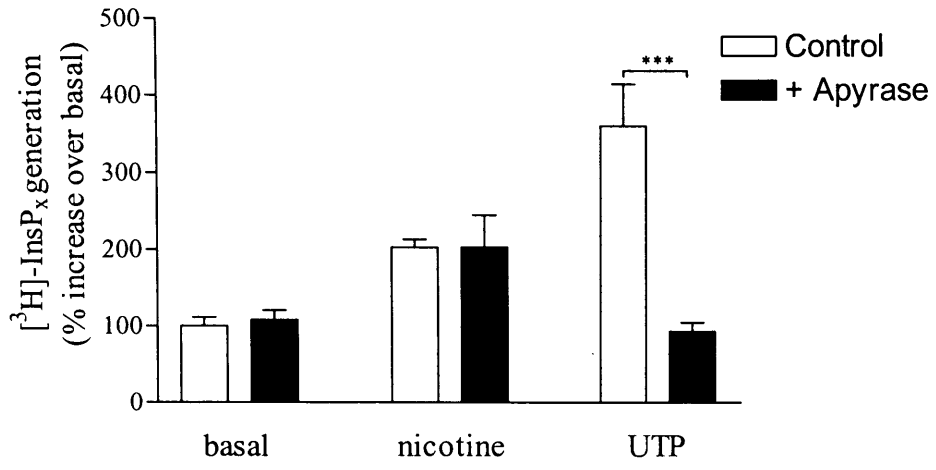


Figure 6.2.16. Effect of apyrase on nicotine- and UTP-mediated stimulation of PLC. Cells were prelabelled with [^{3}H]-inositol for 48h before treatment with apyrase (10 u.ml^{-1}) for 10 min at 37°C in the presence of 10mM Li^+ then stimulated with either nicotine (100μM) or UTP (300μM) for a further 30 min. The accumulation of [^{3}H]-$InsP_x$ was measured as described in "Materials and Methods". Data are presented as percentage increase over basal (211 ± 12 dpm/well). Data are mean + SEM from three different cell preparations (each in triplicate). *** $P<0.001$ vs. control, one-way ANOVA followed by post-hoc Dunnett's test.

Effect of PTX on nicotinic receptor-mediated PLC activation

Opioids are also co-released with catecholamines and nucleotides from chromaffin granules (Livett *et al.*, 1981). Although, opioids activate $G\alpha_i$-coupled receptors, those can in some circumstances activate PLC (Charles *et al.*, 2003; Werry *et al.*, 2003). However, such activation is sensitive to inhibition of $G\alpha_i$ with pertussis toxin PTX.

Chromaffin cells express opioid receptors (Bunn *et al.*, 1988; Margioris *et al.*, 1995). To investigate whether nicotinic receptor-mediated PLC activation is a consequence of released opioids acting on $G\alpha_i$-coupled μ- and δ-opioid receptors the effect of PTX on total [^{3}H]-$InsP_x$ generation mediated by nicotinic receptors was studied.

Treatment of cells with PTX (100 ng.ml^{-1}, 24h) had no significant effect on either basal or nicotine-mediated [^{3}H]-$InsP_x$ accumulation. In contrast, PTX significantly attenuated total [^{3}H]-$InsP_x$ production mediated by angiotensin II (100nM), demonstrating that at least part of this response was mediated through $G\alpha_{i/o}$ (Figure 6.2.17).

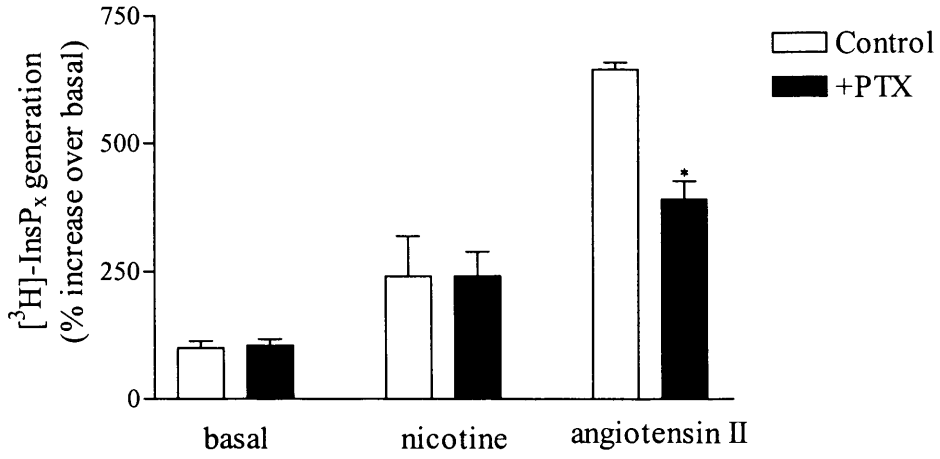


Figure 6.2.17. Effect of pertussis toxin on nicotine- and angiotensin II-mediated stimulation of PLC. Cells were prelabelled with [^{3}H]-inositol for 24h before treatment with pertussis toxin (PTX) (100 ng.ml^{-1}) for a further 24h at 37°C. Cells were then stimulated with either nicotine (100μM) or angiotensin II (100nM) for 30 min at 37°C in the presence of 10mM Li^+. The accumulation of [^{3}H]-$InsP_x$ was measured as described in "Materials and Methods". Data are presented as percentage increase over basal. Data are mean + SEM from three different cell preparations (each in triplicate). * $P<0.05$ vs. control, one-way ANOVA followed by post-hoc Dunnett's test.

Effect of stabilization of cortical F-actin on [^{3}H]-InsP$_x$ accumulation mediated by nicotinic receptors

Although efforts were made to assess the possible contribution of released compounds to nicotine-mediated PLC activation, the experiments do not cover all possible receptor types. As an alternative, jasplakinolide was used. This blocks catecholamine release and is likely therefore to the stimulated release of any other potentially receptor active compounds. The effects of jasplakinolide on nicotinic receptor-mediated PLC activation were therefore investigated.

Pretreatment of cells with jasplakinolide (10μM, 30 min) had no significant effect on total [^{3}H]-InsP$_x$ generation induced by either nicotine (100μM) or angiotensin II (100nM) (Figure 6.2.18).

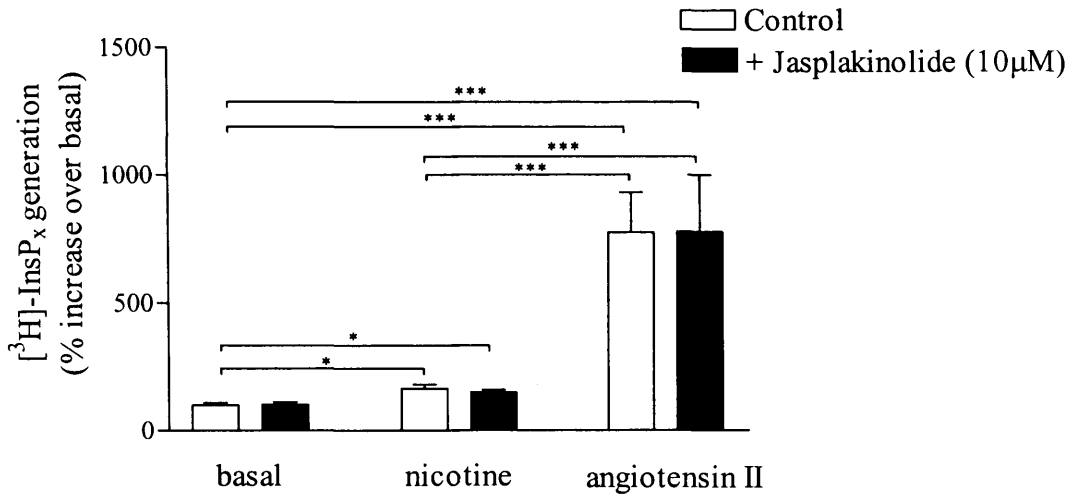


Figure 6.2.18. Effect of jasplakinolide, a cortical F-actin stabilizer, on nicotinic receptor and $G\alpha_{q/11}$-coupled receptor mediated stimulation of PLC. Cells were prelabelled with [^{3}H]-inositol for 48h before incubation with or without jasplakinolide (10μM) for 30 min at 37°C in the presence of 10mM Li^+ and then stimulated with either nicotine (100μM) or angiotensin II (100nM) for a further 30 min. The accumulation of [^{3}H]-$InsP_x$ was measured as described in "Materials and Methods". Data are presented as percentage increase over basal (224 ± 16 dpm/well). Data are mean + SEM from three different cell preparations (each in triplicate). * $P<0.05$, ** $P<0.01$, one-way ANOVA followed by post-hoc Bonferroni's test (not all comparisons are shown).

Effect of U73122 and PMA on nicotine-mediated catecholamine secretion

This experiment aimed to investigate the role of PLC activation in nicotinic receptor-evoked secretion of catecholamines.

The results demonstrated that catecholamine release evoked by nicotinic receptor is derived by PLC not other components. To verify this, the effect of combination of either nicotine (100μM) and U73122 (10μM) or nicotine (100μM), PMA (1μM) and U73122 (10μM) on secretion were tested. Data indicated that the effect of nicotine on secretion markedly reduced in the presence of U73122 (17.68 ± 0.17% vs. 24.22 ± 0.81% of control) (** $P<0.01$ vs. control, as determined by ANOVA followed by Bonferroni's test).

PMA was added to this combination to substitute for inhibited PLC but it was not able to induce any significant changes in catecholamine secretion from that induced by combination of nicotine and U73122 (17.78 ± 0.35% (data of nicotine plus U73122 plus PMA) vs. 17.68 ± 0.17% (data of nicotine plus U73122) (Figure 6.2.19). These data are difficult to interpreted for the following reasons. The inhibition of nicotine-mediated release by U73122 suggests PLC activation is important, possibly for the generation of DAG and the subsequent activation of PKC (or other DAG targets). Any such lack of DAG generation should be overcome for by PMA. The inability of PMA to overcome the inhibition by U73122 suggests either that this compound is mediating its effects via a mechanism independent of PLC or that another aspect of PLC activity (apart from DAG generation) is important. This could include PLC-dependent Ca^{2+} signalling. Indeed the nicotinic receptor-evoked Ca^{2+} response was reduced by both U73122 and thapsigargin, suggesting that a PLC-dependent rise of $[Ca^{2+}]_i$ is an important component for release.

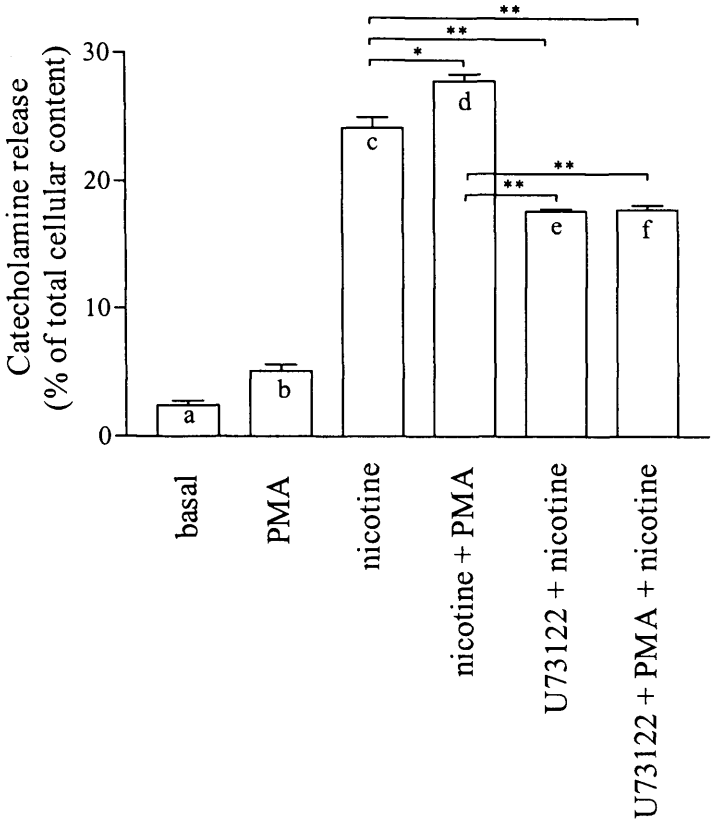


Figure 6.2.19. The effect of U73122 and PMA on nicotine-mediated catecholamine secretion. Chromaffin cells were incubated with U73122 (10μM) for 30 min at 37°C before stimulation with both PMA (1μM) and nicotine (100μM). Catecholamine released over a 10 min stimulation with agonist was measured as described in "Materials and Methods" and is expressed as percentage of total cellular content. Data are mean + SEM from three different cell preparations. * $P<0.05$, ** $P<0.01$, one-way ANOVA followed by Bonferroni's test. b, c, d, e and f are significantly different from a at *** $P<0.001$. (Not all comparisons are shown on the graph).

Effect of thapsigargin on nicotine-mediated Ca^{2+} signalling

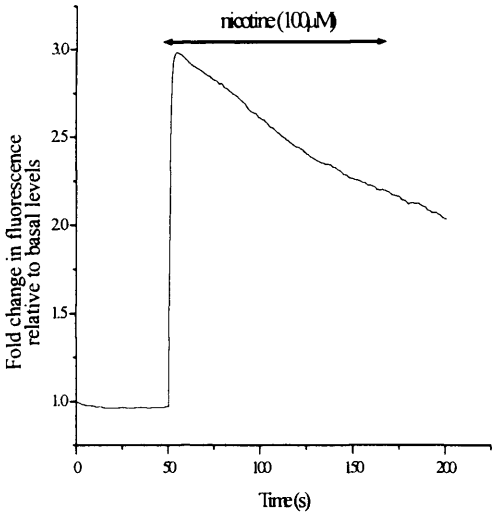
The influx of Ca^{2+} into chromaffin cells appeared to play a major role in regulating PLC activity in response to nicotinic receptor activation (6.2.13). However, it was unclear if nicotine evoked the release of intracellular Ca^{2+} and whether this was involved in promoting secretion. Thus, thapsigargin has been shown to selectively prevent sequestration of Ca^{2+} into the endoplasmic reticulum by inhibiting the Ca^{2+}-ATPase pumps of the endoplasmic reticulum (Thastrup *et al.*, 1990; Lytton *et al.*, 1991). Confocal imaging of Ca^{2+} signals and fluo-3-loaded cells were used to monitor the changes in $[Ca^{2+}]_i$.

Challenge of cells with nicotine (100μM) revealed a robust (3.0 fold over basal), rapid (30s) peak followed by a lower (1.81 fold over basal) sustained phase of elevated $[Ca^{2+}]_i$ (Figure 6.2.20: panel A). Treatment with thapsigargin slightly but significantly reduced peak (2.10 vs. 3.0 of control fold over basal) and plateau (1.36 vs. 1.81 of control, fold over basal) Ca^{2+} response mediated by nicotine (6.2.20: panel B).

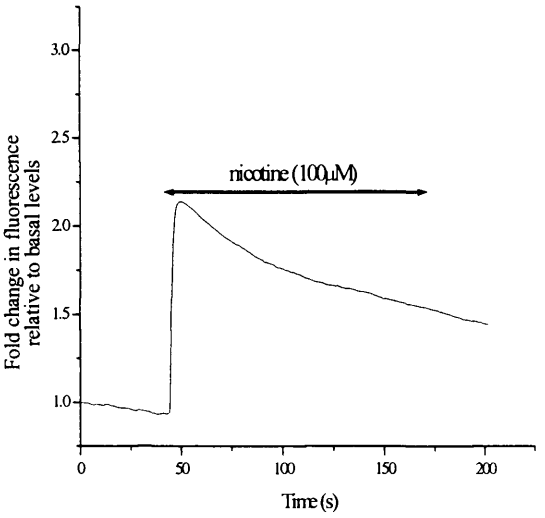
Histamine (100μM), used as a positive control for a response depending upon intracellular Ca^{2+} stores, induced a peak (2.12 fold over basal) and sustained plateau phase (1.50 fold over basal) of Ca^{2+} signalling (Figure 6.2.21: panel A). However, Ca^{2+} signalling evoked by histamine was less robust compared to nicotine. Treatment with thapsigargin abolished both peak and sustained phase Ca^{2+} responses to histamine (6.2.21: panel B).

The mean data of the Ca^{2+} responses are summarized in Figure 6.2.20: panel C.

A. Control



B. Thapsigargin pretreated



C. Summary data

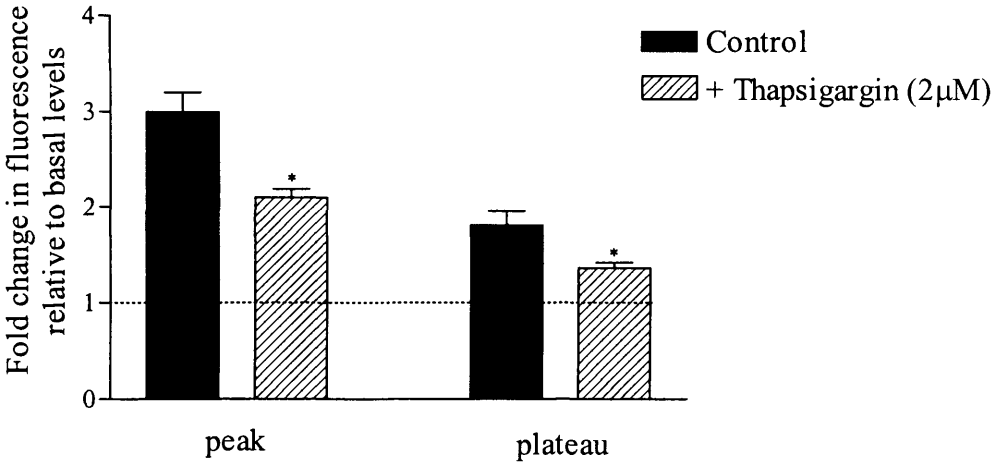
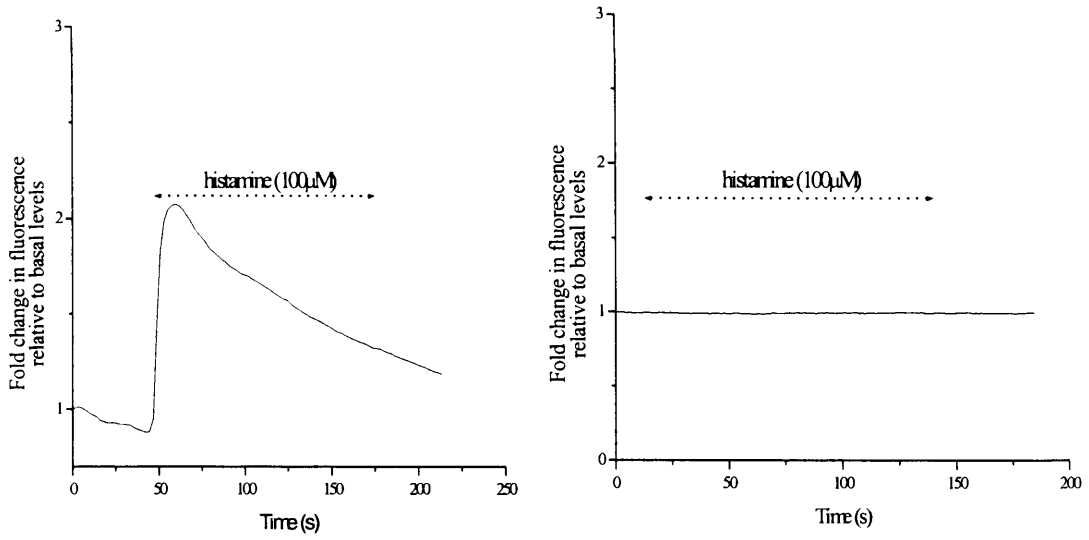


Figure 6.2.20. Effect of thapsigargin on nicotine-mediated Ca^{2+} signalling in bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at λ488 nm and imaged on the confocal microscope. Panel A: cells were challenged with nicotine (100μM) after 30s. Panel B: cells were pretreated with thapsigargin (2μM, 10 min) at 37°C then challenged with nicotine (100μM). Bar indicates the presence of nicotine. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three independent experiments (three adrenal gland preparations, approximately 180 cells in total). Panel C shows the summary data of three independent cell culture preparations. Data are mean + SEM of three independent cell culture preparations. * $P<0.05$ vs. appropriate control (peak or plateau) by unpaired Student's *t*-test.

A. Control

B. Thapsigargin pretreated



C. Summary data

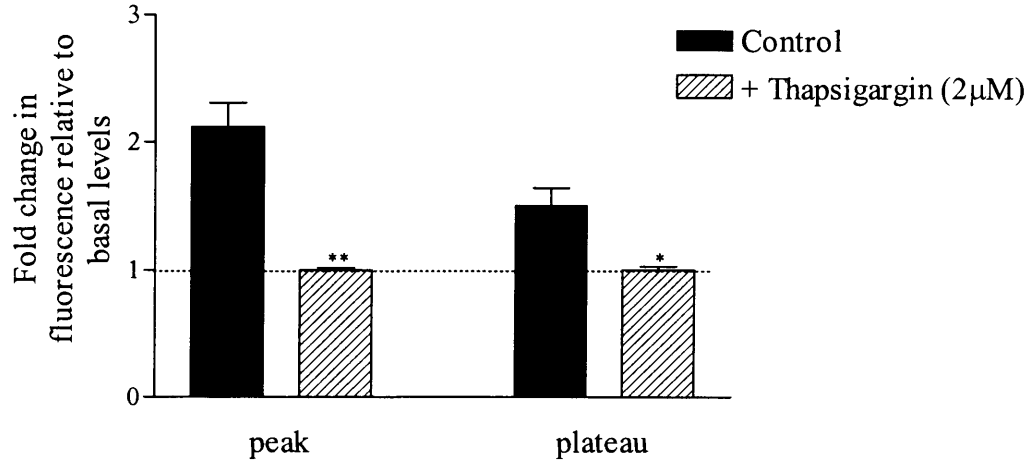


Figure 6.2.21. Effect of thapsigargin on histamine-mediated Ca^{2+} signalling in bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at λ488 nm and imaged on the confocal microscope. Panel A: cells were challenged with histamine (100μM) after 30s. Panel B: cells were pretreated with thapsigargin (2μM, 10 min) at 37°C then challenged with histamine (100μM). Bar indicates the presence of histamine. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three independent experiments (three adrenal gland preparations, approximately 180 cells in total). Panel C shows the summary data of three independent cell culture preparations. Data are mean + SEM of three independent cell culture preparations. * $P<0.05$, ** $P<0.01$ vs. appropriate control (peak or plateau) by unpaired Student's *t*-test.

Effect of U73122 on nicotine-mediated Ca^{2+} signalling

Confocal imaging of Ca^{2+} signalling in fluo-3 loaded cells and U73122 were used to determine if the activation of PLC contributed to the Ca^{2+} signalling mediated by nicotinic receptors.

Pre-treatment of cells for 30 min with U73122 (10μM, 30 min) reduced both the peak (Figure 6.2.22: panel A) and sustained phase (1.05 ± 0.08% vs. 1.81 ± 0.15%) (Figure 6.2.22: panel C) of $[Ca^{2+}]_i$ elevation mediated by nicotine (100μM). U73343 (10μM, 30 min) used as negative control for U73122 had no effect on Ca^{2+} signalling evoked by nicotine (Figure 6.2.22: panel B). Mean data are summarized in Figure 6.2.22: panel D.

Preincubation of cells with U73122 abolished the Ca^{2+} response mediated by histamine (100μM), which was used here as a positive control for PLC activation (Figure 6.2.23: panel A and C). However, U73343 failed to antagonize histamine-mediated Ca^{2+} signalling (Figure 6.2.23: panel B). Mean data are summarized in Figure 6.2.23: panel D.

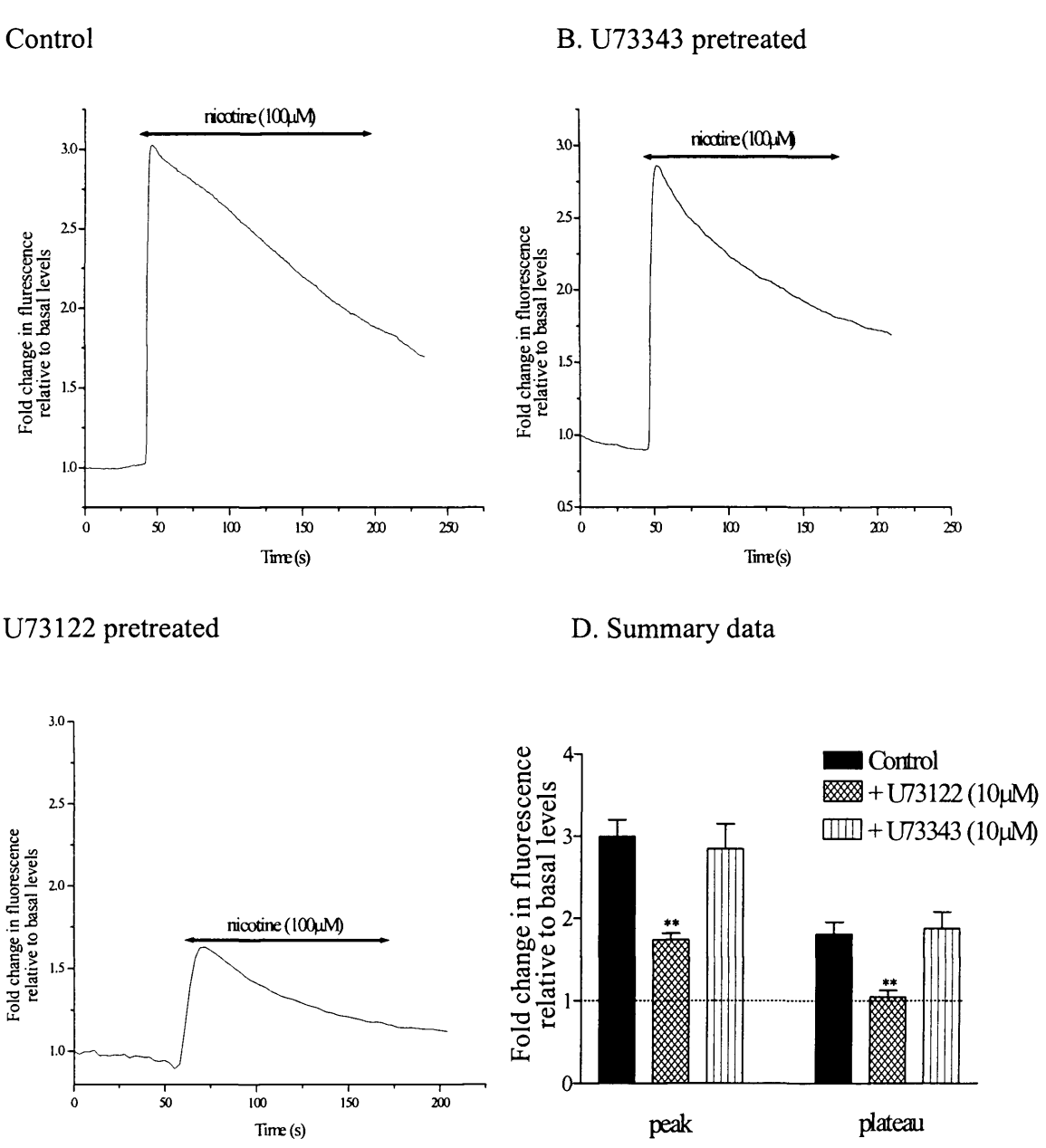


Figure 6.2.22. Effect of U73122 on nicotine-mediated Ca^{2+} signalling in bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at λ488 nm and imaged on the confocal microscope. Panel A: cells were challenged with nicotine (100μM) after 30s. Cells were pretreated with either U-73343 (10μM, panel B) or U-73122 (10μM, panel C) for 30 min at 37°C then challenged with nicotine (100μM, panels B and C). Bars indicate the presence of nicotine. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three independent experiments (three adrenal gland preparations, approximately 150 cells in total for each condition). Panel D shows the summary data of three independent cell culture preparations. Data are mean + SEM and ** P <0.01 vs. appropriate control (peak or plateau) by ANOVA followed by post hoc Dunnett's test.

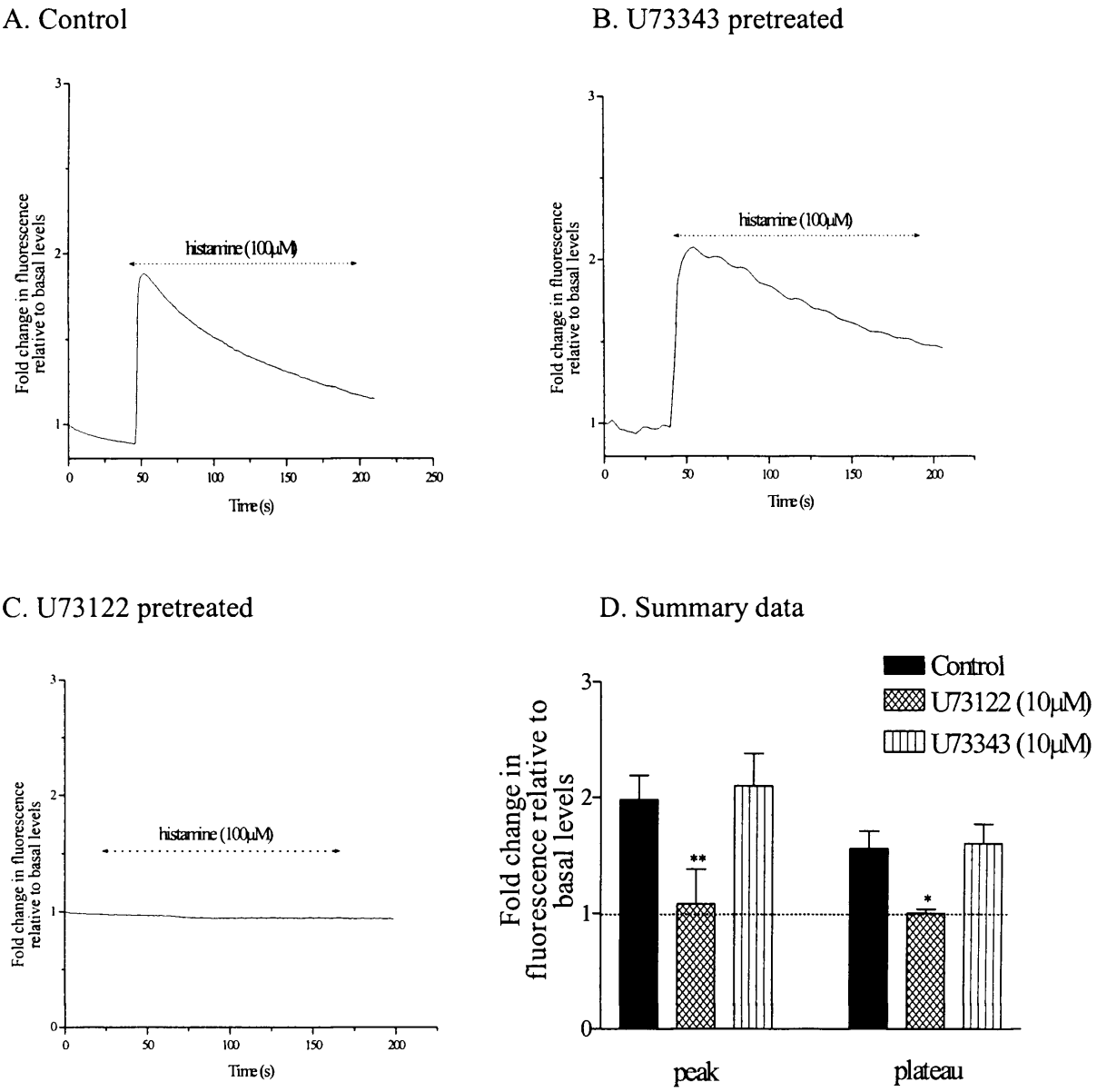


Figure 6.2.23. Effect of U73122 on histamine-mediated Ca^{2+} signalling in cultured bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at λ488 nm and imaged on the confocal microscope. Panel A: cells were challenged with histamine (100μM) after 30s. Cells were pretreated with either U73343 (10μM, panel B) or U73122 (10μM, panel C) for 30 min at 37°C then challenged with histamine (100μM, panel B and C). Bars indicate the presence of histamine. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three independent experiments (three adrenal gland preparations, approximately 150 cells in total for each condition). Panel D shows the summary data of three independent cell culture preparations. Data are mean + SEM and * P <0.05, ** P<0.01 by one-way ANOVA followed by post-hoc Dunnett's test.

Effect of Cd^{2+} on nicotine and KCl mediated Ca^{2+} signalling

The data described previously demonstrated that PLC activation in response to nicotine was Ca^{2+}-dependent (see Figure 6.2.13). As Ca^{2+} entry in response to nicotinic receptor activation could be via either VOCCs or the nicotinic receptor channels themselves (See Chapter 3: Section 3.3.4), these studies sought to distinguish between the contribution of VOCCs and nicotinic receptor channels to Ca^{2+} signalling using confocal imaging of $[Ca^{2+}]_i$ in fluo-3 loaded cells.

Cd^{2+} (100μM) rapidly and reversibly blocks all voltage-activated Ca^{2+} channels but at this concentration does not interfere with activation of nicotinic receptors (Gray *et al.*, 1996). Pre-treatment of cells with Cd^{2+} abolished Ca^{2+} signalling evoked by KCl (40mM) demonstrating the ability of Cd^{2+} at this concentration to block VOCCs (Figure 6.2.24). In contrast, Cd^{2+} failed to inhibit nicotine-mediated Ca^{2+} response (Figure 6.2.25). Mean data are summarized in Figure 6.2.26.

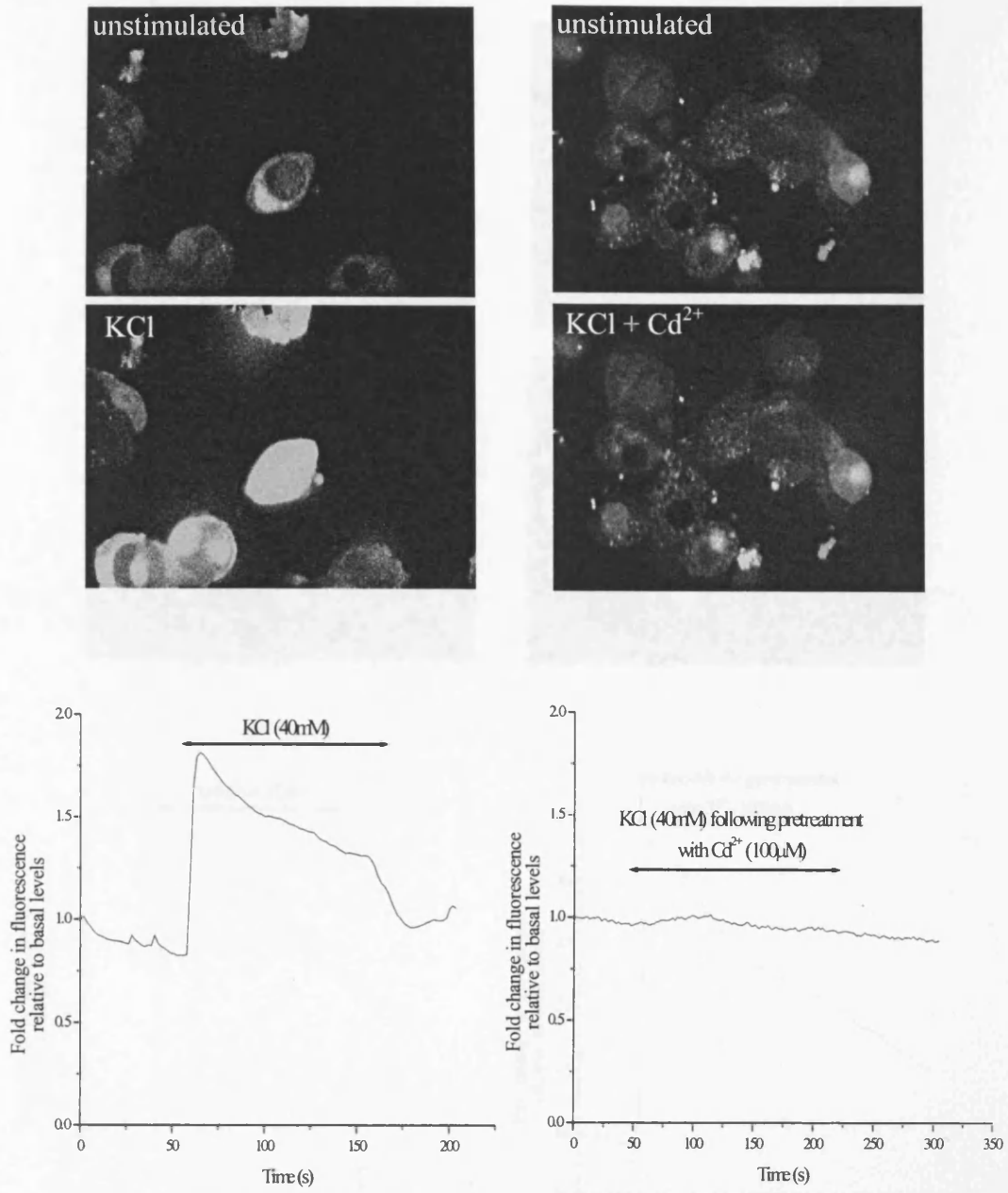


Figure 6.2.24. Effect of Cd^{2+} on KCl-mediated Ca^{2+} signalling in cultured bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at λ488 nm and imaged on the confocal microscope. Cells were challenged with KCl (40mM) in the absence or presence of Cd^{2+} (100μM, 5 min pre-treatment). The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three different cell culture preparations. Mean data are shown in Figure 6.2.26.

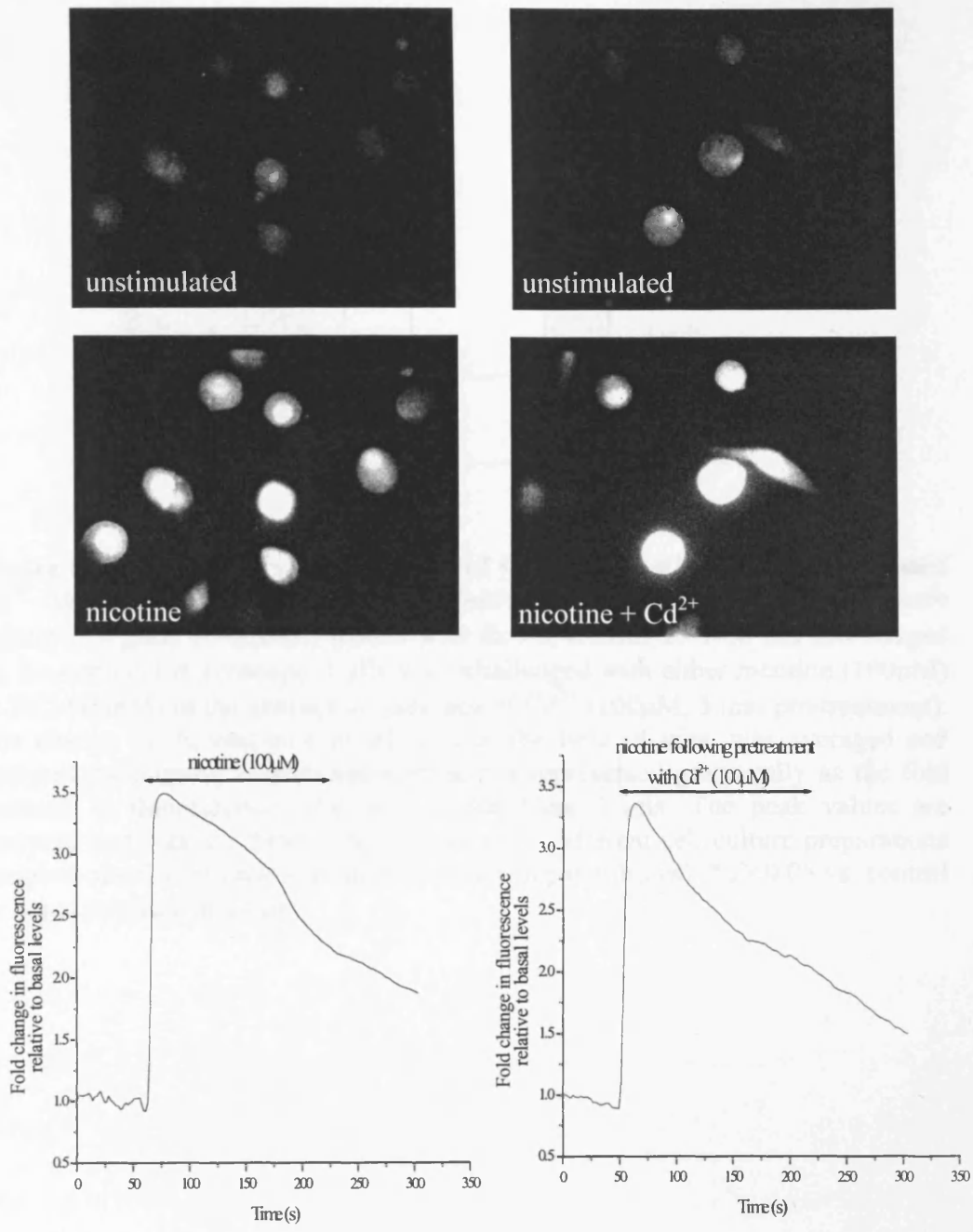


Figure 6.2.25. Effect of Cd^{2+} on nicotine-mediated Ca^{2+} signalling in cultured bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at λ488 nm and imaged on the confocal microscope. Cells were challenged with nicotine (100μM) in the absence or presence of Cd^{2+} (100μM, 5 min pre-treatment). The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three different cell culture preparations. Mean data are shown in Figure 6.2.26.

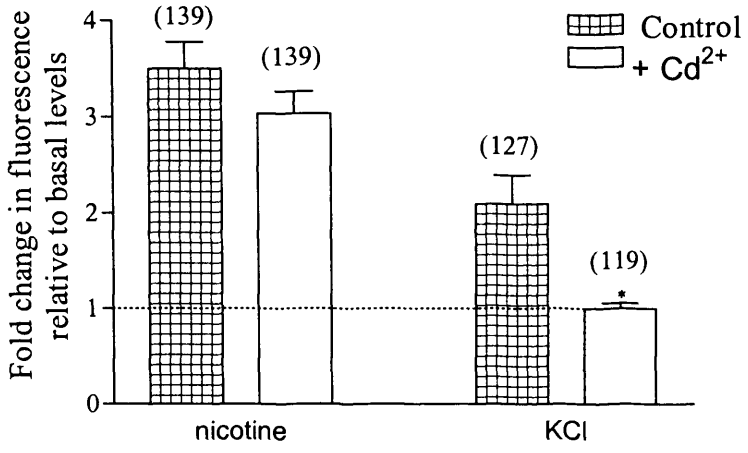


Figure 6.2.26. Summary of the effect of Cd^{2+} on nicotine- or KCl-mediated Ca^{2+} signalling in cultured bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at λ488 nm and imaged on the confocal microscope. Cells were challenged with either nicotine (100μM) or KCl (40mM) in the absence or presence of Cd^{2+} (100μM, 5 min pre-treatment). The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. The peak values are analysed and data are mean + SEM from three different cell culture preparations (number of cells for each condition is shown in parentheses). * $P<0.05$ vs. control by unpaired Student's *t*-test.

Effect of U73122 on nicotine-mediated subcellular re-distribution of PKCα

Previous work described in this thesis demonstrated that PKCα played a major role in nicotinic receptor-evoked secretion of catecholamines (see Chapter 5). Thus, the aim here was to determine if nicotinic receptor-mediated activation of PKCα was dependent upon the activation of PLC. Western blotting of cytosol and membrane fractions followed by densitometry was used to study the effect of PLC inhibition on nicotinic receptor-mediated PKCα translocation as an index of its activation. As described earlier (see Chapter 5), to validate the translocation method, the effect of exposure to PMA (1μM, 10 min) on the subcellular distribution of PKCα was examined. PMA mediated a significant loss of PKCα from the cytosolic fraction and this was accompanied by a marked increase in the membrane fraction (Figure 6.2.27). Pretreatment of cells with either U73122 (10μM) or its inactive analogue U73343 (10μM) failed to inhibit recruitment of PKCα to the plasma membrane in response to PMA (Figure 6.2.27).

Nicotine (100μM) mediated a dramatic translocation of PKCα from the cytosolic fraction to the membrane fraction. Pre-treatment of cells with U73122 markedly inhibited translocation of PKCα-mediated by nicotine (100μM, 10 min) from the cytosolic fraction to the membrane (Figure 6.2.27). However, U73343 was unable to prevent recruitment of PKCα to the plasma membrane triggered by nicotine (Figure 6.2.27: panel B and C).

Both cytosolic and membrane fractions were probed with β-actin antibody to confirm both an equal loading and that the membrane fraction was not contaminated with cytosol during the cell fractionation process.

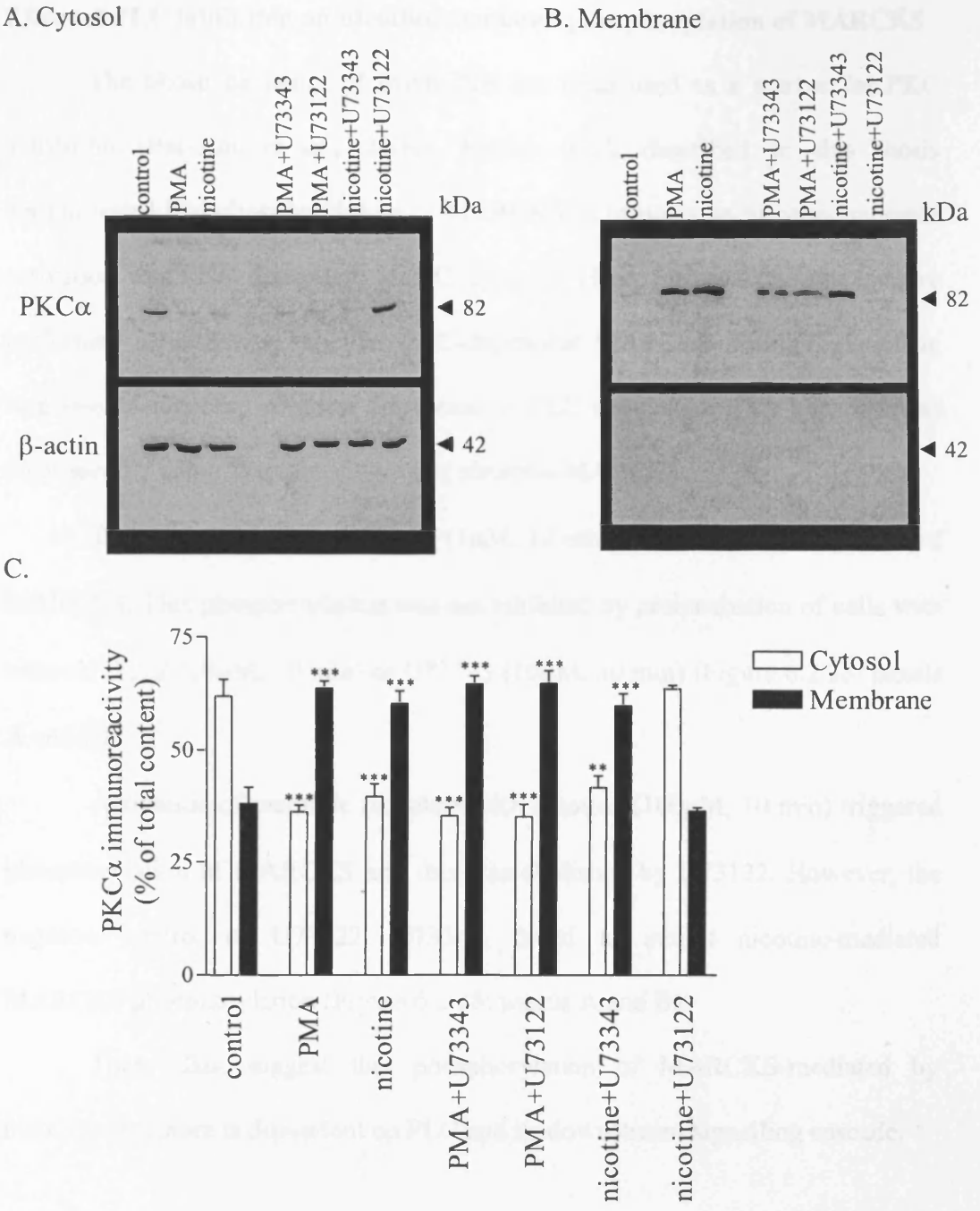


Figure 6.2.27. Effect of U73122 on nicotine-mediated subcellular re-distribution of PKCα in cultured bovine adrenal chromaffin cells. Cells were incubated with either U73122 (10μM) or U73343 (10μM) for 30 min before stimulation with either PMA (1μM) or nicotine (100μM) for 10 min at 37°C. The cytosol and membrane fractions were prepared and immunoblots performed as described in "Materials and Methods". The density of immunoblots in the cytosol and membrane fractions were quantified using Scion Image analysis software. Cytosolic and membrane readings were calculated as a percentage of the total (membrane plus cytosol). ** $P<0.01$, *** $P<0.001$ by one-way ANOVA followed by Dunnet's test vs. the appropriate (cytosol or membrane) control. Data are mean + SEM from three experiments performed on different cell culture preparations.

Effect of PLC inhibition on nicotine-mediated phosphorylation of MARCKS

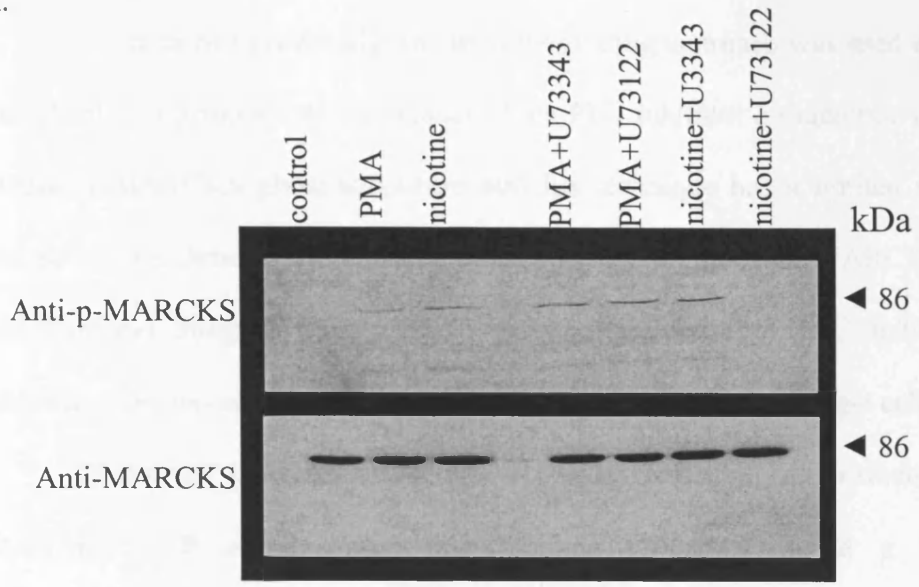
The phosphorylation of MARCKS has been used as a marker for PKC activation (Bar-Am *et al.*, 2004). Earlier work described in this thesis demonstrated that phosphorylation of MARCKS in response to nicotinic receptor activation was PKC dependent (see Chapter 5). Here, further experiments were performed to determine whether PKC-dependent MARCKS phosphorylation in response to nicotinic receptor activation is PLC dependent. This question was addressed by using Western blotting of phospho-MARCKS.

Treatment of cells with PMA (1μM, 10 min) induced phosphorylation of MARCKS. This phosphorylation was not inhibited by preincubation of cells with either U73122 (10μM, 30 min) or U73343 (10μM, 30 min) (Figure 6.2.28: panels A and B).

Activation of nicotinic receptors with nicotine (100μM, 10 min) triggered phosphorylation of MARCKS and this was abolished by U73122. However, the negative control of U73122, U73343, failed to inhibit nicotine-mediated MARCKS phosphorylation (Figure 6.2.28: panels A and B).

These data suggest that phosphorylation of MARCKS-mediated by nicotinic receptors is dependent on PLC and its downstream signalling cascade.

A.



B.

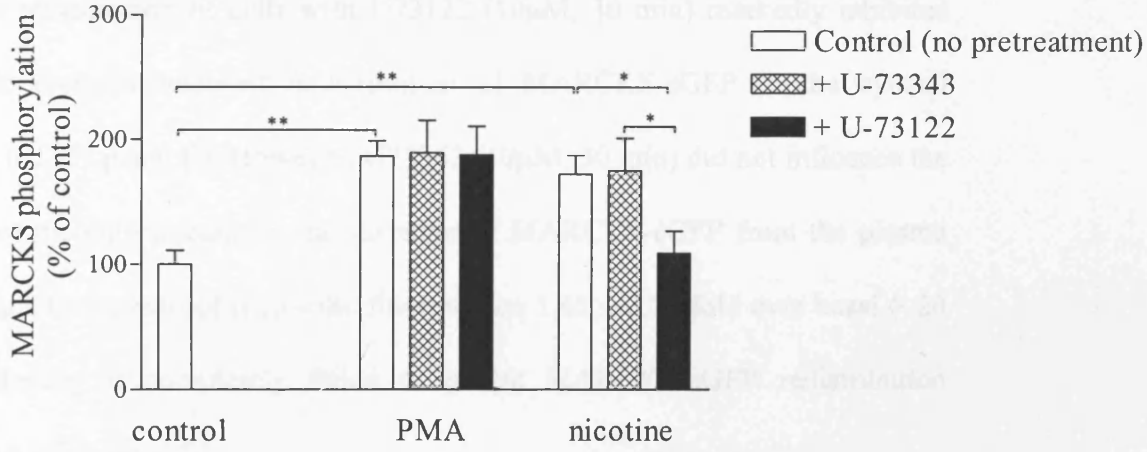


Figure 6.2.28. Effect of PLC inhibition on nicotine-mediated phosphorylation of MARCKS in chromaffin cells. Chromaffin cells were incubated with either U73122 (10μM) or U73343 (10μM) for 30 min at 37°C before stimulation with either nicotine (100μM) or PMA (1μM) for a further 20 min 37°C. Immunoblots of the whole cell lysate were prepared as described in "Materials and Methods". Panel A shows the immunoblot of phospho-MARCKS (p-MARCKS). The amount of phosph-MARCKS was quantified using Scion Image analysis software and expressed as a percentage of control. Panel B shows cumulative data on MARCKS phosphorylation obtained from experiments carried out on four different cell culture preparations. Data are mean + SEM and * $P<0.05$, ** $P<0.01$ by one-way ANOVA followed by Bonferroni's test (not all comparisons are shown on the graph).

Nicotine-mediated redistribution of MARCKS-eGFP: effects of the PLC inhibitor U73122

As described previously, the immunoblotting approach was used earlier in this chapter to demonstrate the impact of the PLC inhibitor on nicotinic receptor-mediated MARCKS phosphorylation but this technique has a limited temporal and spatial resolution. Therefore, another alternative approach, MARCKS-eGFP and confocal imaging were used to examine influence of PLC inhibitor on nicotinic receptor-mediated MARCKS activation in real time at single cell.

Real-time confocal imaging of cells transiently transfected with MARCKS-eGFP demonstrated that nicotine (100μM) caused a transient translocation of MARCKS-eGFP from the plasma membrane to the cytosol (cytosolic fluorescence 1.79 ± 0.09 fold over basal) (Figure 6.2.29: panel A)

Preincubation of cells with U73122 (10μM, 30 min) markedly inhibited nicotinic receptor-mediated redistribution of MARCKS-eGFP to the cytosol (Figure 6.2.29: panel C). However, U73343 (10μM, 30 min) did not influence the ability of nicotine to cause a translocation of MARCKS-eGFP from the plasma membrane to the cytosol (cytosolic fluorescence 1.45 ± 0.07 fold over basal (~20 cells) thereby it completely failed to inhibit MARCKS-eGFP redistribution (Figure 6.2.29: panel B).

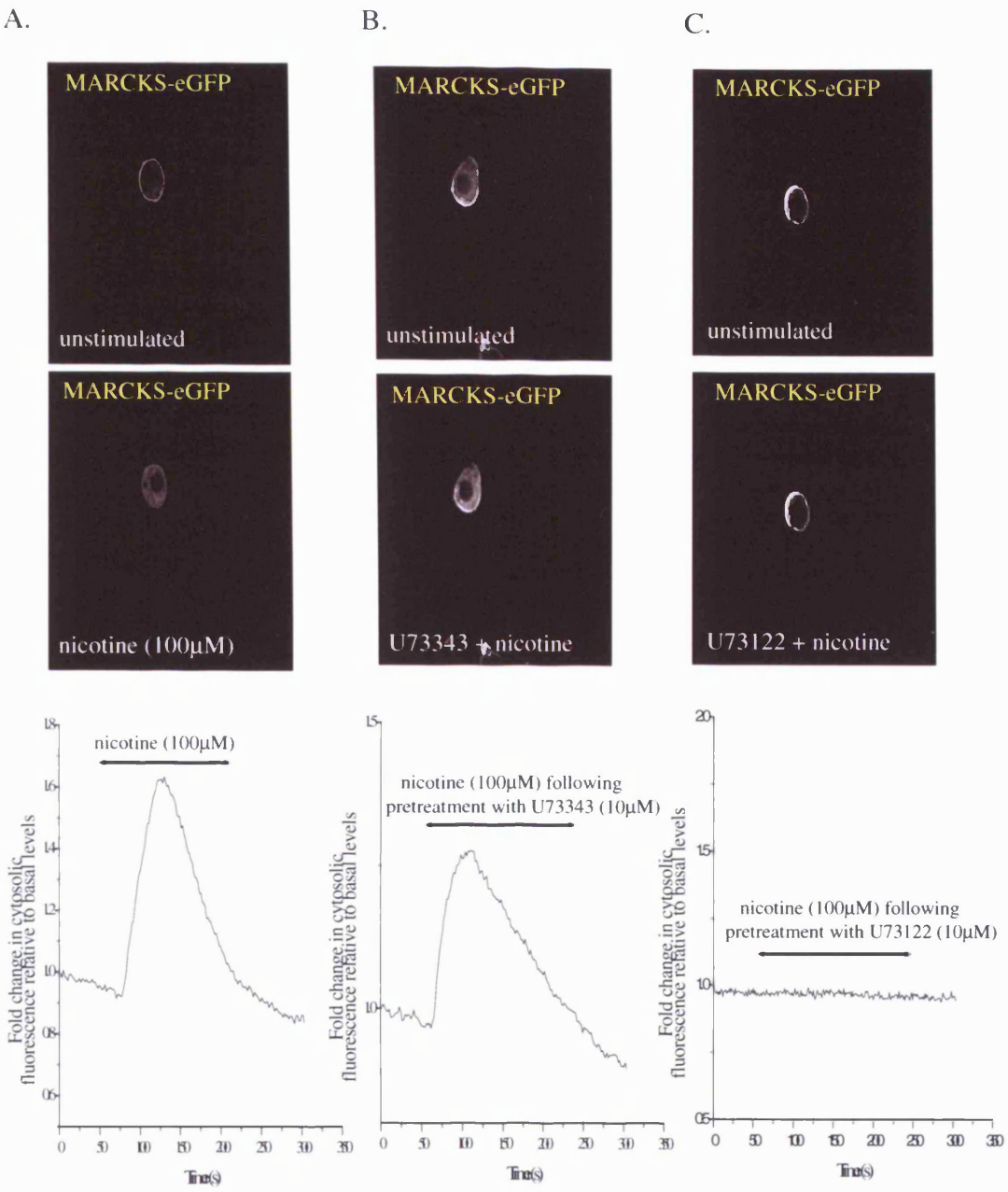


Figure 6.2.29. Single cell imaging of nicotine-mediated redistribution of MARCKS-eGFP: effects of the PLC inhibitor U73122. Cells that had been transiently transfected with MARCKS-eGFP were perfused with nicotine (100μM) in the absence of the PLC inhibitor (panel A). Cells were incubated with either U73122 (10μM; panel C) or U73343 (10μM; panel B) for 30 min at 37°C then challenged with nicotine. The change in cytosolic fluorescence was expressed graphically as the fold increase in fluorescence relative to basal levels. Data are representative of three different cell culture preparations (n=25 cells in total).

Effect of PLC inhibition on cortical F-actin disassembly mediated by nicotinic receptor activation

Earlier experiments showed that PKC is involved in nicotinic receptor mediated cortical F-actin disassembly (see Chapter 5). To investigate whether the disruption of cortical F-actin mediated by nicotinic receptor activation is PLC dependent, the effect of inhibition of PLC on nicotinic receptor mediated cortical F-actin disassembly was studied. As previously described (see Materials and Methods), confocal microscopy was used to examine fixed chromaffin cells stained with rhodamine-phalloidin (as a probe for F-actin) (Vitale *et al.*, 1991).

Chromaffin cell cortical F-actin disruption mediated by cytochalasin D (2μM, 20 min), an F-actin destabilizer, was not inhibited by either U73122 (10μM, 30 min) or its negative control U73343 (10μM, 30 min) based on the proportion of cells showing disruption of the cortical F-actin ring (Figure 6.2.30 and Figure 6.2.31). In contrast, the fragmentation of the cortical F-actin ring caused by nicotine (100μM, 40s) was significantly attenuated by U73122 whereas, its inactive analogue U73343 failed to inhibit this cortical F-actin disassembly (Figure 6.2.30 and Figure 6.2.31). These data suggest that PLC plays a role in mediating F-actin disassembly evoked by nicotinic receptor activation.

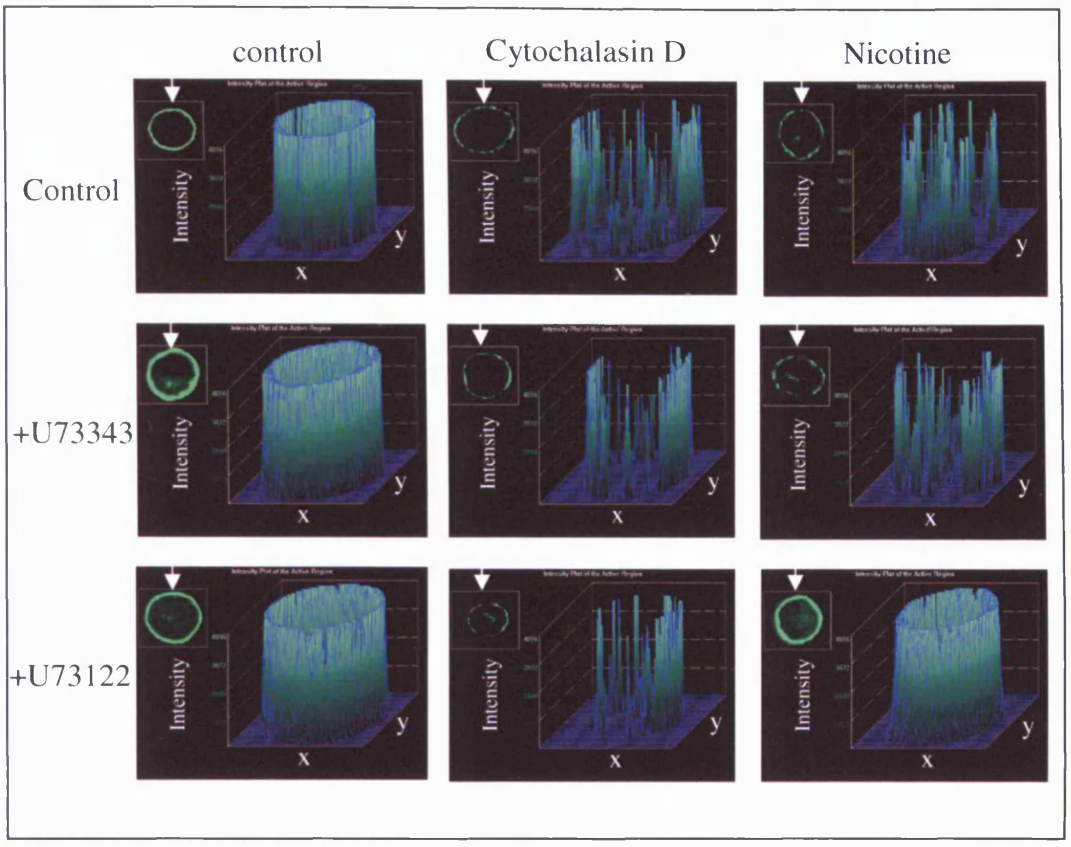


Figure 6.2.30. Effect of PLC inhibition on cortical F-actin disassembly mediated by either cytochalasin D or nicotine. Chromaffin cells were incubated with either cytochalasin D (2μM, 30 min) or nicotine (100μM, 40s) following preincubation with either the PLC inhibitor U73122 (10μM, 30 min) or its negative control U73343 (10μM, 30 min). Following these treatments, the cells were fixed and processed for rhodamine-labelled phalloidin staining as indicated under "Materials and Methods". The arrows at the left corner of panels point out the confocal image of the cell. Pseudo-three-dimensional image analysis were performed using "UltraView confocal software" where the maximum intensity of the fluorescent ring was set to 4096 arbitrary units. Images are representative of six hundred cells from three different cell culture preparations. Although these images are chosen as representative, in some instance, for example, effect of nicotine on cortical F-actin in presence of U73122, the proportion of cells showing F-actin disassembly in response to nicotine is still approximately 50% (see Figure 6.2.31). The images have been chosen, however, to reflect the nature of changes following the inhibitor rather than the degree of inhibition.

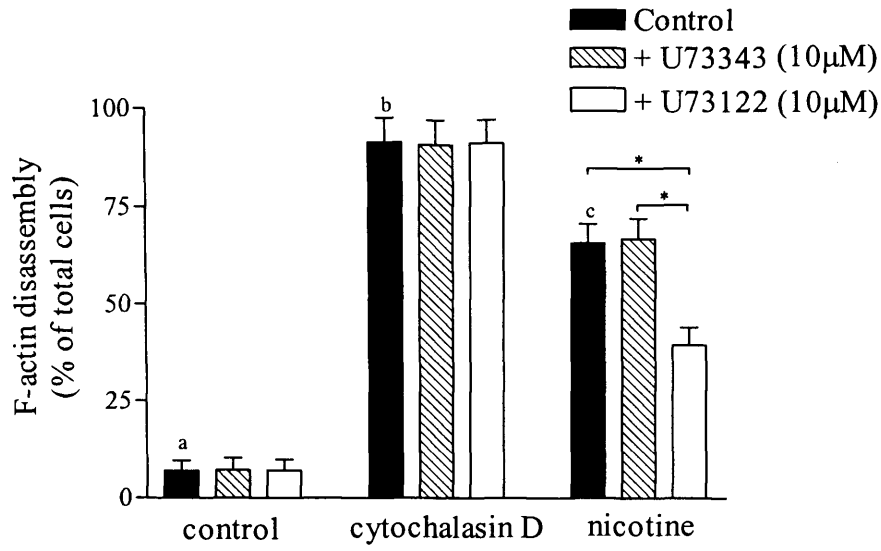


Figure 6.2.31. Effect of PLC inhibition on cortical F-actin disassembly mediated by either cytochalasin D or nicotine. Chromaffin cells were incubated with either cytochalasin D (2μM, 30 min) or nicotine (100μM, 40s) following preincubation with either the PLC inhibitor U73122 (10μM, 30 min) or its negative control U73343 (10μM, 30 min). Following these treatments, the cells were fixed and processed for rhodamine-labelled phalloidin staining as indicated under "Materials and Methods". The rhodamine cortical staining of chromaffin cells was analysed and classified as being continuous or discontinous and the percentage of cells displaying cortical F-actin disassembly (disrupted cortical rhodamine staining) in control and treated preparations was calculated in a single-blind fashion. Six hundred cells for each condition from a total of three different cell cultures were examined. Data shown are mean + SEM. For * $P<0.05$. In addition b and c are significantly different from a at $P<0.001$. All comparisons were by one-way ANOVA followed by post hoc Bonferroni's test. (Not all comparisons are shown on the graph).

Nicotinic receptor-induced redistribution of transiently expressed Munc13-1-eGFP in chromaffin cells

Interestingly, previously published data demonstrated that part of the effect of phorbol esters on synaptic transmission could not be inhibited by PKC inhibitors (O'Dell *et al.*, 1991) suggesting that there may be additional phorbol ester receptors at a synapse such as Munc13 isoforms (Brose *et al.*, 1995). This study aimed to identify whether exocytosis mediated by nicotinic receptor activation may be regulated not only by PKC but also Munc13. This was explored initially by examining the effect of nicotinic receptor activation on the subcellular distribution of recombinant Munc13-1. Under resting (basal, non-stimulated) conditions, Munc13 is located predominantly in the cytosol. However, following activation it rapidly translocates to the plasma membrane (Betz *et al.*, 1998; Ashery *et al.*, 1999; 2000). In this study, cells were transfected with Munc13-1-eGFP and its potential subcellular translocation determined by confocal microscopy before and during agonist application.

Chromaffin cells transiently transfected with cDNA encoding Munc13-1-eGFP showed a uniform distribution of cytoplasmic fluorescence with nuclear sparing (Figure 6.2.32). PMA (1μM, 10 min) induced a gradual and sustained re-distribution of Munc13-1-eGFP to the plasma membrane (cytosolic fluorescence 0.55 ± 0.07 fold of basal (15 cells)). In contrast, application of nicotine (100μM) failed to cause redistribution of Munc13-1-eGFP to the plasma membrane (Figure 6.2.33: panel A). These data suggest that either nicotinic receptor stimulation does not activate Munc13 or alternatively that the methodology is insufficiently sensitive, due, for example to a relatively low level of DAG formation.

In order to address this latter point, an attempt was made to enhance the level of DAG generation in response to nicotinic receptor activation by using a mixture of inhibitors of DAG lipase and kinase. This combination of RHC80267 (30μM, 5 min), an inhibitor of DAG lipase and R59949 ((1μM, 10 min), an inhibitor of DAG kinase, did not affect the inability of nicotine to induce redistribution of Munc13-1-eGFP to the plasma membrane (Figure 6.2.33: panel B).

Histamine (100μM) used as a positive control for PLC activation and downstream signalling pathways (here specifically DAG generation) also failed to induce a re-distribution of Munc13-1-eGFP to the plasma membrane (Figure 6.2.34: panel A). Preincubation of cells with a mixture of DAG lipase and kinase inhibitors did not alter the cytoplasmic distribution of fluorescence intensity or its lack of re-distribution in response to histamine (Figure 6.2.34: panel B). Taken together, these data suggest that either agonist-mediated DAG generation is insufficient to induce the translocation of Munc13 or that recombinant Munc13 is insufficiently sensitive, perhaps due to the overexpression of high levels.

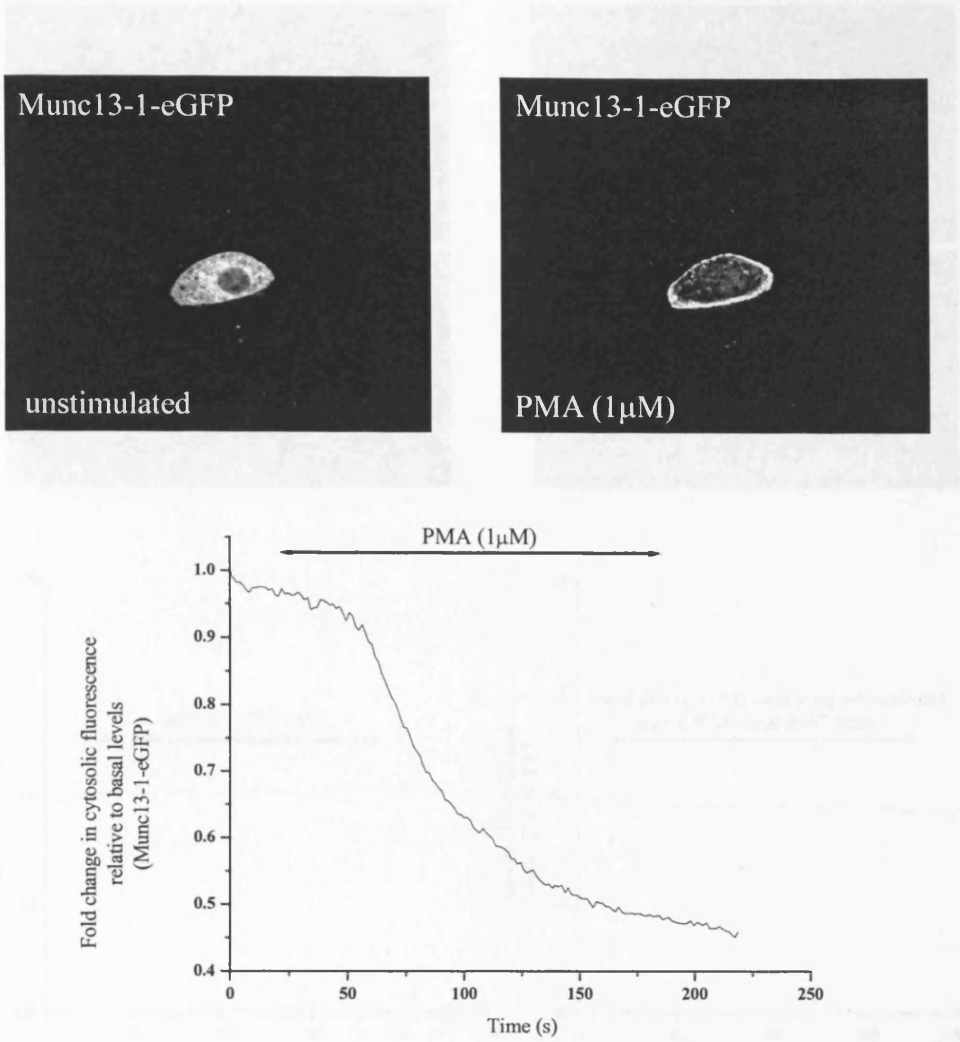


Figure 6.2.32. Single-cell imaging of PMA-induced translocation of transiently expressed Munc13-1-eGFP in chromaffin cells. Cells transiently transfected with Munc13-1-eGFP were challenged with PMA (1μM) after 20s. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of three different cell culture preparations (n=15-20 cells).

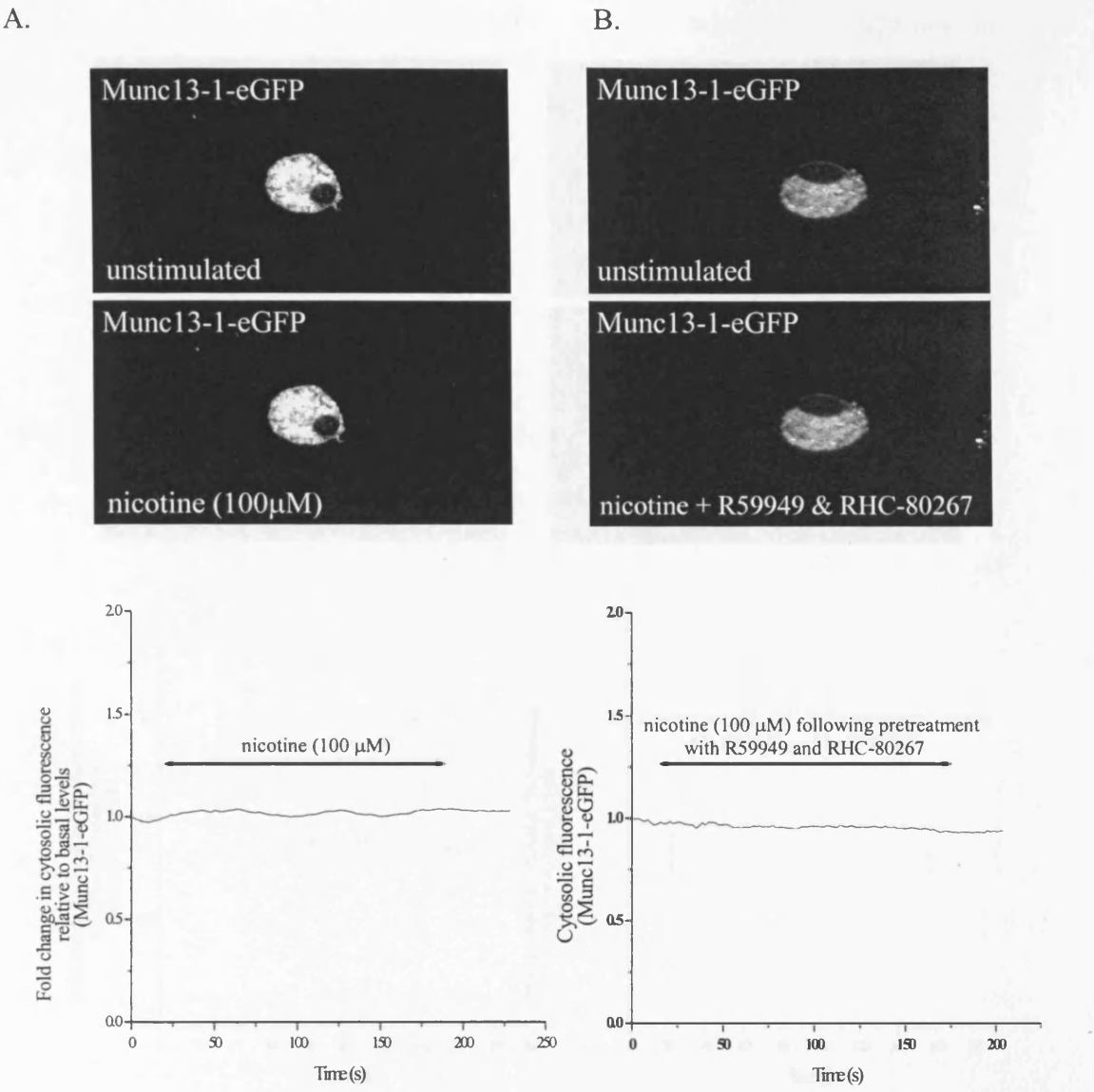


Figure 6.2.33. Single-cell imaging of Munc13-1-eGFP during challenge of cells with nicotine. Chromaffin cells transiently transfected with Munc13-1-eGFP were challenged with nicotine (100μM) after 20s in the absence (A) or presence (B) of R59949 (1μM, 10 min preincubation) (DAG kinase inhibitor) and RHC-80267 (30μM, 5 min preincubation) (DAG lipase inhibitor). The change in cytosolic fluorescence was expressed graphically as the fold change relative to basal level. Data are representative of three different cell culture preparations (n=25-35 cells).

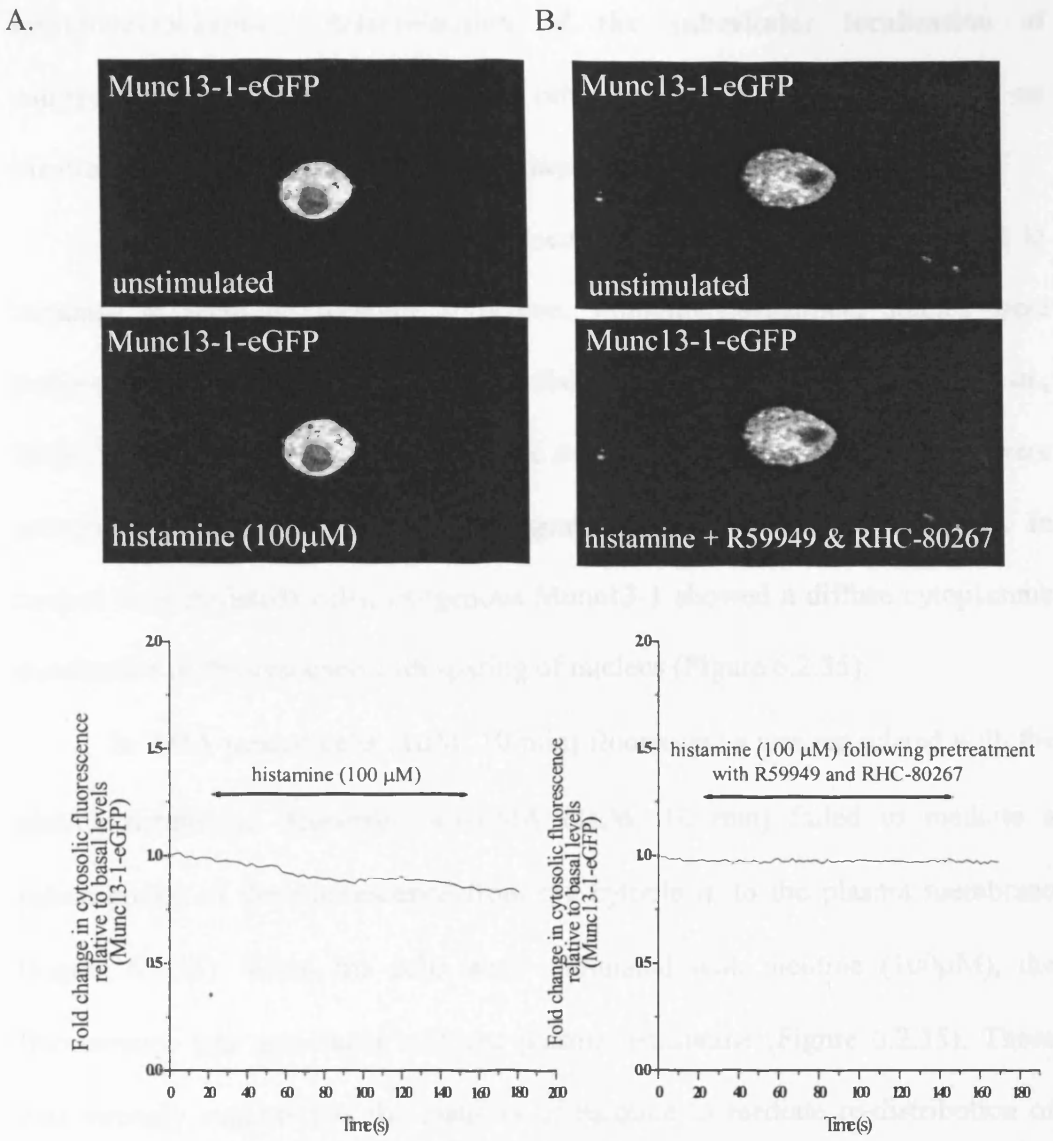


Figure 6.2.34. Single-cell imaging of Munc13-1-eGFP during challenge of cells with histamine. Chromaffin cells transiently transfected with Munc13-1-eGFP were challenged with histamine (100μM) after 20s in the absence (A) or presence (B) of R59949 (1μM, 10 min preincubation) (DAG kinase inhibitor) and RHC-80267 (30μM, 5 min preincubation) (DAG lipase inhibitor). The change in cytosolic fluorescence was expressed graphically as the fold change relative to basal level. Data are representative of three different cell culture preparations (n=15-20 cells).

Immunocytochemical determination of the subcellular localization of endogenous Munc13-1 in chromaffin cells: the effect of PLC inhibition on nicotine-mediated redistribution of endogenous Munc13-1

To characterize the subcellular localization of endogenous Munc13-1 in response to nicotinic receptor activation, immunocytochemical studies were performed with a specific Munc13-1 antibody (Duncan *et al.*, 1999; Sheu *et al.*, 2003; Groffen *et al.*, 2004). To exclude non-specific staining, fixed cells were probed only with a FITC-conjugated goat anti-mouse secondary antibody. In control (unstimulated) cells, exogenous Munc13-1 showed a diffuse cytoplasmic distribution of fluorescence with sparing of nucleus (Figure 6.2.35).

In PMA treated cells (1μM, 10 min) fluorescence was associated with the plasma membrane. However, 4α-PMA (1μM, 10 min) failed to mediate a redistribution of the fluorescence from the cytoplasm to the plasma membrane (Figure 6.2.35). When the cells were stimulated with nicotine (100μM), the fluorescence was associated with the plasma membrane (Figure 6.2.35). These data strongly suggest that the inability of nicotine to mediate re-distribution of Munc13-1-eGFP (Figure 6.2.33) is a consequence of its high expression level. Thus, the effect of PLC inhibition on nicotine-mediated re-distribution of endogenous Munc13-1 was studied using immunofluorescence labelling and confocal microscopy.

Preincubation of cells with either U73122 (10μM, 30 min) or its negative control U73343 (10μM, 30 min) did not alter the cytoplasmic distribution of endogenous Munc13-1 of unstimulated cells (Figure 6.2.36 and Figure 6.2.37). Similarly, neither U73122 nor U73343 (Figure 6.2.36 and Figure 6.2.37) influenced the inability of 4α-PMA to alter the distribution of Munc13-1.

Furthermore, neither U73122 nor U73343 were able to inhibit membrane localization of endogenous Munc13-1 mediated by PMA (Figure 6.2.36 and Figure 6.2.37). In contrast, nicotine (100μM, 10 min) caused a redistribution of endogenous Munc13-1 that was significantly attenuated by pretreating cells with U73122. In contrast, the inactive analogue, U73343, failed to prevent recruitment of endogenous Munc13-1 to the plasma membrane in response to nicotine (Figure 6.2.36 and Figure 6.2.37).

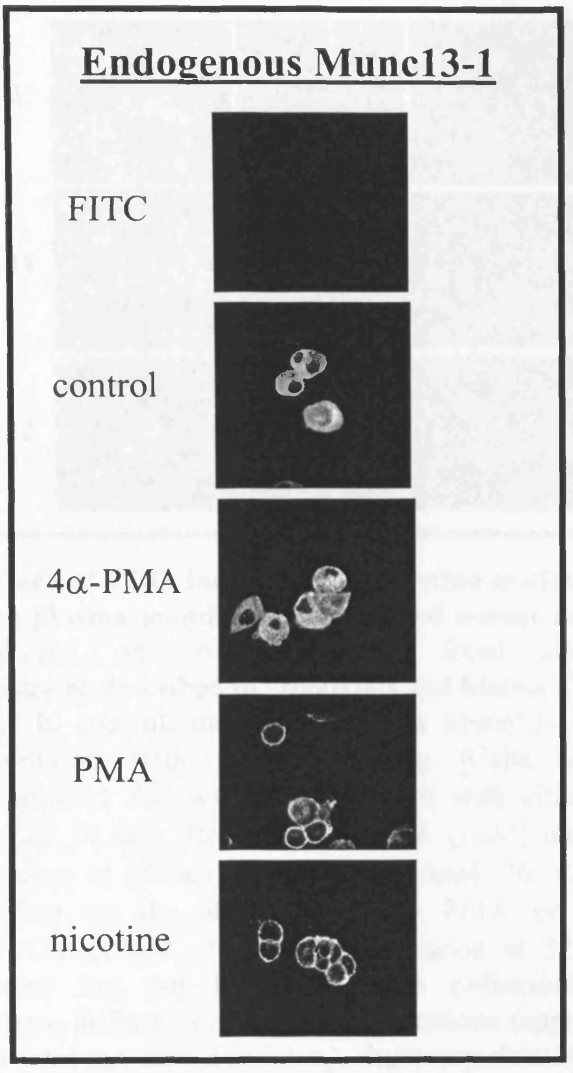


Figure 6.2.35. Immunolocalization of Munc13-1 in cultured bovine adrenal chromaffin cells. Cells were fixed and processed for immunocytochemistry as described in "Materials and Methods" using an antibody against Munc13-1. In unstimulated cells, fluorescence showed a diffuse cytoplasmic distribution with nuclear sparing. PMA (1μM, 10 min) caused a redistribution of fluorescence to the cell periphery whereas 4α-PMA (1μM, 10 min) did not. Nicotine (100μM, 10 min) also caused a redistribution of cytosolic fluorescence to the cell periphery. Pictures are representative of three different cell culture preparations (approximatey 300 cells were examined in total for each condition).

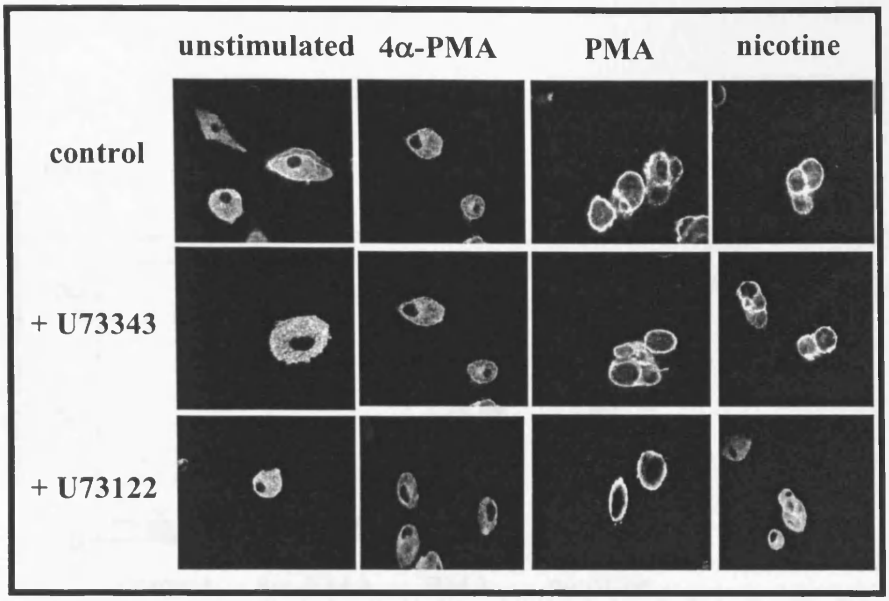


Figure 6.2.36. Effect of PLC inhibitors on nicotine-mediated Munc13-1 re-distribution to the plasma membrane in cultured bovine adrenal chromaffin cells. Cells cultured on coverslips were fixed and processed for immunocytochemistry as described in "Materials and Methods" using an antibody against Munc13-1. In control, unstimulated cells Munc13-1 showed a diffuse cytoplasmic distribution with nuclear sparing. Cells showed membrane localization of Munc13-1 following pre-treatment with either PMA (1μM) or nicotine (100μM) for 10 min. However, 4α-PMA (1μM) did not influence the subcellular distribution of Munc13-1. U73343 (10μM, 30 min preincubation at 37°C) had no effect on the ability of either PMA or nicotine to cause redistribution. U73122 (10μM, 30 min preincubation at 37°C) attenuated the abilities of nicotine but not PMA to cause redistribution. Pictures are representative of three different cell culture preparations (approximatey 300 cells were examined in total for each condition). Summary data are shown in Figure 6.2.37.

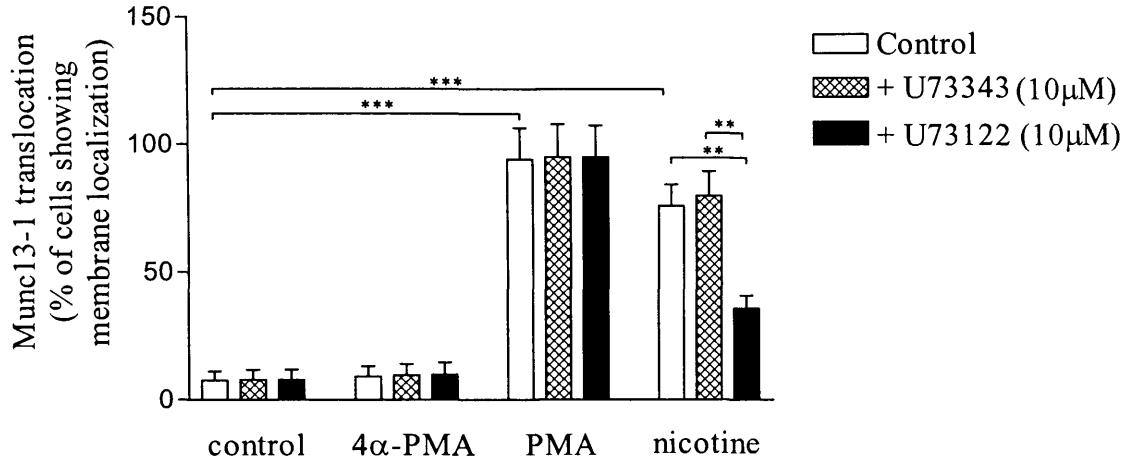


Figure 6.2.37. Effect of the PLC inhibitor U73122 on nicotine-mediated Munc13-1 re-distribution to the plasma membrane in cultured bovine adrenal chromaffin cells. Cells were incubated with either 4α-PMA (1μM), PMA (1μM) or nicotine (100μM) for 10 min at 37°C following preincubation with either vehicle control (Kreb's/HEPES buffer), U73122 (10μM) or U73343 (10μM) for 30 min at 37°C. Following these treatments, the cells were fixed and processed for immunocytochemistry as described in "Materials and Methods". Membrane localization was analysed by calculating the percentage of cells showing membrane localization in control and treated preparations. To avoid personal bias, code numbers were given to coverslips then cells were examined and classified without knowing whether they were from control or treated preparations (single-blind design). A total of three hundred cells from three different cell cultures were examined for each experimental condition. Data shown are mean + SEM and ** $P<0.01$, *** $P<0.001$ by one-way ANOVA followed by post-hoc Bonferroni's test. (Not all comparisons are shown on the graph).

6.3 DISCUSSION

6.3.1 Summary of data

The aim of this chapter was to investigate the mechanisms underlying PKC activation mediated by nicotinic receptor stimulation, particularly to explore whether PLC is involved. Various approaches were used to confirm the capability of nicotinic receptor stimulation to mediate PLC activation. Firstly, the subcellular re-distribution of transiently expressed eGFP-$PH_{PLC\delta1}$ and eGFP-$PKC\gamma Cl_2$ demonstrated nicotinic receptor-mediated generation of $Ins(1,4,5)P_3$ and DAG generation respectively in real time at the single cell level. Nicotinic receptor activation also resulted in the accumulation of total inositol phosphates as an index of total PLC activation. Finally, pharmacological inhibition of PLC reduced nicotinic receptor-mediated inositol phosphate generation and PKC activation showing that PKC activation required the PLC signalling pathway. Further, studies revealed that extracellular Ca^{2+} was required for PLC activation as removal of extracellular Ca^{2+} inhibited [^{3}H]-$InsP_x$ accumulation evoked by nicotine. Ca^{2+} signalling data also suggested that PLC activation is partially driven by Ca^{2+} released from intracellular stores. In addition, the PLC inhibitor U73122 attenuated Ca^{2+} signalling evoked by nicotinic receptors. Data also indicated that nicotinic receptor activation itself was responsible for PLC activation and not the activation of PLC-coupled receptors following the nicotinic receptor-mediated release of agonists from the chromaffin cells. Moreover, Ca^{2+} entry through nicotinic receptor channels constitutes the main source for entry in nicotinic receptor mediated Ca^{2+} signalling as block of VOCC by Cd^{2+} did not interfere with nicotine-mediated Ca^{2+} signalling.

Nicotinic-mediated PLC and PKC activation is likely to be linked to catecholamine secretion by MARCKS activation and subsequent F-actin disassembly, which allows access of secretory vesicles to their release sites at the plasma membrane. In addition to the activation of PKC, the current studies suggest that this DAG (and possibly Ca^{2+}) may also contribute to the activation of Munc13 which is also involved in facilitating exocytosis.

6.3.2 Mechanisms underlying PKC activation

Data in the previous chapter confirmed that nicotinic receptor activation resulted in the activation of PKC-α, -β and -ε but not ι in bovine chromaffin cells. Within Chapter 5 data suggest that these PKC isoforms, particularly PKCα and possibly also PKCβ contribute to catecholamine secretion evoked by nicotinic receptors. Activation of these PKC isoforms, which represent both conventional (PKC-α and -β) and novel (PKCε) PKC classes, requires DAG alone (novel) or in combination with Ca^{2+} (conventional). However, given that DAG is required for activation of members of both of these PKC subfamilies, the activation mechanism was unclear. Thus, under basal, or unstimulated conditions biological membrane contains very little DAG (Hodgkin *et al.*, 1998) and it might be expected that production of DAG is required for PKC activation. One possibility is that DAG generation occurs following nicotinic receptor-mediated activation of PLC. Indeed, previous studies in chromaffin cells indicated that Ca^{2+} influx following nicotinic- or high K^+-mediated depolarisation could increase the activity of PLC (Eberhard and Holz, 1988; 1989; Sasakawa *et al.*, 1989). Activated PLC hydrolyses membrane PtdIns(4,5)P_2, generating both Ins(1,4,5)P_3 and DAG. The increased membrane DAG and intracellular Ca^{2+} could then be the driving force for the activation of PKC. The capability of nicotinic receptor stimulation to

activate PLC was addressed in this study and examined using various approaches. Firstly, measurement of the accumulation of [^{3}H]-InsP_x against a Li^+-block of inositol monophosphatase activity reflects the total PLC activity (Wojcikiewicz *et al.*, 1993; Willars *et al.*, 1998b) and the current study demonstrated that nicotinic receptor activation caused accumulation of [^{3}H]-InsP_x although the extent of [^{3}H]-InsP_x generation was lower than that triggered by $G\alpha_{q/11}$-coupled receptors. Secondly, the generation of Ins(1,4,5)P_3 and DAG in response to nicotinic receptor activation was shown using biosensors. Interestingly, not all cells expressing eGFP-PKCγCl_2 or eGFP-$PH_{PLC\delta1}$ responded to either nicotine, histamine or angiotensin II. This might be a reflection of the need for robust responses to mediate the translocation of the biosensors and that the cultures represent a heterogenous collection of cells. The activation of PLC mediated by nicotinic receptor stimulation was further confirmed by using the PLC inhibitor U73122 which exerted inhibitory effects on [^{3}H]-InsP_x generation evoked by nicotinic receptor stimulation as well as by histamine, which was used as a positive control. Wortmannin also blocked the nicotinic receptor-mediated accumulation of [^{3}H]-InsP_x suggesting that this accumulation does indeed occur as a consequence of the hydrolysis of PtdIns(4,5)P_2.

6.3.3 Mechanisms underlying PLC activation mediated by nicotinic receptor stimulation

Since this study strongly suggests the involvement of PLC in nicotinic receptor-mediated cellular responses, particularly exocytosis, the crucial question that has to be addressed is how do nicotinic receptors activate PLC? The most likely mechanism for activation of PLC by nicotinic receptors is the influx of Ca^{2+} through VOCC or nicotinic receptors themselves. It is, however, possible that

release of GPCR agonists following nicotinic receptor activation could be responsible for the activation of PLC. However, this is unlikely as apyrase, PTX and stabilization of cortical F-actin with jasplakinolide all failed to inhibit nicotinic receptor-mediated [^{3}H]-InsP$_x$ accumulation.

The possible sources of Ca^{2+} entry triggered by nicotine are through VOCCs (Vijayaraghavan *et al.*, 1992; Dajas-Bailador and Wonnacott, 2004) or nAChRs, which can themselves, serve as a Ca^{2+} entry pathway (Decker and Dani, 1990; Zhang *et al.*, 1996b, Harkin and Fox, 1998). The current study reveals that activation of PLC triggered by nicotinic receptors is essentially dependent on extracellular Ca^{2+}. In addition, this study suggests that influx of Ca^{2+} via nicotinic receptors themselves contributes mostly to nicotinic receptor-mediated responses as determined by the ability of Cd^{2+} to block Ca^{2+} responses to KCl but not nicotine. Furthermore, the ability of thapsigargin, an inhibitor of the sarcoplasmic or endoplasmic reticulum ATPase (Thastrup *et al.*, 1990) to attenuate Ca^{2+} signalling mediated by nicotinic receptor activation suggests a contribution of intracellular Ca^{2+} stores to nicotinic receptor-mediated elevation of $[Ca^{2+}]_i$. This Ca^{2+} might therefore participate in PKC activation as suggested in previous studies (Burgoyne, 1991; Mollard *et al.*, 1995; Dajas-Bailador and Wonnacott, 2004). An involvement of intracellular stores was also supported using U73122, which inhibited nicotinic receptor-mediated elevation of $[Ca^{2+}]_i$.

6.3.4 A crucial role for PLC-dependent PKC activation in nicotinic receptor-mediated catecholamine secretion

Elevation of $[Ca^{2+}]_i$ as described previously within this chapter could contribute directly to the activation of PKC, particularly the α and β isoforms. However, a rise in $[Ca^{2+}]_i$ due to Ca^{2+} influx with the subsequent generation of

DAG could contribute to or be an instrumental in the activation of DAG-sensitive PKC isoforms. Thus, the role of nicotinic receptor-mediated PLC activation in the activation of PKC was examined by determining the impact of the PLC inhibitor, U73122, on the activation of PKCα. Western blotting of PKCα in cytosolic and membrane fractions to determine its subcellular localisation as an index of activation showed that U73122 inhibited activation of PKCα in response to nicotinic receptor activation suggesting that PLC and its downstream messengers are crucial to PKCα activation.

As described previously (Chapter 5), activation of PKC, particularly the α isoform in response to nicotinic receptor stimulation may trigger catecholamine secretion by phosphorylation of MARCKS, a major PKC substrate in chromaffin cells (Powis *et al.*, 1996b). Thus, phosphorylated MARCKS loses its ability to cross-link actin filaments (Aderem, 1992; Hartwig *et al.*, 1992) and this would be expected to result in the disorganization of the cortical actin cytoskeleton and eventually migration of secretory vesicles to exocytotic sites at the plasma membrane in preparation for exocytosis (Vitale *et al.*, 1995). Thus, the current study suggests that activation of PKCα, mediated by nicotinic receptors, and all of its subsequent events including MARCKS phosphorylation, F-actin disassembly and catecholamine secretion are crucially dependent on PLC activation.

6.3.5 The role of targets of DAG other than PKC in nicotinic receptor-mediated catecholamine release

Although DAG may contribute significantly to PKC activation and subsequent catecholamine secretion, additional DAG targets include Munc13 (Kazanietz, 2002), which is well known to facilitate vesicle priming (Brose *et al.*, 1995). Thus, Munc13 may act in parallel with PKC to enhance transmitter release

(Betz *et al.*, 1998). This study has tried to address whether nicotinic receptor stimulation mediates Munc13 activation and whether this activation is dependent on PLC activation. In this study, nicotinic receptor activation was unable to mediate redistribution of over-expressed Munc13-1-eGFP in real time at the single cell level and all attempts to elevate the level of endogenous DAG in chromaffin cells by using DAG kinase and DAG lipase inhibitors failed to cause Munc13-1-eGFP redistribution in response to nicotinic receptor activation. This might be a consequence of a massive expression of Munc13-1-eGFP whereby the DAG generated may be insufficient to cause significant translocation. This possibility was supported by the observation that nicotine could cause the redistribution of endogenous Munc13-1 to the plasma membrane. Thus, this study suggests that Munc13-1 might also contribute to catecholamine secretion evoked by nicotinic receptor activation. This is the first demonstration that Munc13-1 activation is downstream of nicotinic receptor activation. Furthermore, the data suggest that PLC-mediated generation of DAG is responsible for the activation of Munc13-1. A number of previous studies have highlighted the potential role of presynaptic Ca^{2+} influx in the activation of PLC with the subsequent generation of DAG and activation Munc13-1 to boost its priming activity (Rhee *et al.*, 2002; Rosenmund *et al.*, 2002).

Munc13-1 is thought to facilitate vesicle priming by localizing to the presynaptic plasma membrane with syntaxin and SNAP-25 (Betz *et al.*, 1997). Priming of vesicles represents a crucial step in Ca^{2+}-dependent exocytosis since only primed vesicles are fusion competent and can respond to an increase $[Ca^{2+}]_i$ by fusing with the plasma membrane. The priming process involves the assembly of syntaxin 1, SNAP-25 and synaptobrevin 2 into the SNARE-complex. The rate-

limiting factor in the SNARE-complex assembly reaction is likely to be the presence of syntaxin 1, which exists in two different conformations. In the closed conformation, the N-terminal H domain folds onto the C-terminal H3 domain, rendering this helical domain inaccessible for SNARE-complex formation (Fernandez *et al.*, 1998; Dulubova *et al.*, 1999). In the open conformation, the N-terminal H domain is not folded back onto the C-terminal H3 domain, leaving the C-terminal H3 domain free to interact with SNAP-25 and synaptobrevin 2 and to form the four bundle of the SNARE complex. Munc18, a cytoplasmic protein can bind syntaxin 1 in the closed conformation and may thereby inhibit SNARE complex formation (Dulubova *et al.*, 1999). Munc13-1 binds the to N-terminus of syntaxin 1 (Betz *et al.*, 1997) and Munc13-1 competes with Munc18 for syntaxin binding (Sassa *et al.*, 1999). This direct interaction of Munc13-1 with the autoinhibitory domain of syntaxin 1 is required for Munc13-1 priming activity. In addition, the presence of three C2 regulatory domains suggests that Munc13-1 might also act by binding Ca^{2+} or phospholipids thereby acting as an exocytotic Ca^{2+} sensor (Aravamudan *et al.*, 1999). Furthermore, DOC2α directly interacts with Munc13-1 in a DAG-dependent manner and this interaction plays an important role in Ca^{2+}-dependent exocytosis (Orita *et al.*, 1997; Groffen *et al.*, 2004). Moreover, its interaction with the cytoskeleton through spectrin (Sakaguchi *et al.*, 1998) might also contribute to Munc13-1 action by rearranging the submembrane cytoskeleton.

In summary, the current data suggest that nicotinic receptor activation facilitates catecholamine secretion through activation of two parallel signalling pathways, PKC and Munc13, which are downstream of nicotinic-receptor stimulation of PLC.

Chapter 7: Summary and concluding discussion

There is substantial evidence in the literature that PKC facilitates exocytosis. Work described within this thesis initially aimed to explore the role of PKC in exocytosis triggered by either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors, and to determine whether different PKC isoforms are involved in the regulation mediated by different receptors. Initially PC12 cells were chosen as a model neurosecretory cell type that is reported to express a range of $G\alpha_{q/11}$-coupled receptors. However, preliminary work indicated that the clone of PC12 cells used in this study, although expressing, for example, ionotropic P2X receptors, did not express the range of $G\alpha_{q/11}$-coupled receptors previously reported. As a result, attention was directed to chromaffin cells, a well-recognised model neurosecretory cell that has also been reported to express a number of different GPCRs and ionotropic receptors. This study demonstrated the expression of receptors for histamine, angiotensin II, acetylcholine and bradykinin that were able to generate $Ins(1,4,5)P_3$, DAG and elevate $[Ca^{2+}]_i$ implying coupling to $G\alpha_{q/11}$. Although, activation of histamine receptors, acetylcholine receptors or bradykinin receptors mediated peak and plateau Ca^{2+} responses, angiotensin II only evoked a peak Ca^{2+} signal. In general, peak Ca^{2+} responses as a result of the activation of $G\alpha_{q/11}$-coupled receptors arise from $Ins(1,4,5)P_3$-mediated release from intracellular Ca^{2+} stores whilst the sustained plateau phase is a consequence of Ca^{2+} entry across the plasma membrane. Such entry across the plasma membrane can result from depletion of intracellular Ca^{2+} stores that activate capacitative Ca^{2+} entry. The data presented here support this model in accord with previous reports (Plevin and Boarder, 1988; Sasakawa *et al*, 1989; Bunn *et al.*,

1990; Cheek *et al.*, 1993; Roberts-Thomson *et al.*, 2000; Marley, 2003). In addition, chromaffin cells express ionotropic nicotinic receptors. These receptors evoked much greater peak and plateau Ca^{2+} responses than those evoked by $G\alpha_{q/11}$-coupled receptors. The general scheme for Ca^{2+} signalling in response to activation of nicotinic receptors would be that activation of nicotinic receptors mediate depolarisation, which subsequently activates VOCCs allowing robust Ca^{2+} entry (Rathouz and Berg, 1994; Dajas-Bailador *et al.*, 2002). In addition, some types of nicotinic receptors are Ca^{2+} permeable; namely those consisting of $\alpha7$ subunits and some consisting of $\alpha3\beta4$ subunits (Costa *et al.*, 1994; Rathouz and Berg, 1994; Ragozzino *et al.*, 1998).

Although, in many cell types a rise in intracellular Ca^{2+} is a key stimulus of regulated exocytosis, PKC also regulates secretion in both chromaffin cells and other secretory cells (Terbush *et al.*, 1988; Terbush and Holz, 1990; Bittner and Holz, 1993; Graham *et al.*, 2000; Taylor *et al.*, 2000). This study reveals that bovine chromaffin cells express PKC-α, -β, -ε and -ι. Interestingly, these PKC isoforms are representative of the three PKC subfamilies (α and β, conventional; ε novel; ι, atypical). Activation of either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors recruited different PKC isoforms. Thus, nicotinic receptor stimulation activated PKC-α, -β and -ε, whilst activation of $G\alpha_{q/11}$-coupled receptors with histamine activated all the expressed PKC and angiotensin II only recruited PKC-α and -ε. Several groups have suggested that PKC has an obligatory role in exocytosis based on studies using a variety of non-selective PKC inhibitors (Knight *et al.*, 1988). In experiments described within this thesis, non-selective PKC inhibitors were also used initially to confirm the role of PKC in catecholamine release mediated either by nicotinic receptors or $G\alpha_{q/11}$-coupled

receptors. These experiments indicated a pivotal role for PKC in catecholamine release mediated by nicotinic receptors. However, in contrast, inhibition of PKC potentiated release of catecholamines induced by $G\alpha_{q/11}$-coupled receptors. The reasons for these contrasting results are most likely due to an inhibitory role of PKC on $G\alpha_{q/11}$-coupled receptor activation and signalling (Lefkowitz, 1993; Willars and Nahorski, 1995b; Ferguson *et al.*, 1996) which is removed by PKC inhibition. This finding is consistent with previous studies; particularly on chromaffin cells, which showed potentiation of $G\alpha_{q/11}$-coupled receptor-mediated signalling by PKC inhibitors (Boarder and Challiss, 1992). Although attempts were made using subtype-selective PKC inhibitors to determine the particular PKC isoforms involved in the regulation of signalling and release, this was not possible and the focus in this thesis was directed to exploring the role of PKC in nicotinic receptor-mediated catecholamine secretion.

Inhibition of PKC markedly inhibited nicotinic receptor-mediated catacholamine release. Many previous studies have used either down-regulation of PKC isoforms or non-selective PKC inhibitors to determine the role of PKC in catecholamine secretion mediated by nicotinic receptor activation (Vainio *et al.*, 1998; Soliakov and Wonnacott, 2001; Mahata *et al.*, 2002). Here this was explored further and the PKC isoforms involved in the facilitation of catecholamine secretion evoked by nicotinic receptors were determined using a range of isoform-selective inhibitors. The present study suggests that PKCα is the predominant isoform involved.

Although, the role of PKC in the secretion of catecholamines is well documented, there is considerable debate about the underlying mechanisms by which PKC enhances exocytosis and the mechanisms underlying PKC activation

in response to nicotinic receptor activation (Waters and Smith, 2000). These issues have been addressed in this study which determined that nicotinic receptors activate PKC, particularly PKCα and that activation of PKCα mediated phosphorylation of MARCKS, a PKC substrate, resulting in an inability to cross-link actin filaments and stabilize the actin network (Hartwig *et al.*, 1992) thereby promoting cortical F-actin disassembly. Although, the effect of PMA on MARCKS phosphorylation and translocation has been extensively studied in chromaffin cells (Vitale *et al.*, 1991; 1995; Rosē *et al.*, 2001; Trifaró, 1989; Trifaró *et al.*, 2002), no study has previously shown a role for PKC in nicotinic receptor-mediated MARCKS activation. Disruption of the cortical F-actin should precede the release process as removal of the cortical F-actin barrier will allow access of secretory vesicles to exocytotic sites at the plasma membrane (Trifaró *et al.*, 1989; Vitale *et al.*, 1991; 2000). Stabilization of cortical F-actin by jasplakinolide markedly inhibited nicotine-mediated catecholamine release highlighting the importance of the cortical F-actin disassembly in the release process. Thus, the data in this thesis emphasize the strong link between nicotinic receptor activation, the PKC-MARCKS pathway and F-actin disassembly followed by substantial increases in catecholamine secretion (Figure 7.1).

Although, it is possible that activation of PKC is triggered by elevation of $[Ca^{2+}]_i$ following activation of nicotinic receptors, evidence provided by this study suggests that the mechanism of PKC activation is rather more complicated. Although Ca^{2+} can activate PKC (Vainio *et al.*, 1998; Soliakov and Wonnacott, 2001; Mahata *et al.*, 2002), the classical isoforms of PKC (including, therefore, PKCα and PKCβ) are activated by Ca^{2+} in combination with DAG, whilst the novel isoforms (represented in this study by PKCε) are activated by DAG alone.

This suggests that Ca^{2+} alone may not be responsible for PKC activation and that it must act in combination with DAG. DAG is present in unstimulated cells at very low concentrations (Hodgkin *et al.*, 1998) suggesting that the generation of DAG could occur and contribute to the activation of PKC. Experiments described in this thesis provide evidence that the source of DAG is from an increase in PLC activity in response to nicotinic receptor activation. Furthermore, the present study suggests that nicotinic receptors mediate entry of extracellular Ca^{2+} directly through the receptors themselves and that this activates PLC to promote the hydrolysis of PtdIns(4,5)P_2 resulting in the generation of both Ins(1,4,5)P_3 and DAG. The current study suggests a key role for PLC-dependent PKC activation mediated by nicotinic receptor stimulation in the cortical F-actin network disassembly by MARCKS. As a consequence of these events, the secretion of catecholamines evoked by nicotinic receptors is extremely dependent on PLC-dependent PKC activation.

This study also suggests that generation of DAG as a consequence of nicotinic receptor-mediated PLC activation could regulate exocytosis through activation of Munc13, which facilitates vesicle priming (Brose *et al.*, 1995; Betz *et al.*, 1998). Indeed, this study strongly suggests that Munc13-1 might contribute to catecholamine secretion evoked by nicotinic receptors in parallel with PKC. The present study proposes that the source of DAG for the activation of Munc13-1 in response to nicotinic receptor stimulation is dependent on the Ca^{2+}-mediated activation of PLC. A model by which PKC facilitates nicotinic receptor-mediated catecholamine release is presented in Figure 7.1. Although much attention has been directed to the release of catecholamines from chromaffin cells, peptide transmitter release remains less studied (Fulop *et al.*, 2005). Catecholamines and

neuropeptides are copackaged in the same granule (Winkler and Westhead, 1980) thus; it is assumed that both types of transmitter are released by a single exocytotic mechanism and this emphasises the importance of future studies in defining the mechanisms underlying peptide release mediated by nicotinic receptor activation.

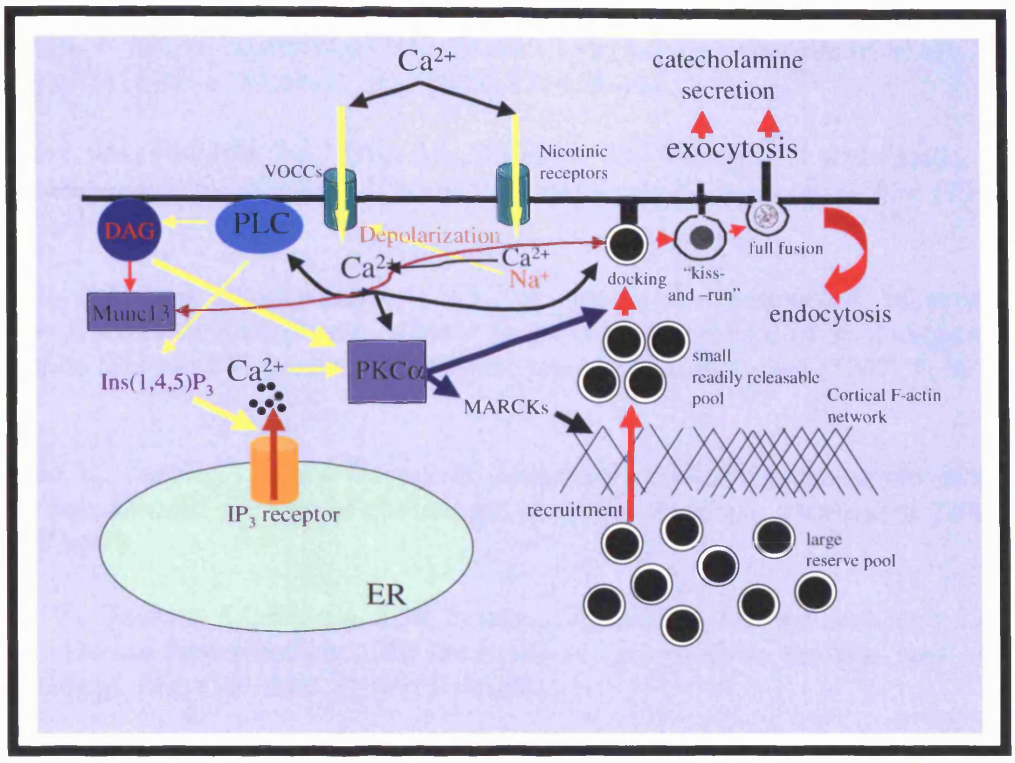


Figure 7.1. Potential mechanisms involved in the secretion of catecholamines mediated by nicotinic receptor activation. Activation of nAChRs by agonist binding causes influx of both Na^+ and Ca^{2+}. The resulting depolarisation triggers influx of Ca^{2+} through VOCCs although data from the present study suggests that a substantial proportion of the Ca^{2+} entry is via the nicotinic receptor itself. Increased $[Ca^{2+}]_i$ may activate PKC directly. The current study also suggests that Ca^{2+} entry promotes activation of PLC with the subsequent generation of DAG, which then contributes significantly to the activation of PKC, particularly PKCα. PKC then phosphorylates MARCKS leading to disruption of the cortical F-actin network and recruitment of secretory vesicles from the reserve pool to the readily releasable pool in preparation for exocytotis. The elevation of $[Ca^{2+}]_i$ and generation of DAG may also be responsible for the activation of Munc13, which will also directly facilitate agonist-mediated exocytosis.

BIBLIOGRAPHY

Aderem, A. Signal transduction and the actin cytoskeleton: the roles of MARCKS and profilin. *Trends Biochem. Sci.* (1992) **17**: 438-443

Aguilar, J.S., Ballesta, J.J., Reig, J.A., Palmero, M., Viniegra, S. and Criado, M. Muscarinic receptor subtypes in bovine adrenal medulla. *Neurochem. Res.* (1992) **17**: 1235-1239

Akers, R.F. and Routtenberg, A. Calcium-promoted translocation of protein kinase C synaptic membranes: relation to the phosphorylation of an endogenous substrate (protein F1) involved in synaptic plasticity. *J. Neurosci.* (1987) **7**: 3976-3983

Alamo, L., García, A.G. and Borges, R. Electrically-evoked catecholamine release from cat adrenals: the role of cholinergic receptors. *Biochem. Pharmacol.* (1991) **42**: 973-978

Ales, E., Tabares, L., Poyato, J.M., Valero, V., Lindau, M. and Al-Varez, D.T. High calcium concentrations shift the mode of exocytosis to the kiss- and –run mechanism. *Nat. Cell. Biol.* (1999) **1**: 40-44

Alessi, D.R., Deak, M., Casamayor, A., Caudwell, F.B., Morrice, N., Norman, D.G., Gaffney, P., Reese, C.B., MacDougall, C.N. and Harbison, D. 3-Phosphoinositide-dependent protein kinase-1 (PDK-1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr. Biol.* (1997) **776**-789

Alexander, D.R., Hexham, J.M., Lucas, S.C., Graves, J.D., Cantrell, D.A. and Crumpton, M.J. A protein kinase C pseudosubstrate peptide inhbits phosphorylation of the CD3 antigen in streptolysin-O-permeabilized human T lymphocytes. *Biochem. J.* (1989) **260**: 893-901

Ali, S.M. and Burgoyne, R.D. The stimulatory effect of calpactin (annexin II) on calcium-dependent exocytosis in chromaffin cells: requirement for both the N-terminal and core domains of p36 and ATP. *Cell signalling* (1990) **2**: 265-276

Ali, S.M., Geisow, M.J. and Burgoyne, R.D. A role for calpactin in calcium-dependent exocytosis in adrenal chromaffin cells. *Nature.* (1989) **340**: 313-315

Allen, L.H. and Aderem, A. A role for MARCKS, the alpha isozyme of protein kinase C and myosin I in zymosan I in zymosan phagocytosis by macrophages. *J. Exp. Med.* (1995) **182**: 829-840

Alvarez, C., Lorenzo, C., Santana, F. and Borges, R. Interaction between G-protein-operated receptors eliciting secretion in rat adrenals: a possible role for protein kinase C. *Biochem. Pharmacol.* (1997) **53**: 317-323

Anborgh, P.H., Dale, L., Seachrist, J. and Ferguson, S.S.G. Differential regulation of β_2-adrenergic and angiotensin II type 1A receptor trafficking and

resensitisation: role of carboxyl-terminal domains. *Mol. Endocrinol.* (2000) **14**: 2040-2053

Appell, K.C. and Barefoot, D.S. Neurotransmitter release from bradykinin-stimulated PC12 cells. *Biochem. J.* (1989) **263**: 11-18

Aravamudan, B., Fergestad, T., Davis, W.S., Rodesch, C.K. and Broadie, K. *Drosophila* UNC-13 is essential for synaptic transmission. *Nat. Neurosci.* (1999) **2**: 965-971

Arbuzova, A., Wang, J., Murray, D., Jacob, J., Cafiso, D.S. and McLaughlin, S. Kinetics of interaction of the myristoylated alanine-rich C kinase substrate, membranes, and calmodulin. *J. Biol. Chem.* (1997) **272**: 27167-27177

Arbuzova, A., Schmitz, A.A. and Vergeres, G. Cross-talk unfolded: MARCKS proteins. *Biochem. J.* (2002) **362**: 1-12

Artalejo, C.R. Adams, M.E. and Fox, A.P. Three types of Ca^{2+} channel trigger secretion with different efficacies in chromaffin cells. *Nature.* (1994) **367**: 72-76

Artalejo, C.R., Ariano, M.A., Perlman, R.L. and Fox, A.P. Activation of facilitation calcium channels in chromaffin cells by D1 dopamine receptors through a cAMP/protein kinase A-dependent mechanism. *Nature* (1990) **348**: 239-242

Artalejo, C.R., Elhamdani, N. and Palfrey, H.C. Secretion: dense-core vesicles can kiss-and-run too. *Curr. Biol.* (1998) **8**: R62-R65

Ashery, U., Varoqueaux, F., Voets, T., Betz, A., Thakur, P., Koch, H., Neher, E., Brose, N. and Rettig, J. Munc13-1 acts as a priming factor for large dense-core vesicles in bovine chromaffin cells. *EMBO J.* (2000) **19**: 3586-3596

Ashery, U., Betz, A., Xu, T., Brose, N. and Rettig, J. An efficient method for infection of adrenal chromaffin cells using the Semliki Forest Virus gene expression system. *Eur. J. Cell. Biol.* (1999) **78**: 525-532

Augustin, I., Rosenmund, C., Südhof, T.C. and Brose, N. Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. *Nature* (1999a) **400**: 457-461

Augustin, I., Betz, A., Herrmann, C., Jo, T. and Brose, N. Differential expression of two novel Munc13 proteins in rat brain. *Biochem. J.* (1999b) **337**: 363-371

Augustine, G.J. and Neher, E. Calcium requirements for secretion in bovine chromaffin cells. *J. physiol.* (1992) **450**: 247-271

Axelrod, J. Purification and properties of phenylethanolamine-N-methyl transferase. *J. Biol. Chem.* (1962) **237**: 1657-1660

Azzi, A., Boscoboinik, D. and Hensey, C. The protein kinase C family. *Eur. J. Biochem.* (1992) **208**: 547-557

Baird, J.G., Lambert, D.G., Mcbain, J. and Nahorski, S.R. Muscarinic receptors coupled to phosphoinositide hydrolysis and elevated cytosolic calcium in human neuroblastoma cell line SK-N-SH. *Br. J. Pharmacol.* (1989) **98**: 1328-1334

Baldwin, J.M. Structure and function of receptors coupled to G proteins. *Curr. Opin. Cell. Biol.* (1994) **6**: 180-190

Bar-Am, O., Yogev-Falach, M., Amit, T., Sagi, Y. and Youdim, M.B. Regulation of protein kinase C by the anti-parkinson drug, MAO-B inhibitor, rasagiline and its derivative, in vivo. *J. Neurochem.* (2004) **89**: 1119-1125

Barak, L.S., Ferguson, S.S.G., Zhang, J., Martenson, C., Meyer, T. and Caron, M.G. Internal trafficking and surface mobility of a functionally intact β_2-adrenergic receptor-green fluorescent protein conjugate. *Mol. Pharmacol.* (1997) **51**: 177-184

Barclay, J.W., Craig, T.J., Fisher, R.J., Ciufo, L.F., Evans, G.J.O., Morgan, A. and Burgoyne, R.D. Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis. *J. Biol. Chem.* (2003) 278: 10538-10545

Barker, S., Kapas, S., Fluck, R.J. and Clark, A.J.L. Effects of the selective protein kinase C inhibitor R031-7549 on human angiotensin II receptor desensitisation and intracellularcalcium release. *FEBS Lett.* (1995) **369**: 263-266

Barritt, G.J. Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca^{2+} signalling requirements. *Biochem. J.* (1999) **337**: 153-1569

Barry, V. and Cheek, T. Extracellular ATP triggers two functionally distinct calcium signaling pathways in PC12 cells. *J. Cell. Sci.* (1994) **107**: 451-462.

Bartlett, P.J., Young, K.W., Nahorski, S.R. and Challiss, R.A.J. Single cell analysis and temporal profiling of agonist-mediated inositol 1,4,5-trisphosphate, Ca^{2+}, diacylglycerol and protein kinase C signalling using fluorescent biosensors. *J. Biol. Chem.* (2005) **280**: 21837-21846

Basu, A. The potential of protein kinase C as a target for anticancer treatment. *Pharmacol. Ther.* (1993) **59**: 257-280

Bayerdorffer, E., Streb, H., Eckhardt, L., Haase, W. and Schulze, I. Effect of inositol-1,4,5-trisphosphate on isolated subcellular fractions of rat pancreas. *J. Membr. Biol.* (1984) **81**: 241-253

Bean, A.J., Zhang, X. and Hokfelt, T. Peptide secretion: What do we know? *FASEB J.* (1994) **8**: 630-638

Behn-Krappa, A. and Newton, A.C. The hydrophobic phosphorylation motif of conventional protein kinae C is regulated by autophosphorylation. *Curr. Biol.* (1999) **9**: 728-737

Belluardo, N., Mudo, G., Blum, M. and Fux, K. Central nicotinic receptors, neurotrophic factors and neuroprotection. *Behav. Brain. Res.* (2000) **113**: 21-34

Benavides, A., Calvo, S., Tornero, D., Gonzalez-Garcia, C., Cena, V. Adrenal medulla calcium channel population is not conserved in bovine chromaffin cells in culture. *Neuroscience.* (2004) **128**: 99-109

Benfenati, F., Onofri, F. and Giovedi, S. Protein-protein interactions and protein modules in the control of neurotransmitter release. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* (1999) **354**: 243-257

Bernstein, F.W. and Bamburg, J.R. Reorganization of actin is depolarised synaptosomes. *J. Neurosci.* (1985) **5**: 2565-2569

Berridge, M.J. Inositol trisphosphate and calcium signalling. *Nature* (1993) **361**: 315-325

Betz, A., Ashery, U., Rickmann, M., Augustin, I., Neher, E., Südhof, T.C., Rettig, J. and Brose, N. Munc13-1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release. *Neuron* (1998) **21**: 123-136

Betz, A., Okamoto, M., Benseler, F. and Brose, N. Direct interaction of the rat Unc13 homologue Munc13-1 with the N-terminus of Syntaxin. *J. Biol. Chem.* (1997) **272**: 2520-2526

Biondi, R.M., Cheung, P.C., Casamayor, A., Deak, M., Currie, R.A. and Alessi, D.R. Identification of a pocket in the PDK-1 kinase domain that interacts with RIF and the C-terminal residues of PKC. *EMBO J.* (2000) **19**: 979-988

Bit, R.A., Davies, P.D., Elliott, L.H., Harris, W., Hill, C.H., Keech, E., Kumar, H., Lawton, G., Maw, A., Nixon, J.S., Vesey, D.R., Wadsworth, J. and Wilkinson, S.E. Inhibitors of protein kinase C. 3. potent and highly selective bisindolymaleimides by conformational restriction. *J. Med. Chem.* (1993) **36**: 21-29

Bittner, M.A. and Holz, R.W. Protein kinase C and clostridial neurotoxins affect discrete and related steps in the secretory pathway. *Cell. Mol. Neurobiol.* (1993) **13**: 649-664

Bittova, L., Stahelin, R.V. and Cho, W. Role of ionic residues of the C1 domain in protein kinase C-alpha activation and the origin of phosphatidylserine specificity. *J. Biol. Chem.* (2000) **11**: 11

Blackshear, P.J. The MARCKS family of cellular protein kinase C substrates. *J. Biol. Chem.* (1993) **268**: 1501-1504

Blumberg, P.M. Complexities of the protein kinase C pathway. *Mol. Carcinogen.* (1991) **4**: 339-344

Blumberg, P.M., Jaken, S., Konig, B., Sharkey, N.A., Leach, K.L., Jeng, A.Y. and Yeh, E. Mechanism of action of the phorbol ester tumor promoters: specific receptors for lipophilic ligands. *Biochem. Pharmacol.* (1984) **33**: 933-940

Boarder, M.R. and Challiss, R.A.J. Role of protein kinase C in the regulation of histamine and bradykinin stimulated inositol polyphosphate turnover in adrenal chromaffin cells. *Br. J. Pharmacol.* (1992) **107**: 1140-1145

Bock, J.B. and Scheller, R.H. Protein transport. A fusion of new ideas. *Nature.* (1997) **387**: 133-135

Bogi, K., Lorenzo, P.S., Acs, P., Szallasi, Z., Wagner, G.S. and Blumberg, P.M. Comparison of the roles of the C1a and C1b domains of protein kinase C alpha in ligand induced translocation in NIH 3T3 cells. *FEBS. Lett.* (1999) **456**: 27-30

Borges, R., Machado, J.D., Betancor, G. and Camacho, M. Pharmacological regulation of the late steps of exocytosis. *Ann. N. Y. Acad. Sci.* (2002) **971**: 184-192

Bormann, J. and Clapham, D.E. GABA receptor channels in adrenal chromaffin cells: a patch-clamp study. *Proc. Natl. Acad. Sci. U.S.A.* (1985) **82**: 2168-2172

Borner, C., Filipuzzi, I., Wartmann, M., Eppenberger, U. and Fabbro, D. Biosynthesis and posttranslational modifications of protein C in human breast cancer cells. *J. Biol. Chem.* (1989) **264**: 13902-13909

Bossu, J.L., Elhamdani, A., Feltz, A., Tanzi, F., Aunis, D., Thierse, D. Voltage-gated Ca entry in isolated bovine capillary endothelial cells: evidence of a new type of BAY K 8644-sensitive channel. *Pflugers. Arch.* (1992) **420**: 200-207

Bottari, S.P., De Gasparo, M., Steckelings, U.M., Levens, N.R. Angiotensin II receptor subtypes: characterization, signalling mechanisms, and possible physiological implications. *Front. Neuroendocrinol.* (1993) **14**:123-171

Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* (1976) **72**: 248-254

Braun, M., Wendt, A., Birnir, B., Broman, J., Eliasson, L., Galvanovskis, J., Gromada, J., Mulder, H. and Rorsman, P. Regulated exocytosis of GABA-contanining synaptic-like microvesicles in pancreatic β-cells. *J. Gen. Physiol.* (2004) **123**: 191-204

Brenner, S. The genetics of *Caenorhabditis elegans*. *Genetics* (1974) **77**: 71-94

Brenner, S.L. and Korn, E.D. Substoichiometric concentrations of cytochalasin D inhibits actin polymerisation. *J. Biol. Chem.* (1979) **254**: 9982-9985

Brocklehurst, K.W., Morita, K. and Pollard, H.B. Characterization of protein kinase C and its role in catecholamine secretion from bovine adrenal medullary cells. *Biochem. J.* (1985) **228**: 35-42

Brooks, J.C. and Treml, S. Catecholamine secretion by chemically skinned cultured chromaffin cells. *J. Neurochem.* (1983) **40**: 468-473

Brose, N. and rosenmund, C. More over protein kinase C, you've got company: alternative cellular effects of diacylglycerol and phorbol esters. *J. Cell. Sci.* (2002) **115**: 4399-4411

Brose, N., Hofmann, K., Hata, Y. and Südhof, T.C. Mammalian homologues of *C. elegans* Unc13 gene define novel family of C-domain proteins. *J. Biol. Chem* (1995) **270**: 25273-25280

Brose, N., Rosenmund, C. and Rettig, J. Regulation of transmitter release by Unc13 and its homologues. *Curr. Opin. Neurobiol.* (2000) **10**: 303-311

Brose, N. and Neher, E. Specificity emerges in the dissection of diacylglycerol- and protein kinase C-mediated signalling pathways. *Proc. Natl. Acad. Sci. U.S.A.* (2002) **99**: 16522-16523

Buchner, K., Adamec, E., Berrmann, M.L. and Nixon, R.A. Isoform-specific translocation of PKC following glutamate administration in primary hippocampal neurons. *Mol. Brain Res.* (1999) **64**: 222-235

Buisson, B. and Bertrand, D. Allosteric modulation of neuronal nicotinic acetylcholine receptors. *J. Physiol. Paris.* (1998) **92**: 89-100

Bunn, S.J., Marley, P.D. and Livett, B.G. Effects of opioid compounds on basal and muscarinic induced accumulation of inositol phosphates in cultured bovine chromaffin cells. *Biochem. Pharmacol.* (1988) **37**: 395-399

Bunn, S.J. and Boyd, T.L. Characterization of histamine-induced catecholamine secretion from bovine adrenal medullay chromaffin cells. *J. Neurochem.* (1992) **58**: 1602-1610

Bunn, S.J. The effect of protein kinase inhibitors on histamine-stimulated phospholipase C activity. In: 7th international symposium on chromaffin cell biology and pharmacology. *Montebello Quebec Canada* (1993) **July 18-23**: 79

Bunn, S.J., Marley, P.D. and Livett, B.G. Receptor stimulated formation of inositol phosphates in cultures of bovine adrenal medullary cells: the effects of bradykinin, bombesin and neurotensin. *Neuropeptides* (1990) **15**: 187-194

Burgoyne, R.D. and Cheek, T.R. Reorganization of pheripheral actin filaments as a preclude of exocytosis. *Biosci. Rep.* (1987) **7**: 281-288

Burgoyne, R.D. and Morgan, A. Analysis of regulated exocytosis in adrenal chromaffin cells: insights into NSF/SNAP/SNARE function. *Bioessays* (1998a) **20**: 328-335

Burgoyne, R.D. and Morgan, A. Ca^{2+} and secretory vesicle dynamics. *Trends Neurosci.* (1995) **18**: 191-196

Burgoyne, R.D. and Morgan, A. Regulated exocytosis. *Biochem. J.* (1993a) **293**: 305-316

Burgoyne, R.D. and Morgan, A. Secretory granule exocytosis. *Physiol Rev.* (2003) **83**: 581-632

Burgoyne, R.D. and Morgan, Calcium sensors in regulated exocytosis. *Cell Calcium* (1998b) **24**: 367-376

Burgoyne, R.D. Control of exocytosis in adrenal chromaffin cells. *Biochim. Biophys. Acta.* (1991) **1071**: 174-202

Burgoyne, R.D. Mechanisms of secretion from adrenal chromaffin cells. *Biochim. Biophys. Acta.* (1984a) **779**: 201-216

Burgoyne, R.D. The relationship between secretion and intracellular free calcium in bovine adrenal chromaffin cells. *Biosci. Rep.* (1984b) **4**: 605-611

Burgoyne, R.D., Fisher, R.J., Graham, M.E., Haynes, L.P. and Morgan, A. Control of membrane fusion dynamics during regulated exocytosis. *Biochem. Soc. Trans.* (2001) **29**: 467-472

Burgoyne, R.D., Geisow, M.J. and Barron, J. Dissection of stages in exocytosis in the adrenal chromaffin cells with use of trifluoperazine. *Proc. R. Soc. Lond. (B)* (1982) **216**: 111-115

Burgoyne, R.D., Morgan, A. and O'Sullivan, A.J. A major role for protein kinase C in calcium-activated exocytosis in permeabilized adrenal chromaffin cells. *FEBS Lett.* (1988) **238**: 151-155

Burgoyne, R.D., Morgan, A. and O'Sullivan, A.J. The control of cytoskeletal actin and exocytosis in intact and permeabilized adrenal chromaffin cells: role of calcium and protein kinase C. *Cell. Signal.* (1989) **1**: 323-334

Burgoyne, R.D., Morgan, A., Barnard, R.J.O., Chamberlain, L.H., Glenn, D.E. and Kibble, A.V. SNAPs and SNAREs in exocytosis in chromaffin cells. *Biochem. Soc. Trans.* (1996) **24**: 653-657

Burgoyne, R.D., Morgan, A., Robinson, I., Pender, N. and Cheek, T.R. Exocytosis in adrenal chromaffin cells. *J. Anat.* (1993b) **183**: 309-314

Calakos, N. and Scheller, R.H. Synaptic vesicle biogenesis docking, and fusion: A molecular description. *Physiol. Rev.* (1996) **76**: 1-29

Campos-Caro, A., Smillie, F.I., Dominguez del Toro, E Rovira, J.C., Sala, S., Sala, F., Ballesta, J.J. and Criado, M. Neuronal nicotinic acetylcholine receptors on bovine chromaffin cells: cloning, expression, and genomic organization of receptor subunits. *J. Neurochem.* (1997) **68**: 488-497

Casabona, G.C. Intracellular signal modulation: a pivotal role for protein kinase C. *Prog. Neuropsychopharmacol. Biol. Psychiatry.* (1997) **21**: 407-425

Castagna, M., Takai, Y. Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* (1982) **257**: 7847-7851

Catterall, W.A. Structure and regulation of voltage-gated Ca^{2+} channels. Annu. *Rev. Cell. Dev. Biol.* (2000) **16**: 521-555

Challiss, R.A.J., Jones, J.A., Owen, P.J. and Boarder, M.R. Changes in inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate mass accumulations in cultured adrenal chromaffin cells in response to bradykinin and histamine. *J. Neurochem.* (1991) **56**: 1083-1086

Chamberlain, L.H., Henry, J. and Burgoyne, R.D. Cysteine string proteins are associated with chromaffin granules. *J. Biol. Chem.* (1996) **271**: 19514-19517

Chang, R.S.L., Tran, V.T. and Snyder, S.H. Characteristics of histamine H_1-receptors in peripheral tissues labelled with [3H] mepyramine. *J. Pharmacol. Exp. Ther.* (1979) **209**: 437-442

Charles, A.C., Mostovskaya, N., Asas, K., Evans, C.J., Dankovich, M.L. and Hales, T.G. Coexpression of delta-opioid receptors with microreceptors in GH3 cells changes the functional response to microagonist from inhibitory to excitatory. *Mol. Pharmacol.* (2003) **63**: 89-95

Cheek, T.R. and Burgoyne, R.D. Effect of activation of muscarinic receptors on intracellular free calcium and secretion in bovine adrenal chromaffin cells. *Biochim. Biophys. Acta.* (1985) **846**: 167-174

Cheek, T.R. and Burgoyne, R.D. Nicotine-evoked disassembly of cortical actin filaments in adrenal chromaffin cells. *FEBS Lett.* (1986) **207**: 110-114

Cheek, T.R. and Thastrup, O. Internal Ca^{2+} mobilization and secretion in bovine adrenal chromaffin cells. *Cell Calcium* (1989) **10**: 213-221

Cheek, T.R., Jackson, T.R., O'Sullivan, A.J., Moreton, R.B., Berridge, M.J. and Burgoyne, R.D. Stimultaneous measurement of cytosolic calcium and secretion in single bovine adrenal chromaffin cells by fluorescent imaging of fura-2 in co-cultured cells. *J. cell Biol.* (1989) **109**: 1219-1227

Cheek, T.R., Morgan, A., O'Sullivan, A.J., Moreton, R.B., Berridge, M.J. and Burgoyne, R.D. Spatial localization of agonist-induced Ca^{2+} entry in bovine

adrenal chromaffin cells. Different patterns induced by histamine and angiotensin II and relationship to catecholamine release. *J. Cell Sci.* (1993) **105**: 913-921

Chen, Y.A. and Scheller, R.H. SNARE-mediated membrane fusion. *Nat. Rev. Mol. Cell. Biol.* (2001) **2**: 98-106

Chiesa, A., Rapizzi, E., Tosello, V., Pinto, P., de Virgilio, M., Fogarty, K.E. and Rizzuto, R. Recombinant aequorin and green fluorescent protein as valuable tools in study of cell signalling. *Biochem. J.* (2001) **355**: 1-12

Choi, A.Y., Cahill, A.L., Perry, B.D. and Perlman, R.L. Histamine evokes greater increases in phosphatidylinositol metabolism and catecholamine secretion in epinephrine-containing than in norepinephrine-containing chromaffin cells. *J. Neurochem.* (1993) **61**: 541-549

Choi, A.Y., Fukui, H. and Perlman, R.L. Glucocorticoids enhance histamine-evoked catecholamine secretion from bovine chromaffin cells. *J. Neurochem* (1995) **64**: 206-212

Chou, M.M., Hou, W., Johnson, J., Graham, L.K., Lee, M.H., Chen, C.S., Newton, A.C., Schaffhausen, B.S. and Toker, A. Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. *Curr. Biol.* (1998) **8**: 1069-1077

Chung, S.H., Takai, Y. and Holz, R.W. Evidence that the Rab3a-binding protein, rabphilin3a, enhances regulated secretion. Studies in adrenal chromaffin cells. *J. Biol. Chem.* (1995) **270**: 16714-16718

Cobbold, P.H., Cheek, T.R., Cuthbertson, K.S.R. and Burgoyne, R.D. *FEBS Lett.* (1987) **211**: 44-48

Codignola, A., Tarroni, P., Cattaneo, M.G., Vicentini, L.M., Clementi, F. and Sher, E. Serotonin release and cell proliferation are under the control of α-bungarotoxin-sensitive nicotinic receptors in small-cell lung carcinoma cell lines. *FEBS Lett.* (1994) **342**: 286-290

Colby, K.A. and Blaustein, M.P. Inhibition of voltage-gated K channels in synaptosomes by sn-1,2-dioctanoylglycerol, an activator of protein kinase C. *J. Neurosci.* (1988) **8**: 4685-4692

Corringer, P.J., Le Novere, N. and Changeux, J.P. Nicotinic receptors at the amino acid level. *Annu. Rev. Pharmacol. Toxicol.* (2000) **40**: 431-458

Costa, A.C.S., Patrick, J.W. and Dani, J.A. Improved technique for studying ion channels expressed in Xenopus Oocyte, including fast superfusion. *Biophys. J.* (1994) **67**: 395-401

Coussens, L., Parker, P.J., Rhee, L., Yang-Feng, T.L., Chen, E., Waterfield, M.D., Francke, U. and Ullrich, A. Multiple, distinct forms of bovine and human protein kinase suggest diversity in cellular signalling pathways. *Science* (1986) **233**: 859-866

Couturier, S., Bertrand, D., Matter, J.M., Bertrand, S., Millar, N., Valera, S., Barkas, T. and Ballivet, M. A neuronal nicotinic acetylcholine receptor subunit (α 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. *Neuron.* (1990a) **5**: 847-856

Couturier, S., Erkman, L., Valera, S., Rungger, D., Bertrand, S., Boulter, J., Ballivet, M. and Bertrand, D. α5, α 3, and non-α 3. Three clustered avian genes encoding neuronal nicotinic acetylcholine receptor-related subunits. *J. Biol. Chem.* (1990b) **265**: 17560-17567

Cox, M.E. and Parsons, S.J. Roles for protein kinase C and mitogen-activated protein kinase in nicotine-induced secretion from bovine adrenal chromaffin cells. *J. Neurochem.* (1997) **69**: 1119-1130

Craig, T.J., Evans, G.J.O. and Morgan, A. Physiological regulation of Munc18/nSec1 phosphorylation on serine-313. *J. Neurochem.* (2003) **86**: 1450-1457

Criado, M., Alamo, L. and Navarro, A. Primary structure of an agonist binding subunit of the nicotinic acetylcholine receptor from bovine adrenal chromaffin cells. *Neurochem. Res.* (1992) **17**: 281-287

Csukai, M., Chen, C.H., De Maheis, M.A. and Mochly-Rosen, D. The coatomer protein beta'-COP, a selective binding protein (RACK) for protein kinase Cepsilon. *J. Biol. Chem.* (1997) **272**: 29200-29206

Cubitt, A.B., Heim, R., Adams, S.R., Boyd, A.E., Cross, L.A. and Tsien, R.Y. Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* (1995) **20**: 448-455

Cuchillo-Ibāñez, O., Lejen, T., Albillos, A., Rosē, S.D., Olivares, R., Villarroya, M., García, A.G. and Trifaró, J.M. Mitochondrial calcium sequestration and protein kinase C cooperate in the regulation of cortical F-actin disassembly and secretion in chromaffin cells. *J. Physiol.* (2004) **560**: 63-76

Cunningham, J.M., Lennon, V.A., Lambert, E.H. and Scheithauer, B. Acetylcholine receptors in small cell carcinomas. *J. Neurochem.* (1985) **45**: 159-167

Curdova, E., Jechova, V. and Hostalek, Z. Properties of apyrase and inorganic pyrophosphatase in *Streptomyces aureofaciens. Folia Microbiol (Praha.)* (1982) **72**: 159-166

Currie, K.P. and Fox, A.P. ATP serves as a negative feedback inhibitor of voltage-gated Ca^{2+} channel currents in cultured bovine adrenal chromaffin cells. *Neuron* (1996) **16**: 1027-1036

Dahmer, M.K. and Perlman, R.L. Bovine chromaffin cells have insulin-like growth factor-I (IGF-I) receptors: IGF-I enhances catecholamine secretion. *J. Neurochem.* (1988) **51**: 321-323

Dajas-Bailador, F. and Wonnacott, S. Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends. Pharmacol. Sci.* (2004) **25**: 317-324

Dajas-Bailador, F.A., Mogg, A.J. and Wonnacott, S. Intracellular Ca^{2+} signals evoked by stimulation of nicotinic acetylcholine receptors in SH-SY5Y cells: contribution of voltage-operated Ca^{2+} channels and Ca^{2+} stores. *J. Neurochem.* (2002a) **81**: 606-614

Dajas-Bailador, F.A., Soliakov, L. and Wonnacott, S. Nicotine activates the extracellular signal-regulated kinase 1/2 via the α7 nicotinic acetylcholine receptor and hippocampal neurones. *J. Neurochem.* (2002b) **80**: 520-530

Davis, P., Elliott, L., Harris, W., Hill, C., Hurst, S., Keech, E., Kumar, M.K.H., Lawton, G., Nixon, J. and Wilkinson, S. Inhibitors of protein kinase C. 2. substituted bisindolylmaleimides with improved potency and selectivity. *J. Med. Chem.* (1992) **35**: 994-1001

Dawson-Scully, K., Bronk, P., Atwood, H.L. and Zinsmaier, K.E. Cysteine-string protein increases the calcium sensitivity of neurotransmitter exocytosis in Drosophila. *J. Neurosci.* (2000) **20**: 6039-6047

Decker, E.R. and Dani, J.A. Calcium permeability of nicotinic acetylcholine receptor: the single-channel calcium influx is significant. *J. Neurosci.* (1990) **10**: 3413-3420

Decker, M.W., Curzon, P., Holladay, M.W., Nikkel, A.L., Bitner, R.S., Bannon, A.W., Donnelly-Roberts, D.L., Puttfarcken, P.S., Kuntzweiler, T.A., Briggs, C.A., Williams, M. and Arneric, S.P. The role of neuronal nicotinic acetylcholine receptors in antinociption: effects of ABT-594. *J. Physiol.* (Paris) (1998) **92**: 221-224

Dekker, L.V., De Graan, P.N. and Gispen, W.H. Transmitter release: target of regulation by protein kinase C? *Prog. Brain Res.* (1991) **89**: 209-233

Dekker, L.V. and Parker, P.J. Protein kinase C- a question of specificity. *Trends. Biochem. Sci.* (1994) **19**: 73-77

Disatnik, M.H., Buraggi, G. and Mochly-Rosen, D. Localization of protein kinase C isozymes in cardiac myocytes. *Exp. Cell. Res.* (1994) **210**: 287-297

Diviani, D., Lattion, A.L. and Cotecchia, S. Characterization of the phosphorylation sites involved in G protein-coupled receptor kinase- and protein kinase C mediated desensitisation of the α_{1B}-adrenergic receptor. *J. Biol. Chem.* (1997) **272**: 28712-28719

Dohlman, H.G. and Thorner, J. RGS proteins and signalling by heterotrimeric G proteins. *J. Biol. Chem.* (1997) **272**: 3871-3874

Dominguez Del Toro, E., Juiz, J., Peng, X., Lindstrom, J. and Criado, M. Immunocytochemical localization of the α 7 subunit of the nicotinic acetylcholine receptor in the rat central nervous system. *J. Comp. Neurol.* (1994) **349**:325-342

Donald, A.N., Wallace, D., Donald, A. and McKenzie, S. and Marley, P.D. Phospholipase C-mediated signalling is not required for histamine-induced catecholamine secretion from bovine chromaffin cells. J. Neurochem. (2002) **81**: 1116-1129

Douglas, W.W. and Rubin, R.P. The role of calcium in the secretory response to the adrenal medulla to acetylcholine. *J. Physiol.* (1961) **159**: 40-57

Douglas, W.W. Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *Br. J. Pharmacol.* (1968) **34**: 451-474

Doussau, F. and Augustine, G.J. The actin cytoskeleton and neurotransmitter release: an overview. *Biochemie.* (2000) **82**: 353-363

Downing, G.J., Kim, S., Nakanishi, S., Catt, K.J. and Balla, T. Characterization of a soluble adrenal phosphatidylinositol 4-kinase reveals wortmannin sensitivity of type III phosphatidylinositol kinases. *Biochemistry* (1996) **35**: 3587-3594

Dulubova, I Sugita, S., Hill, S., Hosaka, M., Fernadez, I., Sudhof, T.C. and Rizo, J. A conformational switch in symaxin during exocytosis: Role of munc18. *EMBO. J.* (1999) **18**: 4372-4382

Duncan, R.R., Betz, A., Shipston, M.J., brose, R.H. and Chow, N. Transient, phorbol ester-induced DOC2-Munc13 interactions in vivo. *J. Biol. Chem.* (1999) **274**: 27347-27350

Dutil, E.M. and Newton, A.C. Dual role of pseudosubstrate in the coordinated regulation of protein kinase C by phosphorylation and diacylglycerol. *J. Biol. Chem.* (2000) **275**: 10697-10701

Dutil, E.M., Toker, A. and Newton, A.C. Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). *Curr. Biol.* (1998) **8**: 1366-1375

Easom, R.A. B-granule transport and exocytosis. *Semin. Cell. Dev. Biol.* (2000) **11**:253-266

Eaton, M.J. and duplan, H. Useful cell lines derived from the adrenal medulla. *Mol. Cell. Endocrinol.* (2004) **228**: 39-52

Eberhard, D.A. and Holz, R.W. Cholinergic stimulation of inositol phosphate formation in bovine adrenal chromaffin cells: distinct nicotinic and muscarinic mechanisms. *J. Neurochem.* (1987) **49**: 1634-1643

Eberhard, D.A. and Holz, R.W. Intracellular Ca^{2+} activates phospholipase C. *Trends Neurosci.* (1988) **11**: 517-520

Edwards, A.S., Faux, M.C., Scott, J.D. and Newton, A.C. Carboxyl-terminal phosphorylation regulates the function and subcellular localization of protein kinase CβII. *J. Biol. Chem.* (1999) **274**: 6461-6468

Efendiev, R., Bertorello, A.M. and Pedemonte, C.H. PKC-β and PKC-ξ mediate opposing effects on proximal tubule Na^+, K^+-ATPase activity. *FEBS Lett.* (1999) **456**: 45-48

Eichholtz, T., de Bont, D.B.A., de Widt, J., Liskamp, R.M.J. and Ploegh, H.L. A myristoylated pseudosubstrate peptide, a novel protein kinase C inhibitor. *J. Biol. Chem.* (1993) **268**: 1982-1986

Eilers, H., Schaeffer, E., Bickler, P.E. and forsayeth, J.R. Functional deactivation of the major neuronal nicotinic receptor caused by nicotine and a protein kinase C-dependent mechanism. *Mol. Pharmacol.* (1997) **52**: 1105-1112

El Far, O., Charvin, N., Leveque, C., Martin-Moutot, N., Takahashi, M. and Seagar, M.J. Interaction of a synaptobrevin (VAMP)-syntaxin complex with presynaptic calcium channels. *FEBS Lett.* (1995) **361**: 101-105

Elgoythen, A.B., Johnson, D.S., Boulter, J., Vetter, D.E. and Heinemann, S. Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell.* (1994) **79**: 705-715

Elhamdani, A., Martin, T.F., Kowalchyk, J.A. and Artalejo, C.R. Ca^{2+}-dependent activator protein for secretion is critical for the fusion of dense-core vesicles with the membrane in calf adrenal chromaffin cells. *J. Neurosci.* (1999) **19**: 7375-7383

Erickson, J.D. and Eiden, L.E. Functional identification and molecular cloning of a human brain vesicle monoamine transporter. *J. Neurochem.* (1993) **61**: 2314-2317

Evans, G.J.O., and Morgan, A. Phosphorylation-dependent interaction of the synaptic vesicle proteins cysteine string protein and synaptotagmin I. *Biochem. J.* (2002) **364**: 343-327

Exton, J.H. Mechanisms of action of calcium-mobilizing agonists: some variations on a young theme. *FASEB. J.* (1988) **2**: 2670-2676

Farago, A. and Nishizuka, Y. Protein kinase C in transmembrane signalling. *FEBS. Lett.* (1990) **268**: 350-354

Fasolato, C., Pandiella, A., Meldolesi, J. and Pozzan, T. Generation of inositol phosphates, cytosolic Ca^{2+} and ionic fluxes in PC12 cells treated with bradykinin. *J. Biol. Chem.* (1988) **263**: 17350-17359

Feldberg, W. and Lewis, G.P. the action of peptides on the adrenal medulla. release of adrenaline by bradykinin and angiotensin. *J. Physiol.* (1964) **171**: 98-108

Feng, X. and Hannun, Y.A. An essential role for autophosphorylation in the dissociation of activated protein kinase C from the plasma membrane. *J. Biol. Chem.* (1998) **273**: 26870-26874

Ferguson, S.S., Barak, L.S., Zhang, J. and Caron, M.G. G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins. *Can. J. Physiol. Pharmacol.* (1996) **74**: 1095-1110

Fernandez, I., Ubach, J., Dulubova, I., Zhang, X., sudhof, T.C. and rizo, J. Tree-dimensional structure of an evolutionarily conseved N-terminaldomain of symtaxin 1A. *Cell.* (1998) **94**: 841-849

Fischer-Colbrie, R., Laslop, A. and Kirchmair, R. Secretogranin II: molecular properties, regulation of biosynthesis and processing to the neuropeptide secretoneurin. *Prog. Neurobiol.* (1995) **46**: 49-70

Flanagan, M.D. and Lin, S. Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. *J. Biol. Chem.* (1980) **225**: 835-838

Flatmark, T., Almas, B. and Ziegler, M.G.C. Catecholamine metabolism: an update on key biosynthetic enzymes and vesicular monoamine transporters. *Ann. N. Y. Acad. Sci.* (2002) **971**: 69-75

Flores, C.M., Rogers, S.W., Pabreza, L.A., Wolfe, B.B. and kellar, K.J. A subtype of nicotinic cholinergic receptor in rat brain is composed of α 4 and β2 subunits and is up-regulated by chronic nicotine treatment. *Mol. Pharmacol.* (1992) **41**: 31-37

Fomina, A.F. and Nowycky, M.C. A current activated on depletion of intracellular Ca^{2+} stores can regulate exocytosis in adrenal chromaffin cells. *J. Neurosci.* (1999) **19**: 3711-3722

Fontainhas, A.M., Obukhov, A.G. and Nowycky, M.C. Protein kinase Cα modulates depolarisation-evoked change of intracellular Ca^{2+} concentration in rat pheochromocytoma cell line. *Neuroscience* (2005) **133**: 393-403

Forsberg, E.J., Rojas, E. and Pollard, H.B. Muscarinic receptor enhancement of nicotine-induced catecholamine secretion may be mediated by phosphoinositide metabolism in bovine adrenal chromaffin cells. *J. Biol. Chem.* (1986) **261**: 4915-4920

Fredriksson, R., Lagerstrom, M.C., Lundin, L.G. and Schioth, H.B. The G-protein-coupled receptors in the human genome form five main families: phytogenetic analysis, paralogon groups and fingerprints. *Mol. Pharmacol.* (2003) **63**: 1256-1272

Fredriksson, R. and Schiöth, H.B. The repertoire of G-protein-coupled receptors in fully sequenced genomes. *Mol. Pharmacol.* (2005) **67**: 1414-1425

Freedman, N.J. and Lefkowitz, R.J. Desensitisation of G protein-coupled receptors. *Recent Frog Hormone Res.* (1996) **51**: 319-351

Fucile, S. Ca^{2+} permeability of nicotinic acetylcholine receptors. *Cell Calcium* (2004) **35**: 1-8

Fucile, S., Renzi, M., Lax, P. and Eusebi, F. Functional Ca^{2+} current through human neuronal alpha7 nicotinic acetylcholine receptors. *Cell Calcium* (2003) **34**: 205-209

Fujita, Y., Sasaki, T., Fukui, K., Kontani, H., Kimura, T., Hata, Y., Südhof, T.C., Scheller, R.H. and Takai, Y. Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C. *J. Biol. Chem.* (1996) **271**: 7265-7268

Fulop, T., Radabaugh, S. and Smith, C. Activity-dependent differential transmitter release in mouse adrenal chromaffin cells. *J. Neurosci.* (2005) **25**: 7324-7332

Garcia-Guzman, M., Sala, F., Sala., S., Campos-Caro, A., Stuhmer, W., Gutierrez, L.M. and Griado, M. alpha-Bungarotoxin-sensitive nicotinic receptors on bovine chromaffin cells: molecular cloning, functional expression and alternative splicing of the alpha 7 subunit. *Eur. J. Neurosci.* (1995) **7**: 647-655

Garcia-Guzman, M., Sala, S., Capos-Caro, A., Criado, M. Role of two acetylcholine receptor subunit domains in homomer formation and intersubunit recognition, as revealed by alpha 3 and alpha 7 subunit chimeras. *Biochemistry.* (1994) **33**:15198-15203

Garry, D.J., Appel, N.M., Garry, M.G. and Sorenson, R.L. Cellular and subcellular immunolocalization of L-glutamate decarboxylase in rat pancreatic islets. *J. Histochem. Cytochem.* (1988) **36**: 573-580

Geeraert, V., Dupont, J.L., Grant, N.J., Huvet, C., Chasserot-Golaz, S., Janoshazi, A. and Procksch, O., de Barry, J. F-actin does not modulate the initial steps of the protein kinase C activation process in living nerve cells. *Exp. Cell. Res.* (2003) **289**: 222-236

Gerzanich, V., Wang, F., Kuryatov, A. and Lindstrom, J. $\alpha 5$ subunit alters desensitisation pharmacology, Ca^{2+} permeability and Ca^{2+} modulation of human neuronal $\alpha 3$ nicotinic receptors. *J. Pharmacol. Exp. Ther.* (1998) **286**: 311-320

Gescher, A. Towards selective pharmacological modulation of protein kinase C--opportunities for the development of novel antineoplastic agents. *Br. J. Cancer.* (1992) **66**: 10-19

Gether, U. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocrine reviews* (2000) **21**: 90-113

Gillis, K.D. and Chow, R.H. Kinetics of exocytosis in adrenal chromaffin cells. *Semin. Cell Dev. Biol.* (1997) **8**: 133-140

Gillis, K.D., Mossner, R. and Neher, E. Protein kinase C enhances exocytosis from chromaffin cells by increasing the size of readily releasable pool of secretory granules. *Neuron* (1996) **16**: 1209-1220

Gilman, A.G. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* (1987) **56**: 615-649

Goodall, A.R., Turner, N.A., Walker, J.H., Ball, S.G. and Vaughan, P.F.T. Activation of protein kinase C-α and translocation of myristoylated alanine-rich C-kinase substrate correlate with phorbol ester-enhanced noradrenaline release from SH-SY5Y human neuroblastoma cells. *J. Neurochem.* (1997) **68**: 392-401

Gopalakrishna, R., Barsky, S.H., Thomas, T.P. and Anderson, W.B. Factors influencing chelator-stables detergent-extractable, phorbol diester-induced membrane association of protein kinase C. *J. Biol. Chem.* (1986) **261**: 16438-16445

Gosink, E.C. and Forsberg, E. J. The effects of ATP and bradykinin on endothelial cell Ca^{2+} homeostasis and formation of CGMP and prostacyclin. *Am. J. Physiol.* (1993) **265**: C1620-C1629

Gotti, C., Fornasari, D. and clementi, F. Human neuronal nicotinic receptors. *Prog. Neurobiol.* (1997) **53**: 199-237

Graff, J.M., Young, T.N., Johnson, J.D. and Blackshear, P.J. Phosphorylation-regulated calmodulin binding to a prominent cellular substrate for protein kinase C. *J. Biol. Chem.* (1989) **264**: 21818-21823

Graham, M.E. and Burgoyne, R.D. Comparison of cysteine string protein (Csp) and mutant alpha-SNAP overexpression reveals a role for Csp in late steps of membrane fusion in dense-core granule exocytosis in adrenal chromaffin cells. *J. Neurosci.* (2000) **20**: 1281-1289

Graham, M.E., Fisher, R.J. and Burgoyne, R.D. Measurement of exocytosis by amperometry in adrenal chromaffin cells: effects of clostridial neurotoxins and activation of protein kinase C on fusion pore kinetics. Biochimie (2000) 82: 469-479

Gray, R., Rajan, A.S., Radcliffe, K.A., Yakehiro, M. and Dani, J.A. Hippocampal synaptic transmission enhanced by low concentrations of nicotine. *Nature* (1996) **383**: 713-716

Green, L.A. and Tischler, A.S. Establishment of noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U.S.A.* (1976) **73**: 2424-2428

Groffen, A.J.A, Brian, E.C., dudok, J.J., Kampmeijer, J., Toonen, R.F. and Verhage, M.C. Ca^{2+}-induced recruitment of the secretory vesicle protein DOC2B to the target membrane. *J. Biol. Chem.* (2004) **279**: 23740-23747

Grynkiewicz, G., Poenie, M. and Tsien, R.Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* (1985) **260**: 3440-3450

Gschwendt, M., Kittstein, W. and Marks, F. Protein kinase C activation by phorbol esters: do cysteine-rich regions and pseudosubstrate motifs play a role? *Trends Biochem. Sci.* (1991) **16**: 167-169

Gueorguiev, V.D., Zeman, R.J., Hiremagalur, B., Menezes, A. and sabban, E.L. Differing temporal roles of Ca^{2+} and cAMP in nicotine-elicited elevation of tyrosine hydroxylase mRNA. *Am. J. Physiol.* (1999) **27**: C54-C65

Gueorguiev, V.D., Zeman, R.J., Meyer, E.M. and Sabban, E.L. Involvement of α7 nicotinic acetylcholine receptors in activation of tyrosine hydroxylase and dopamine β-hydroxylase gene expression in PC12 cells. *J. Neurochem.* (2000) **75**: 1997-2005

Haghighi, A.P. and Cooper, E. A molecular link between inward rectification and calcium permeability of neuronal nicotinic acetylcholine alpha3beta4 and alpha4beta2 receptors. *J. Neurosci.* (2000) **20**: 529-541

Hannun, Y.A., Loomis, C.R. and Bell, R.M. Protein kinase C activation in mixed micelles: mechanistic implications of phospholipid, diacylglycerol, and calcium interdependencies. *J. Biol. Chem.* (1986) **261**: 7184-1790

Harkins, A.B. and Fox, A.P. Activation of nicotinic acetylcholine receptors augments calcium channel-mediated exocytosis in rat pheochromocytoma (PC12) cells. *J. Gen. Physiol.* (1998) **111**: 257-269

Harris, T.E., Persaud, S.J., Saermark, T. and Jones, P.M. A myristoylated pseudosubstrate peptide inhibitor of protein kinase C: effects on glucose- and carbachol-induced insulin secretion. *Mol. Cell. Endocrinol.* (1996) **121**: 133-141

Hartwig, J.H., Thelen, M., Rosen, A., Janmey, P.A., Nairn, A.C. and Aderem, A. MARCKS is an actin filament cross-linking protein regulated by protein kinase C and calcium-calmodulin. *Nature* (1992) **356**: 618-622

Heinemann, C., Von Rüdenel., Chow, R.H. and Neher, E. A two-step model of secretion control in neuroendocrine cells. *Pflügers Arch. Eur. J. physiol.* (1993) **424**: 105-112

Henry, J.P., Bohon, D., Sagne, C., Isambert, M.F., Desnos, C., Blanchard, V., Raisman-Vozari, R., Krejci, E., Massoulie, J. and Gasnier, B. Biochemistry and molecular biology of the vesicular monoamine transporter from chromaffin granules. *J. Exp. Biol.* (1994) **196**: 251-262

Hepler, J.R. Emerging roles for RGS proteins in cell signalling. *Trends Pharmacol. Sci.* (1999) **20**: 376-382

Hermans, E., Vanisberg, M.A., Geurts, M. and Maloteaux, J.M. Down-regulation of neurotensin receptors after ligand-induced internalisation in rat primary cultured neurons. *Neurochem. Int.* (1997) **31**: 291-299

Hermans, E. Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacol. Ther.* (2003) **99**: 25-44

Herrington, J., Solaro, C.R., Neely, A. and Lingle, C.J. The suppression of Ca^{2+}- and voltage-dependent outward K^+ current during mAChR activation in rat adrenal chromaffin cells. *J. Physiol.* (1995) **485**: 297-318

Heuser, J.E. The role of coated vesicles in recycling of synaptic vesicle membrane. *Cell. Biol. Int. Rep.* (1989) **13**: 1063-1076

Hilfiker, S., Pieribone, V.A., Norstedt, C., Greengard, P. and Czernik, A.J. Regulation of synaptotagmin I phosphorylation by multiple protein kinases. *J. Neurochem.* (1999) **73**: 921-932

Hirose, K., Kadowaki, S., Tanabe, M., Takeshima, H. and Lino, M. Spatiotemporal dynamics of inositol 1,4,5-trisphosphate that underlies complex Ca^{2+} mobilization patterns. *Science* (1999) **284**: 1527-1530

Hodel, A., Schafer, T., Gerosa, D. and Burger, M.M. In chromaffin cells, the mammalian Sec1p homologue is a syntaxin 1A-binding protein associated with chromaffin granules. *J. Biol. Chem.* (1994) **269**: 8623-8626

Hodgkin, M.N., Pettitt, T.R., Martin, A., Michell, R.H., Pemberton, A.J. and Wakelam, M.J. Diacylglycerol and phosphatidates: which molecular species are intracellular messengers? *Trends Biochem. Sci.* (1998) **23**: 200-204

Hofmann, J. The potential for isoenzyme-selective modulation of protein kinase C. *FASEB. J.* (1997) **11**: 649-669

Hogg, R.C., Raggenbass, M. and Bertrand, D. Nicotinic acetylcholine receptors: from structure to brain function. Rev. Physiol. Biochem. Pharmacol. (2003) **147**: 1-46

Hogg, R.C. and Bertrand, D. Regulating the regulators: the role of nicotinic acetylcholine receptors in human epilepsy. *Drug News Perspect.* (2003) **16**: 261-266

Hollinger, S. and Hepler, J.R. Cellular regulation of RGS proteins: Modulators and itergrators of G protein signalling. *Pharmacol. Rev.* (2002) **54**: 527-559

Holz, R.W., Bittner, M.A. and Senter, R.A. Regulated exocytotic fusion I: chromaffin cells and PC12 cells. *Methods Enzymol.* (1992) **219**: 165-178

Holz, R.W., Brondyk, W.H., Senter, R.A., Kuizon, L. and Macara, I.G. Evidence for the involvement of Rab3A in Ca^{2+}-dependent exocytosis from adrenal chromaffin cells. *J. Biol. Chem.* (1994) **269**: 10229-10234

Houchi, H., Yoshizumi, M., Minakuchi, K., Ishimura, Y., Morita, K., Tamaki, T. and Oka, M. Potentiation of histamine-induced catecholamine secretion by ouabain in cultured bovine adrenal chromaffin cells is dependent on calcium and sodium influx. *Life Sci.* (1997) **60**: 2051-2058

House, C. and Kemp, B.E. Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. *Science* (1987) **238**: 1726-1728

Hug, H. and Sarre, T.F. Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.* (1993) **291**: 329-343

Huganir, R.L. Regulation of the nicotinic acetylcholine receptor by serine and tyrosine protein kinases. *Adv. Exp. Med. Biol.* (1991) **287**:279-294

Hur, E.M. and Kim, K.T. G protein-coupled receptors signalling and cross-talk: achieving rapidly and specificity. *Cell Signal.* (2002) **14**: 397-405

Hurley, J.H., Newton, A.C., Parker, P.J., Blumberg, P.M. and Nishizuka, Y. Taxonomy and function of C1 protein kinase C homology domains. *Protein. Sci.* (1997) **6**: 477-480

Huttner, W.B. and Natori, S. Regulated secretion. Helper proteins for neuroendocrine secretion. *Curr. Biol.* (1995) **5**: 242-245

Huwiler, A., Fabbro, D. and Pfeilshifter, J. Possible regulatory functions of protein kinase C-α and ε isoenzymes in rat mesangial cells: stimulation of prostaglandin synthesis and feedback inhibition of angiotensin II-stimulated phosphoinositide hydrolysis. *Biochem. J.* (1991) **279**: 441-445

Ikeda, M., Fahien, L.A. and Udenfriend, S. A kinetic study of bovine adrenal tyrosine hydroxylase. *J. Biol. Chem.* (1966) **241**: 4452-6

Ikeda, S.R. and Dunlap, K. Voltage-dependent modulation of N-type calcium channels: role of G protein subunits. *Adv. Second Messenger phosphoprotein Res.* (1999) **33**: 131-151

Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. *J. Biol. Chem.* (1977) **252**: 7610-7616

Ioannides, C.G., Freedman, R.S., Liskamp, R.M., Ward, N.E. and O'Brian, C.A. Inhibition of IL-2 receptor induction and IL-2 production in the human leukemic cell line jurkat by a novel peptide inhibitor of protein kinase C. *Cell. Immunol.* (1990) **131**: 242-252

Ishii, H., Jirousek, M.R., Koya, D., Takagi, C., Xia, P., Clermont, A., Bursell, S.E., Kern, T.S., Ballas, L.M., Heath, W.F., Stramm, L.E., Feener, E.P. and King,

G.L. Amelioration of vascular dysfunction in diabetic rats by an oral PKC beta inhbition. *Science* (1996) **272**: 728-731

Isobe, K., Nakai, T. and Takuwa, Y. Ca^{2+}-dependent effect of pituitary adenylate cyclase-activating polypeptide on catecholamine secretion from cultured porcine adrenal medullary chromaffin cells. *Endocrinology* (1993) **132**: 1757-1765

Izrael, M., Van der Zee, E.A., Slotkin, T.A. and Yanai, J. Cholinergic synaptic signalling mechanisms underlying behavioural teratogenicity: effects of nicotine, Chlorpyrifos, and heroin coverage on protein kinase C translocation in the intermediate part of the hyperstriatium ventrale and on impairing behavior in avian model. *J. Neurosci. Res.* (2004) **78**: 499-507

Jagger, D.J., Griesinger, C.B., Rivolta, M.N., Holly, M.C. and Ashmore, J.F. Calcium signalling mediated by the 9 acetylcholine receptor in a cochlear cell line from the immorlomouse. *J. Physiol.* (2000) **15**: 49-54

Jaken, S. Protein kinase C isozymes and substrates. *Curr. Opin .Cell. Biol.* (1996) 8: 168-173

Jan, L.Y. and Stevens, C.F. Signalling mechanisms: a decade of signalling. *Curr. Opin. Neurobiol.* (2000) **10**: 625-630

Janigro, D., Maccaferri, G. and Meldolesi, J. Calcium channels in undifferentiated PC12 rat pheochromocytoma cells. *FEBS Lett.* (1989) **255**: 398-400

Jirousek, M.R., Gillig, J.R., Gonzalez, C.M., Heath, W.F., McDonald, J.H., Neel, D.A., Rito, C.J., Singh, U., Stramm, L.E., Melikian-Badalian, A., Baevsky, M., Ballas, L.M., Hall, S.E., Winneroski, L.L. and Faul, M.M. (S)-13-[Dimethylamino)methyl]-10, 11, 14, 15-tetrahydro-4, 9: 16, 21-dimetheno-1H, 13H-dibenzo[e, k] pyrrolo [3, 4-h] [1,4,13] oxadiazacyclohexadecene-1, 3 (2H)-dione (LY333531) and related analogues isozyme selective inhibitors of protein kinase Cβ. *J. Med. Chem.* (1996) **39**: 2664-2671

Johnson, J.A., Gary, M.O., Chen, C.H., Mochly-Rosen, D. A protein kinase C translocation inhibitor as isoenzyme-selective antagonist of cardiac function. *J. Biol. Chem.* (1996) **271**: 24962-24966

Johnson, L.N. and O'Reilly, M. Control by phosphorylation. *Curr. Opin. Struct. Biol.* (1996) **6**: 762-769

Johnson, J.E., Giorgione, J., Newton, A.C. The C1 and C2 domains of protein kinase C are independent membrane targeting modules with specificity for phosphatidylserine conferred by C1 domain. *Biochemistry* (2000) **39**: 11360-11369

Johnson, R.G. Accumulation of biological amines into chromaffin granules: a model for hormone and neurotransmitter transport. *Physiol. Rev.* (1988) **68**: 232-307

Johnstone, S.A., Hubaishy, I. and Waisman, D.M. Phosphorylation of annexin II tetramer by protein kinase C inhibits aggregation of lipid vesicles by the protein. *J. Biol. Chem.* (1992) **267**: 25976-25981

Junge, H.J., Rhee, J.S., Jahn, O., Varoqueaux, F., Spiess, J., Waxham, M.N., Rosenmund, C. and Brose, N. Calmodulin and Munc13 form a Ca^{2+} sensor/effector complex that controls short-term synaptic plasticity. *Cell* (2004) **118**: 389-401

Kalcheim, C., Langley, K. and Unsicker, K. From the neural crest to chromaffin cells: introduction to a session on chromaffin cell development. *Ann. N. Y. Acad. Sci.* (2002) **971**: 544-546

Kanner, B.I. and Schuldiner, S. Mechanism of storage and transport of neurotransmitters. *CRC. Crit. Rev. Biochem.* (1987) **22**: 1-38

Kao, L.S. and Schneider, A.S. Calcium mobilization and catecholamine secretion in adrenal chromaffin cells. A Quin-2 fluorescent study. *J. Biol. Chem.* (1986) **261**: 4881-4888

Kashiwada, Y., Huang, L., Ballas, L.M., Jiang, J.B., Janzen, W.P. and Lee, K.H. New hexahydroxybiphenyl derivatives as inhibitors of protein kinase C. *J. Med. Chem.* (1994) **37**: 195-200

Katz, B., Katz, M., Wolfson, Wolfson, W.O., Cohn, C. and Rubenstein, B.B. Increased adrenal cortical function accompanying prolonged remission induced by ACTH in myasthenia gravis. *J. Clin. Endocrinol. Metab.* (1950) **10**: 835-836

Katz, B. Quantal mechanism of neural transmitter release. *Science.* (1971) **173**: 123-126

Kavran, J.M., Klein, D.E., Lee, A., Falasca, M., Isakoff, S.J., Skolnik, E.Y. and Lemmon, M.A. Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains. *J. Biol. Chem.* (1998) **273**: 30497-30508

Kazanietz, M.G. Novel "nonkinase" phorbol ester receptors: the C1 domain connection. *Mol. Pharmacol.* (2002) **61**: 759-767

Keenan, C. and Kelleher, D. Tropical Review protein kinase C and the cytoskeleton. *Cell Signal.* (1998) **10**: 225-232

Kelly, R.B. Storage and release of neurotransmitters. *Cell* (1993) **72**: 45-53

Keranen, L.M., Dutil, E.M., Newton, A.C. Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. *Curr. Biol.* (1995) **5**: 1394-1403

Kiley, S.C. and Jaken, S. Protein kinase C: interactions and consequences. *Trends. Cell. Biol.* (1994) **4**: 223-227

Kilpatrick, D.L., Slepetis, R. and Kirshner, N. Ion channels and membrane potential in stimulus-secretion coupling in adrenal medulla cells. *J. Neurochem.* (1981) **36**: 1245-1255

Kilpatrick, D.L., Slepetis, R.J., Corcoran, J.J. and Kirschner, N. Ca^{2+} uptake and catecholamine secretion by cultured bovine adrenal medulla cells. *J. Neurochem.* (1982) **38**: 427-435

Kim, K.T. and Westhead, E.W. Cellular responses to Ca^{2+} from extracellular and intracellular sources are different as shown by simultaneous measurements of cytosolic Ca^{2+} and secretion from bovine chromaffin cells. *Proc. Natl. Acad. Sci. U.S.A.* (1989) **86**: 9881-9885

Kiselyov, K., Shin, D.M. and Muallem, S. Signalling specificity in GPCR-dependent Ca^{2+} signalling. *Cell. Signal.* (2002) **15**: 243-253

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.* (1980) **255**: 2273-2276

Klabunde, T. and Hessler, G. Drug design strategies for targeting G-protein-coupled receptors. *Chem. Bio. Chem.* (2002) **3**: 928-944

Knight, D.E. and Kesteven, N.T. Evoked transient intracellular free Ca^{2+} changes and secretion in isolated bovine adrenal medullary cells. *Proc. R. Soc. Lond. B. Biol. Sci.* (1983) **218**: 177-1799

Knight, D.E., Sugden, D. and Baker, P.F. Evidence implicating protein kinase C in exocytosis from electropermeabilized bovine chromaffin cells. *J. Membr. Biol.* (1988) **104**: 21-34

Knight, D.E., Von Grafenstein, H. and Athayde, C.M. Calcium-dependent and calcium-independent exocytosis. *Trend Neurosci.* (1989) **12**: 451-458

Koch, H., Hofmann, K. and Brose, N. Definition of munel3-homology-domains and characterization of novel ubiquitously expressed munel3 isoform. *Biol, Chem. J.* (2000) **349**: 247-253

Koffer, A., Tatham, P.E.R. and Gompers, B.D. Changes in the state of actin during the exocytotic reaction in the mast cells. *J. Cell Biol.* (1990) **111**: 919-927

Koizumi, S., Rosa, P., Willars, G.B., Challiss, R.A.J., Taverna, E., Francolini, M., Bootman, M.D., Lipp, P., Inoue, K., Roder, J. and Jeromin, A. Mechanisms underlying the neuronal calcium sensor-1-evoked enhancement of exocytosis in PC12 cells. *J. Biol. Chem.* (2002) **277**: 30315-30324

Kotsonis, P. and Majewski, H. The structural requirements for phorbol esters to enhance noradrenaline and dopamine release from rat brain cortex. *Br. J. Pharmacol.* (1996) **119**: 115-125

Kowluru, R.A., Jirousek, M.R., Stramm, L., Farid, N., Engerman, R.L. and Kern, R.S. Abnormalities of retinal metabolism in diabetes or experimental galactosemia: V. relationship between protein kinase C and ATPases. *Diabetes* (1998) **47**: 464-469

Kraft, A.S., Anderson, W.B., Cooper, H.L. and Sando, J.J. Decrease in cytosolic calcium/phospholipid-dependent protein kinase C activity following phorbol ester treatment of El4 thymoma cells. *J. Biol. Chem.* (1982) **257**: 13193-13196

Krejci, E., Gasnier, B., Botton, D., Isambert, M.F., Sagnē, C., Gagnon, J., Massouliē, J. and Henry, J.P. Expression and regulation of the bovine vesicular monoamine transporter gene. *FEBS Lett.* (1993) **335**: 27-32

Kretzschmar, S., Volknandt, W. and Zimmermann, H. Colocalization on the same synaptic vesicles of Syntaxin and SNAP-25 with synaptic vesicle proteins: a re-evaluation of functional models required? *Neurosci. Res.* (1996) **26**: 141-148

Kristiansen, K. Molecular mechanisms of ligand binding, signalling, and regulation within the superfamily of G-protein-coupled receptors: molecular modelling and mutagenesis approaches to receptor structure and function. *Pharmacol. Ther.* (2004) **103**: 21-80

Krupnick, J.G. and Benovic, J.L. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu. Rev. Pharmacol. Toxicol.* (1998) **38**: 289-319

Kudlacek, O., Just, H., Korkhov, V.M., Vartian, N., Klinger, M., Pankevych, H., Yang, Q., Nanoff, C., Freissmuth, M. and Boehm, S. The human D_2 dopamine receptor synergizes with the A_{2A} adenosine receptor to stimulate adenylyl cyclase in PC12 cells. *Neuropsychopharmacology* (2003) **28**: 1317-1327

Kuryatov, A., Olale, F.A., Choi, C. and Lindstrom, J. Acetylcholine receptor extracellular domain determines sensitivity to nicotine-induced inactivation. *Eur. J. Pharmacol.* (2000) **393**: 11-21

Kuwashima, H., Matsumura, C. and Kimura, T. Differential secretion of adrenaline and noradrenaline in response to various secretagogues from bovine chromaffin cells. *Clin. Exp. Pharmacol. Physiol.* (2000) **27**: 494-499

Lara, B., Gandia, L., Martinez-Sierra, R., Torres, A. and Garcia, A.G. Q-type Ca^{2+} channels are located closer to secretory sites than L-type channels: functional evidence in chromaffin cells. *Pflügers Arch.* (1998) **435**: 472-478

Laslop, A. and Mahata, S.K. Neuropeptides and chromogranins: session overview. *Ann. N. Y. Acad. Sci.* (2002) **971**: 294-299

Lawrence, G.W., Foran, P. and Dolly, J.O. Insights into a basis for incomplete inhibition by botulinum toxin A of Ca^{2+}-evoked exocytosis from permeabilised chromaffin cells. *Toxicology* (2002) **181**: 249-253

Lax, P., Fucile, S. and Eusebi, F. Ca^{2+} permeability of human heteromeric nAChRs expressed by transfection in human cells. *Cell Calcium* (2002) **32**: 53-58

Lee, R.W.H. and Trifaro, J.M. Characterization of anti-actin antibodies and their use in immunocytochemical studies on the localization of actin in adrenal chromaffin cells in culture. *Neuroscience.* (1981) **6**: 2087-2108

Leenders, A.G.M. and Sheng, Z.H. Modulation of neurotransmitter release by the second messenger-activated protein kinases: implications for presynaptic plasticity. *Pharmacol. Ther.* (2005) **105**: 69-84

Lefkowitz, R.J. G protein-coupled receptor kinases. *Cell* (1993) **74**: 409-412

Lelkes, P.I., Friedman, J.E., Rosenheck, K. and Optaka, A. Destabilization of actin filaments as a requirement for the secretion of catecholamines from permeabilized chromaffin cells. *FEBS. Lett.* (1986) **208**: 357-363

Lemons, P.P., Chen, D., Bernstein, A.M., Bennett, M.K. and Whiteheart, S.W. Regulated secretion in platelets: identification of elements of the platelet exocytosis machinery. *Blood* (1997) **90**: 1490-1500

Leonard, S., Adler, L.E., Benhammou, K., Berger, R., Breese, C.R., Drebing, C., Gault, A.J., Lee, M.J., Logel, J., Olincy, A., Ross, R.G., Stevens, K., Sullivan, B., Vianzon, R., virnich, D.E., Waldo, M., Walton, K. and freeman, R. Smoking and mental illness. *Pharmacol. Biochem. Behav.* (2001) **70**: 561-570

Leonard, S. and Bertrand, D. Neuronal nicotinic receptors: from structure to function. *Nicotine and Tobacco research* (2001) **3**: 203-223

Levitt, M. Spector, S., Sjoerdsma, A. and Udenfriend, S. elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. *J. Pharmacol. Exp. Ther.* (1965) **148**: 1-8

Li, C., Ullrich, B., Zhang, J.Z., Anderson, R.G.W., Brose, N. and Sudhof, T.C. Ca^{2+}-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature.* (1995) **375**: 594-599

Li, L. and Chin, L.S. The molecular machinery of synaptic vesicle exocytosis. *Cell. Mol. Life. Sci.* (2003) **60**: 942-960

Li, Q., Ho, C.S., Marinescu, V., Bhatti, H., Bokoch, G.M., Ernst, S.A., Holz, R.W. and Stuenkel, E.L. Facilitation of Ca^{2+}-dependent exocytosis by Rac1-GTPase in bovine chromaffin cells. *J. Physiol.* (2003) **550**: 431-445

Liang, M., Eason, M.G., Jewell-Motz, E.A., Williams, M.A., Theiss, C.T., Dorn, G.W. and Liggett, S.B. Phosphorylation and functional desensitisation of the α_{1A}-adrenergic receptor by protein kinase C. *Mol. Pharmacol.* (1998) **54**: 44-49

Lin, R.C. and Scheller, R.H. Mechanisms of synaptic vesicle exocytosis. *Ann. Rev. Cell Dev. Biol.* (2000) **16**: 19-49

Lindau, M. and Gomperts, B.D. Techniques and concepts in exocytosis: focus on mast cells. *Biochim. Biophys. Acta.* (1991) **1071**: 429-471

Lindau, M. and Almers, W. Structure and function of fusion pores in exocytosis and ectoplasmic membrane fusion. *Curr. Opin. Cell. Biol.* (1995) **7**: 509-517

Linden, D.J. and Routtenberg, A. The role of protein kinase C in long-term potentiation: a testable model. *Brain Res. Rev.* (1989) **14**: 279-296

Liu, W.S. and Heckman, C.A. The sevenfold way of PKC regulation. *Cell. Signal.* (1998) **10**: 529-542

Livett, C.F., Dean, D.M., Whelan, L.G., Undenfriend, S. and Rossier, J. Co-release of enkephalin and catecholamines from cultured adrenal chromaffin cells. *Nature* (1981) **289**: 317-319

Livett, B.G. Adrenal medullary chromaffin cells in vitro. *Physiol. Rev.* (1984) **64**:1103-1161

Livett, B.G. and Marley, P.D. Effects of opioides and morphine on histamine induced catecholamine secretion from cultured bovine adrenal chromaffin cells. *Br. J. Pharmacol.* (1986) **89**: 327-334

Lohse, M.J., Benovic, J.L., Codina, J., Caron, M.G. and Lefkowitz, R.J. β-arrestin: a protein that regulates β-adrenergic receptor function. *Science* (1990) **248**: 1547-1550

López, M.G., Albillos, A., de La Fuente, M.T., Borges, R., Gandia, L., Carbone, E., Gorcia, A.G and Artalejo, A.R. Localized L-type calcium channels control exocytosis in cat chromaffin cells. *Pflügers Arch.* (1994a) **427**: 348-354

López, M.G., Montiel, C., herrero, C.J., Garcia-Palomero, E., mayorgas, I., Guijo, J.M., Villarroya, M., Olivares, R., Gandia, L., McIntosh, J.M., Olivera, B.M. and Garcia, A.G. Unmasking the functions of the chromaffin cell alpha7 nicotinic receptor by using short pulses of acetylcholine and selective blockers. *Proc. Natl. Acad. Sci. USA.* (1998) **95**: 14184-14189

Luetje, C.W. and Patrick, J. Both alpha- and beta-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. *J. Neurosci.* (1991) **11**:837-845

Lytton, J., Westlin, M. and Hanley, M.R. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase family of calcium pumps. *J. Biol. Chem.* (1991) **266**: 17067-17071

MacDermott, A.B., Role, L.W. and Siegelbaum, S.A. Presynaptic ionotropic receptors and the control of transmitter release. *Annu. Rev. Neurosci.* (1999) **22**: 443-485

Maeno-Hikichi, Y., Chang, S., Matsumura, K., Lai, M., Lin, H., Nakagawa, N., Kuroda, S. and Zhang, I.F. A PKC epsilon-ENH-channel complex specificity modulates N-type Ca^{2+} channels. *Nat. Neurosci.* (2003) **6**: 468-475

Mahata, M., Mahapatra, N.R., O'Connor, D.T. and Mahata, S.K. Chromaffin cell catecholamine secretion: bisindolylmaleimide compounds exhibit novel and potent antagonist effects at the nicotinic cholinergic receptor in pheochromocytoma cells. *Mol. Pharmacol.* (2002) **61**: 1340-1347

Majewski, H. and Iannazzo, L. Protein kinase C: a physiological mediator of enhanced transmitter output. *Prog. Neurobiol.* (1998) **55**: 463-475

Majewski, H., kotsonis, P., Iannazzo, L., Murphy, T.V. and Musgrave, I.F. Protein kinase C and transmitter release. *Clin. Exp. Pharmacol. Physiol.* (1997) **24**: 619-623

Maneu, V., Rojo, J., Mulet, J., Valor, L.M., Sala, F., Criado, M., Garcia, A.G. and Gandia, L. A single neuronal nicotinic receptor $\alpha3\alpha7\beta4$ is present in the bovine chromaffin cell. *Ann. N. Y. Acad. Sci.* (2002) **971**: 165-167

Margioris, A.N., Venihaki, M., Stournaras, C. and Gravanis, A. PC12 cells as a model to study the effects of opioids on normal and tumoral adrenal chromaffin cells. *Ann. N. Y. Acad. Sci.* (1995) **771**: 166-172

Markos, F., Healy, V. and Harvey, B.J. Aldosterone rapidly activate N^+/H^+ exchange in M-1 cortical collecting duct cells via a PKC-MAPK pathway. *Nephron. Physiol.* (2005) **99**: 1-9

Marley, P.D. and Livett, B.G. Differences between the mechanism of adrenaline and noradrenaline secretion from isolated bovine adrenal chromaffin cells. *Neurosci. Lett.* (1987) **77**: 81-86

Marley, P.D. Mechanisms in histamine-mediated secretion from adrenal chromaffin cells. *Pharmacol. Ther.* (2003) **98**:1-34

Marley, P.D. New insights into the non-nicotinic regulation of adrenal medullary functions. *Trends Pharmacol. Sci.* (1987) **8**: 411-413

Marley, P.D., Wallace, D., Donald, M. and McKenzie, S. How does histamine evoke catecholamine secretion from bovine chromaffin cells? *Ann. N.Y. Acad. Sci.* (2002) **971**: 148-149

Mathur, A. and Vallano, M.L. 2, 2`, 3, 3`, 4,4`-hexahydroxy-1,1-biphenyl-6, 6-dimethanol dimethyl ether (HBDDE)-induced neuronal apoptosis independent of classical protein kinase Cα or γ inhibition. *Biochem. Pharmacol.* (2000) **60**: 809-815

Mayne, G.C. and Murray, A.W. Evidence that protein kinase Cε mediates phorbol ester inhibition of calphostin C- and tumor necrosis factor-α-induced apoptosis in U937 histiocytic lymphoma cell. *J. Biol. Chem.* (1998) **273**: 24115-24121

McGehee, D.S. and Role, L.W. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu. Rev. Physiol.* (1995) **57**: 521-546

Medkova, M. and Cho, W. Interplay of C1 and C2 domains of protein kinase C-alpha in its membrane binding and activation. *J. Biol. Chem.* (1999) **274**: 19852-19861

Mellor, H. and Parker, P.J. The extended protein kinase C superfamily. *Biochem. J.* (1998) **332**: 281-292

Messing, R.O., Stevens, A.M., Kiyasu, E. and Sneade, A.B. Nicotinic and muscarinic agonists stimulate rapid protein kinase C translocation in PC12 cells. *J. Neurosci.* (1989) **9**: 507-512

Miledi, R., Parker, I. and Schalow, G. Transmitter induced calcium entry across the post-synaptic membrane at frog endplates measured using arsenazo III. *J. Physiol. (Camb.)* (1980) **300**: 197-212

Mochly-Rosen, D. and Gordon, A.S. Anchoring proteins for protein kinase C: a means for isozyme selectivity. *FASEB. J.* (1998) **12**: 35-42

Mochly-Rosen, D. Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science* (1995) **268**: 247-251

Mochly-Rosen, D., Khaner, H. and Lopez, J. Identification of intracellular receptor proteins for activated protein kinase C. *Proc. Natl. Acad. Sci. U.S.A.* (1991a) **88**: 3997-4000

Mochly-Rosen, D., Henrich, C.J., Cheever, L., Khaner, H. and Simpson, P.C. A protein kinase isozyme is translocates to cytoskeletal elements on activation. *Mol. Biol. Cell* (1990) **1**: 693-706

Mochly-Rosen, D., Khaner, H., Lopez, J. and Smith, B.L. Intracellular receptors for activated protein kinase C. Identification of a binding site for the enzyme. *J. Biol. Chem.* (1991b) **266**: 14866-14868

Mogami, H., Zhang, H., Suzuki, Y., Urano, T., Saito, N., Kojima, I. And Petersen, O.H. Decoding of short-lived Ca^{2+} influx signals into long term substrate phosphorylation through activation of two distinct classes of protein kinase C. *J. Biol. Chem.* (2003) **278**: 9896-9904

Mollard, P., Seward, E.P and Nowycky, M.C. Activation of nicotinic receptors triggers exocytosis from bovine chromaffin cells in the absence of membrane depolarization. Proc. *Natl. Acad. Sci. U. S. A.* (1995) **92**: 3065-3069

Morgan, A and Burgoyne, R.D. Common mechanisms for regulated exocytosis in the chromaffin cell and the synapse. *Semin. Cell. Dev. Biol.* (1997) **8**: 141-149

Mosior, M., McLaughlin, S. Peptides that mimic the pseudosubstrate region of protein kinase C bind to acidic lipids in membranes. *Biophys. J.* (1991) **60**: 149-159

Mulle, C., Benoit, P., Pinsel, C., Roa, M. and Changeux, J.P. Calcitonin gene-related peptide enhances the rate of desensitization of the nicotinic acetylcholine receptor in cultured mouse muscle cells. *Proc. Natl. Acad. Sci. USA.* (1988) **85**: 5728-5732

Muller, G. Towards 3D structures of G protein-coupled receptors: a multidisciplinary approach. *Curr. Med. Chem.* (2000) **7**: 861-888

Murthy, V.N. and De Camilli, P. Cell biology of the presynaptic terminal. *Annu. Rev. Neurosci.* (2003) **26**: 701-728

Murthy, V.N. and Stevens, C.F. Synaptic vesicles retain their identity through the endocytic cycle. *Nature* (1998) **392**: 497-501

Nagy, G., Matti, U., Nehring, R.B., Binz, T., Rettig, J., Neher, E. and Sϕrensen, J.B. Protein kinase C-dependent phosphorylation of synaptosome-associated protein of 25 kDa at Ser187 potentiates vesicle recruitment. *J. Neurosci.* (2002) **22**: 9278-9286

Nahorski, S.R., Young, K.W., Challiss, R.A.J. and Nash, M.S. Visualizing phosphoinositide signalling in single neurons gets a green light. *Trends Neurosci.* (2003) **26**: 444-452

Nakaki, T., Sasakawa, N., Yamamoto, S. and Kato, R. Functional shift from muscarinic to nicotinic cholinergic receptors involved in inositol trisphosphate and cyclic GMP accumulation during the primary culture of adrenal chromaffin cells. *Biochem. J.* (1988) **251**: 397-403

Nakanishi, S., Catt, K.J. and Balla, T.A. Wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositol phospholipids. *Proc. Natl. Acad. Sci. U.S.A.* (1995) **92**: 5317-5321

Nalefski, E.A. and Falke, J.J. The C2 domain calcium-binding motif: structural and functional diversity. *Protein. Sci.* (1996) **5**: 2375-2390

Nardone, J., Gerald, C., Rimawi, L. and Hogan, P.G. Identification of a B_2 bradykinin receptor expressed by PC12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. U.S.A.* (1994) **91**: 4412-4416

Nash, M.S., Young, K.W., Willars, G.B., Challiss, R.A.J. and Nahorski, S.R. Single-cell imaging of graded Ins(1,4,5)P_3 production following G-protein-coupled-receptor activation. *Biochem. J.* (2001) **356**: 137-142

Nassar-Gentina, V., Catalan, L. and Luxoro, M. Nicotinic and muscarinic components in acetylcholine stimulation of porcine adrenal medullary cells. *Mol. Cell. Biochem.* (1997) **169**:107-113

Neer, E.J. Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* (1995) **80**: 249-257

Neher, E. and Zucker, R.S. Multiple calcium dependent processes related to secretion in bovine chromaffin cells. *Neuron* (1993) **10**: 21-30

Newton, A.C. and Johnson, J.E. Protein kinase C: a paradigm for regulation of protein function by two membrane-targeting modules. *Biochim. Biophys. Acta.* (1998) **1376**: 155-172

Newton, A.C. and Keranen, L.M. Phosphatidyl-L-serine is necessary for protein kinase C is high-affinity interaction with diacylglycerol-containing membranes. *Biochemistry* (1994) **33**: 6651-6658

Newton, A.C. Interaction of proteins with lipid headgroups: lessons from protein kinase C. *Annu. Rev. Biophys. Biomol. Struct.* (1993) **22**: 1-25

Newton, A.C. Protein kinase C: Protein kinase C. Seeing two domains. *Curr. Biol.* (1995) **5**: 973-976

Newton, A.C. Regulation of protein kinase C. *Curr. Opin. Cell. Biol.* (1997) **9**: 161-167

Newton, A.C. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem. Rev.* (2001) **101**: 2353-23564

Newton, A.C. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *Biochem. J.* (2003) **370**: 361-371

Nie, Z., Ranjan, R., Wenniger, J.J., Hong, S.N., Bronk, P. and Zinsmaier, K.E. Overexpression of cysteine-string proteins in Drosophila reveals interactions with syntaxin. *J. Neurosci.* (1999) **19**: 10270-10279

Ninomiya, Y., Kishimoto, T., Yamazawa, T., Ikeda, H., Miyashita, Y. and Kasai, H. Kinetic diversity in the fusion of exocytotic vesicles. *EMBO J.* (1997) **16**: 929-934

Nishizuka, Y., Inoue, M., Kishimoto, A. and Takai, Y. Studies on a cyclic nucleotide-independent protein kinase C and its proenzyme in mammalian tissues. *J. Biol. Chem.* (1977) **252**: 7610-7616

Nishizuka, Y. Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. *Science* (1992) **258**: 607-614

Nishizuka, Y. Protein kinase C and lipid signalling for sustained cellular responses. *FASEB J.* (1995) **9**: 484-496

Nishizuka, Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* (1988) **334**: 661-665

Noble, E.P., Bommer, M., Liebisch, D. and Herz, H. H_1-histaminergic activation of catecholamine release by chromaffin cells. *Biochem. Pharmacol.* (1988) **37**: 221-228

Noble, E.P., Bommer, M., Sincini, E., Costa, T. and Herz, A. H_1-histaminergic activation stimulates inositol-1-phosphate accumulation in chromaffin cells. *Biochem. Biophys. Res. Commun.* (1986) **135**: 566-573

Nusse, O. and Lindau, M. The dynamics of exocytosis in human neutrophils. *J. Cell. Biol.* (1988) **107**: 2117-2123

O'Connell, G.C., Douglas, S.A. and Bunn, S.J. The involvement of specific Phospholipase C isozymes in catecholamine release from digitonin permeabilized bovine adrenal medullary chromaffin cells. *Neurosci. Lett.* (2003) **342**: 1-4

O'Dell, T.J., Kandel, E.R. and Grant, S.G.N. Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature* (1991) **353**: 558-560

O'Farrell, M. and Marley, P.D. Different contributions of voltage-sensitive Ca^{2+} channels to histamine-induced catecholamine release and tryrosine hydroxylase activation in bovine adrenal chromaffin cells. *Cell Calcium* (1999) **25**: 209-217

O'Sullivan, A.J. and Burgoyne, R.D. A comparison of bradykinin, angiotensin II and muscarine stimulation of cultured bovine adrenal chromaffin cells. *Biosc. Rep.* (1989) **9**: 243-252

O'Sullivan, A.J., Cheek, T.R., Moreton, R.B., Berridge, M.J. and Burgoyne, R.D. Localization and heterogeneity of agonist-induced changes in cytosolic calcium concentration in single bovine adrenal chromaffin cells from video-imaging of fura-2. *EMBO J.* (1989) **8**: 401-411

Oancea, E. and Meyer, T. Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. *Cell.* (1998) **95**: 307-318

Oheim, M. and Stuhmer, W. Tracking chromaffin granules on their way through the actin cortex. *Eur. Biophys. J.* (2000) **29**: 67-89

Ohmori, S., Sakai, N., Shirai, Y., Yamamoto, H., Miyamoto, E., Shimizu, N. and Saito, N. Importance of protein kinase C targeting for the phosphorylation of its substrate, myristoylated alanine-rich C-kinase substrate. *J. Biol. Chem.* (2000) **275**: 26449-26457

Ohta, T., Morishita, M., Mori, Y. and Ito, S. Ca^{2+} store-independent augmentation of $[Ca^{2+}]_i$ responses to G-protein coupled receptor activation in recombinantly TRPC5-expressed rat pheochromocytoma (PC12) cells. *Neurosci. Lett.* (2004) **358**: 161-164

Okabe, T., sugimoto, N. and Matsuda, M. Calmodulin is involved in catecholamine secretion from digitonin-permeabilized bovine adrenal medullary chromaffin cells. *Biochem. Biophys. Res. Commun.* (1992) **186**: 1006-1011

Okada, T., Sakuma, L., Fukui, Y., Hazeki, O. and Ui, M. Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as result of selective inhibition of phosphatidylinositol 3-kinase. *J. Biol. Chem.* (1994) **269**: 3563-3567

Olivares-Reyes, J.A., Smith, R.D., Hunyady, L., Shah, B.H. and Catt, K.J. Agonist-induced signalling, desensitisation, and internalisation of phosphorylation-deficient AT1A angiotensin receptor. *J. Biol. Chem.* (2001) **276**: 37761-37768

Olivier, A.R. and Parker, P.J. Identification of multiple PKC isoforms in Swiss 3T3 cells: differential downregulation by phorbol ester. *J. Cell. Physiol.* (1992) **152**: 240-244

Ono, Y., Fujii, T, Iganashi, K., Kuno, T., Tanaka, C., Kikkawa, U. and Nishizuka, Y. Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. *Proc. Natl. Acad. Sci. USA.* (1989) **86**: 4868-4871

Orci, L., Gabbay, K.H. and Malaisse, W.J. Pancreatic beta-cell web: its possible role in insulin secretion. *Science* (1972) **175**: 1128-1130

Orita, S., Naito, A., Sakaguchi, G., Maeda, M., Igarashi, H., Sasaki, T. and Takai, Y. Physical and functional interaction of Doc2 and Munc13 in Ca^{2+}-dependent exocytotic machinery. (1997) **272**: 16081-16084

Orr, J.W. and Newton, A.C. Interaction of protein kinase C with phosphatidylserine 2: specificity and regulation. *Biochemistry* (1992) **31**: 4667-4673

Orr, J.W., Keranen, L.M. and Newton, A.C. Reversible exposure of the pseudosubstrate domain of protein kinase C by phosphatidylserine and diacylglycerol. *J. Biol. Chem.* (1992) **267**: 15263-15266

Osada, S., Mizuno, K., Saido, T.C., Suzuki, K., Kuroki, T. and Ohno, S. A new member of the protein kinase C family, nPKC theta, predominantly expressed in skeletal muscle. *Mol. Cell. Biol.* (1992) **12**: 3930-3938

Ouimet, C.C., Wang, J.K., Walaas, S.I., Albert, K.A. and Greengard, P. Localization of the MARCKS (87 kDa) protein, a major specific substrate for protein kinase Cs in rat brain. *J. Neurosci.* (1990) **10**: 1683-1698

Pak, Y., O'Dowd, B.F., Wang, J.B. and George, S.R. Agonist-induced G-protein-dependent and –independent down-regulation of the mu opioid receptor. The receptor is a direct substrate for protein-tyrosine kinase. *J. Biol. Chem.* (1999) **274**: 27610-27616

Palfrey, H.C. and Artalejo, C.R. Vesicle recycling revisited: rapid endocytosis may be the first step. *Neuroscience* (1998) **83**: 969-989

Palmer, R.H., Ridden, J. and Parker, P.J. Cloning and expression patterns of two members of a novel protein-kinase-C-related kinase family. *Eur. J. Biochem.* (1995) **227**: 344-351

Parmer, R.J. and Zinder, O. Catecholaminergic pathways, chromaffin cells, and human disease. *Ann. N. Y. Acad. Sci.* (2002) **971**: 497-505

Pavlović-Šurjančev, B., Cahill, A.L. and Perlman, R.L. Staurosporine activates a 60,000Mr protein kinase in bovine chromaffin cells that phosphorylates myelin basic protein in vitro. *J. Neurochem.* (1993) **61**: 697-703

Penberthy, W.T. and Dahmer, M.K. Insulin-like growth factor-I-enhanced secretion is abolished in protein kinase C-deficient chromaffin cells. *J. Neurochem.* (1994) **62**: 1707-1715

Pender, N. and Burgoyne, R.D. Histamine-stimulates exocytosis a sub-population of bovine adrenal chromaffin cells. *Neurosci Lett.* (1992) **144**: 207-210

Perlman, R.L. and Chalfie, M. Catecholamine release from the adrenal medulla. *Clin. Endocrinol. Metab.* (1977) **6**: 551-576

Perrin, D. and Aunis, D. Reorganization of α-fodrin induced by stimulation of secretory cells. *Nature* (1995) **315**: 589-592

Pierce, K.L., Premont, R.T. and Lefkowitz, R.J. Seven-transmembrane receptors. *Mol. Cell. Biol. (Nature rev.)* (2002) **3**: 639-649

Plevin, R. and Boarder, M.R. Stimulation of formation of inositol phosphates in primary cultures of bovine adrenal chromaffin cells by angiotensin II, histamine, bradykinin, and carbachol. *J. Neurochem.* (1988) **51**: 634-641

Ponting, C.P. and Parker, P.J. Extending the C2 domain family: C2s in PKCs delta, epsilon, eta, theta, phospholipases, GAPs, and perforin. *Protein. Sci.* (1996) **5**: 162-166

Powell, A.D., Teschemacher, A.G. and Seward, E.P. P2Y purinoceptors inhibit exocytosis in adrenal chromaffin cells via modulation of voltage-operated calcium channels. *J. Neurosci.* (2000) **20**: 606-616

Powis, D.A., Clark, C.L. and O'Brien, K.J. Depleted internal store-activated Ca^{2+} entry can trigger neurotransmitter release in bovine chromaffin cells. *Neurosci. Lett.* (1996a) **204**: 165-8

Powis, D.A., O'Brien, K.J., Harrison, J.M., Jarvie, P.E. and Dunkley, P.R. Mn^{2+} can substitute for Ca^{2+} in causing catecholamine secretion but not for increasing tyrosine hydroxylase phosphorylation in bovine adrenal chromaffin cells. *Cell Calcium.* (1996b) **19**: 419-429

Pozzan, T., Di Virgilio, F., Vicentini, L.M. and Meldolesi, J. Activation of muscarinic receptors in PC12 cells: stimulation of Ca^{2+} influx and redistribution. *Biochem. J.* (1986) **234**: 547-553

Prentki, M., Biden, T.J. Janjic, D., Irvine, R.F., Berridge, M.J. and Wollheim, C.B. Rapid mobilization of Ca^{2+} from rat insulinoma microsomes by inositol-1,4,5-trisphosphate. *Nature.* (1984) **309**: 562-564

Putney, J.W. A model for receptor-regulated calcium entry. *Cell. Calcium.* (1986) **7**: 1-12

Putney, J.W. and McKay, R.R. Capacitative calcium entry channels. *Bioessays.* (1999) **21**: 38-46

Putney, J.W. Capacitative calcium entry revisited. *Cell Calcium* (1990) **11**: 611-624

Ragozzino, D., Barabino, B., Fucile, S. and Eusebi, F. Ca^{2+} permeability of mouse and chick nicotinic acetylcholine receptors expressed in transiently infected human cells. *J. Physiol.* (1998) **507**: 749-757

Ranjan, R., Bronk, P. and Zinsmaier, K.E. Cysteine string protein is required for calcium secretion coupling of evoked neurotransmission in drosophila but not for vesicle recycling. *J. Neurosci.* (1998) **18**: 956-964

Rathouz, M.M. and Berg, D.K. Synaptic-type acetylcholine receptor raise intracellular calcium levels in neurons by two mechanisms. *J. Neurosci.* (1994) **14**: 6935-6945

Rathouz, M.M., Vijayaraghavan, S. and Berg, D.K. Elevation of intracellular calcium levels in neurons by nicotinic acetylcholine receptors. *Mol. Neurobiol.* (1996) **12**: 117-131

Régnier-Vigouroux, A., Tooze, S.A. and Huttner, W.B. Newly synthesized synaptophysin is transported to synaptic-like microvesicles via constitutive secretion and the plasma membrane. *EMBO J.* (1991) **10**: 3589-3601

Reetz, A., Solimena, M., Matteoli, M., Folli, F., Takei, K. and De Camilli, P. GABA and pancreatic β-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. *EMBO J.* (1991) **10**: 1275-1284

Rhee, S.G. Regulation of phosphoinositide-specific Phospholipase C. *Annu. Rev. Biochem.* (2001) **70**: 281-312

Rhee, J.S., Betz, A., Pyott, S., Reim, K., Varoqueaux, F., Agustin, I., Hesse, D., Sudhof, T.C., Takahashi, M., Rosenmund, C. and Brose, N. β phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by munc13s and not by PKCs. *Cell.* (2002) **108**: 121-133

Rhee, S.G., Suh, P.G., Ryu, S.H. and Lee, S.Y. Studies of inositol phospholipid-specific phospholipase C. *Science* (1989) **244**: 546-550

Rhee, S.G. and Bae, Y.S. Regulation of phosphoinositide-specific phospholipase C isozymes. *J. Biol. Chem.* (1997) **272**: 15045-15048

Rhoads, A.R., Parui, R., Vu, N.D., Cadogan, R. and Wagner, P.D. ATP-induced secretion in PC12 cells and photoaffinity labelling of receptors. *J. Neurochem.* (1993) **61**: 1657-1666

Richards, E.M., Raizada, M.K., Gelband, C.H. Sumners, C. Angiotensin II type 1 receptor-modulated signalling pathways in neurons. *Mol. Neurobiol.* (1999) **19**: 25-41

Richmond, J.E., Davis, W.S. and Jorgensen, E.M. Unc-13 is required for synaptic vesicle fusion in *C. elegans*. *Nat. Neurosci.* (1999) **2**: 959-964

Richmond, J.E., Weimer, R.M. and Jorgensen, E.M. An open form of Syntaxin bypasses the requirement for the Unc13 in vesicle priming. *Nature* (2001) **412**: 338-341

Richmond, J.E. and Broadie, K.S. The synaptic vesicle cycle: exocytosis and endocytosis in Drosophila and C.elegens. *Curr. Opin. Neurobiol.* (2002) **12**: 499-507

Rink, T.J., Sanchez, A. and Hallam, T.J. Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. *Nature* (1983) **305**: 317-319

Ritchie, A.K. Catecholamine secretion in a rat pheochromocytoma cell line: two pathways for calcium entry. *J. Physiol. (Lond.)* (1979) **286**: 541-561

Roberts-Thomson, E.L., Saunders, H.I., Palmer, S.M., Powis, D.A., Dunkley, P.R. and Bunn, S.J. Ca^{2+} influx stimulated Phospholipase C activity in bovine adrenal chromaffin cells: responses to K^{+} depolarisation and histamine. *Eur. J. Pharmacol.* (2000) **398**: 199-207

Robinson, I.M. and Burgoyne, R.D. Characterisation of distinct inositol 1,4,5 trisphosphate-sensitive and caffeine-sensitive calcium stores in digitonin-permeabilised adrenal chromaffin cells. *J. Neurochem.* (1991) **56**: 1587-1593

Robinson, I.M., Cheek, T.R. and Burgoyne, R.D. Ca^{2+} influx induced by the Ca^{2+}-ATPase inhibitors 2,5-di-(tert-butyl)-1,4-benzohydroquinone and thapsigargin in bovine adrenal chromaffin cells. *Biochem. J.* (1992) **288**: 457-463

Robinson, P.J. The role of protein kinase and its neuronal substrates dephosphin, B-50 and MARCKS in neurotransmitter release. *Mol. Neurobiol.* (1992) **5**: 87-129

Rodríguez Del Castillo, A., Lemaire, S., Tchakarov, L., Jeyapragasan, M., Doucet, J.P., Vitale, M.L. and Trifaró, J.M. Chromaffin cell Scinderin: a novel calcium-dependent actin-filament severing protein. *EMBO J.* (1990) **9**: 43-52

Rodríguez Del Castillo, A., Vitale, M.L. and Trifaró, J.M. Ca^{2+} and PH determine the interaction of chromaffin cell Scinderin with phosphatidylserine and phosphatidylinositol 4,5-bisphosphate and its cellular distribution during nicotinic-receptor stimulation and protein kinase C activation. *J. Cell. Biol.* (1992) **119**: 797-810

Role, L.W. Diversity in primary structure and function of neuronal nicotinic acetylcholine receptor channels. *Curr. Opin. Neurobiol.* (1992) **2**: 54-62

Ron, D. and Kazanietz, M.G. New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.* (1999) **13**: 1658-1676

Ron, D., Chen, C.H., Caldwell, J., Jasmieson, L., Orr, E. and Mochly-Rosen, D. Cloning of an intracellular receptor for protein kinase C: a homolog of the beta subunit of G proteins. *Proc. Natl. Acad. Sci. U.S.A.* (1994) **91**: 839-843

Ron, D.C., Luo, J. and Mochly-Rosen, D. C2 region-derived peptides inhibit translocation and function of β protein kinase C in vivo. *J. Biol. Chem.* (1995) **270**: 24180-24187

Rong, M.A., Patrick, E., Kudlace, K. and Sansom, S.C. Protein kinase Cα participates in activation of store-operated Ca^{2+} channels in human glomerular mesangial cells. *Am. J. Physiol. Cell Physiol.* (2002) **283:** C1390-C1398

Rosē, S.D., Lejen, T., Casaletti, L., Larson, R.E., Pene, T.D. and Trifaró, J.M. Molecular motors involved in chromaffin cell secretion. *Ann. N.Y. Acad. Sci.* (2002) **971**: 222-231

Rosē, S.D., Lejen, T., Zhang, L. and Trifaró, J.M. Chromaffin cell F-actin disassembly and potentiation of catecholamine release in response to protein kinase C activation by phorbol ester is mediated through myristoylated alanine-rich C kinase substrate phosphorylation. *J. Biol. Chem.* (2001) **276**: 36757-36763

Rosenmund, C., Sigler, A., Augustin, I., Rein, K., Brose, N. and Rhee, J.S. Differential control of vesicle priming and short-term plasticity by Munc13 isoforms. *Neuron.* (2002) **33**: 411-424

Roth, D. and Burgoyne, R.D. Stimulation of catecholamine secretion from adrenal chromaffin cells by 14,3,3 proteins is due to reorganisation of the cortical actin network. *FEBS. Lett.* (1995) **374**: 77-81

Ryan, T.A. Inhibitors of myosin light chain kinase block synaptic vesicle pool mobilization during action potential firing. *J. Neurosci.* (1999) **19**: 1317-1323

Ryves, W.J., Evans, A.T., Olivier, A.R., Parker, P.J. and Evans, F.J. Activation of PKC-isotypes α, βI, γ, δ and ε by phorbol esters of different biological activities. *FEBS Lett.* (1991) **288**: 5-9

Saido, T.C., Shibata, M., Takenawa, T., Murofushi, H. and Suzuki, K. Positive regulation of mu-calpain action by polyphosphoinositides. *J. Biol. Chem.* (1992) **267**: 24585-24590

Sakaguchi, G., Orita, S., naito, A., Maeda, M., Igarashi, H., sasaki, T. and Takai, Y. A novel brain-specific isoform of beta spectrin: isolation and its interaction with Munc13. *Biochem. Biophys. Res. Commun.* (1998) **248**: 846-851

Sakai, N., Sasaki, K., Ikegaki, N., Shirai, Y., Ono, Y. and Saito, N. Direct visualization of the translocation of the gamma-subspecies of protein kinase C in living cells using fusion proteins with green fluorescent protein. *J. Cell. Biol.* (1997) **139**: 1465-1476

Samain, E., Bouillier, H., Perret, C., Safar, M. and Bagher, G. angiotensin II-induced Ca^{2+} increase in smooth muscle cells from SHR is regulated by actin and microtubule networks. *Am. J. Physiol.* (1999) **277**: H834-H841

Sargent, P.B. The diversity of neuronal nicotinic acetylcholine receptors. *Annu. Rev. Neurosci.* (1993) **16**: 403-443

Sasakawa, N., Nakaki, T., Yamamoto, S. and Kato, R. Calcium uptake-dependent and –independent mechanisms of inositol trisphosphate formation in adrenal chromaffin cells: comparative studies with high K^+, Carbamylcholine and angiotensin II. *Cell Signal.* (1989) **1**: 75-84

Sassa, T., Harada, S., Ogawa, H., Rand, J.B., Maruyama, I.N., Hosono, R. Regulation of the Unc-18-caenorhabditis elegans syntaxin comlex by Unc-13. *J. Neurosci.* (1999) **19**: 4772-4777

Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K. and Lai, A. PLCζ: a sperm-specific trigger of Ca^{2+} oscillation in eggs and embryo development. *Development* (2002) **129**: 3533-3544

Schiavo, G. and Stenbeck, G. Molecular analysis of neurotransmitter release. *Essays in Biochemistry* (1997) **32**: 29-41

Schiavo, G., Stenbeck, G., Rothman, J.E. and Söller, T.H. Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapse. *Proc. Natl. Acad. Sci.* (1997) **94**: 997-1001

Schliwa, M. Action of cytochalasin D on cytoskeleton networks. *J. Cell. Biol.* (1982) **92**: 79-91

Schoch, S., Castillo, P.E., Jo, T., Mukherjee, K., Gerppert, M., Wang, Y., Schmitz, F., Malenka, R.C. and Sudhof, T.C. RIM1alpha forms a protein scaffold

for regulating neurotransmitter release at the active zone. *Nature.* (2002) **415**: 321-326

Schroeder, G.E., Kotsonis, P., Musgrave, I.F. and Majewski, H. Protein kinase C involvement in maintenance and modulation of noradrenaline release in the mouse brain cortex. *Br. J. Pharmacol.* (1995) **116**: 2757-2763

Schwarz, H., Villiger, P.M., Von Kempis, J. and Lotz, M. Neuropeptide Y is an inducible gene in the human immune system. *J. Neuroimmunol.* (1994) **51**: 53-61

Séguéla, P., Wadiche, J., Dineley-Millers, K., dani, J.A. and Patrick, J.W. Molecular cloning, functional properties, and distribution of rat brain α 7: a nicotinic cation channel highly permeable to calcium. *J. Neurosci.* (1993) **13**: 596-604

Sena, C.M. Santos, R.M., Standen, N.B., Boarder, M.R. and Rosario, L.M. Isoform-specific inhibition of voltage-sensitive Ca^{2+} channels by protein kinase C in adrenal chromaffin cells. *FEBS. Lett.* (2001) **492**: 146-150

Sena, C.M., Rosário, L.M., Parker, P.J., Patel, V. and Boarder, M.R. Differential regulation of histamine- and bradykinin-stimulated phospholipase C in adrenal chromaffin cells: evidence for involvement of different protein kinase C isoforms. *J. Neurochem.* (1996) **66**: 1086-1094

Seward, E.P. and Nowycky, M.C. Kinetics of stimulus-coupled secretion in dialyzed bovine chromaffin cells in response to trains of depolarizing pulses. *J. Neurosci.* (1996) **16**:553-62

Shafer, T.J. and Atchison, W.D. Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. *Neurotoxicology* (1991) **12**: 473-492

Shapira, R., Silberberg, S.D., Ginsburg, S. and Rahamimoff, R. Activation of protein kinase C augments evoked transmitter release. *Nature* (1987) **1325**: 58-60

Sharkey, N.A. and Blumberg, P.M. Kinetic evidence that 1,2-diolein inhibits phorbol ester binding to protein kinase C via a competitive mechanism. *Biochem. Biophys. Res. Commun.* (1985) **133**: 1051-1056

Sharma, G. and Vijayaraghavan, S. Nicotinic receptor signaling in nonexcitable cells. *J. Neurobiol.* (2002) **53**: 524-34

Shen, L. and Glazer, R.I. Induction of apoptosis in glioblastoma cells by inhibition of protein kinase C and its association with the rapid accumulation of p53 and induction of the insulin-like growth factor-1-binding protein-3. *Biochem. Pharmacol.* (1998) **55**: 1711-1719

Sher, E., Giovannini, F., Boot, J. and Lang, B. Peptide neurotoxins, small-cell lung carcinoma and neurological paraneoplastic syndromes. *Biochimie* (2000) **82**: 927-936

Sheu, L., Pasyk, E.A., Ji, J., Huang, X., Gao, X., Varoqueaux, F., Brose, N. and Gaisano, H.Y. Regulation of insulin exocytosis by Munc13-1. *J. Biol. Chem.* (2003) **278**: 27556-27563

Shimazaki, Y., Nishiki, T.I., Omori, A., Sekiguchi, M., Kamata, Y., Kozaki, S. and Takahashi, M. Phosphorylation of 25-kDa synaptosome-associated protein. *J. Biol. Chem.* (1996) **271**: 14548-14553

Shirai, Y., Kashiwagi, K., Yagi, K., Sakai, N. and Saito, N. Distinct effects of fatty acids on translocation of gamma- and epsilon-subspecies of protein kinase C. *J. Cell. Biol.* (1998) **143**: 511-521

Shoji-Kasai, Y., Morishima, M., Kuwahara, R., Kondo, S., Itakura, M. and Takahashi, M. Establishment of variant PC12 subclones deficient in stimulation-secretion coupling. *Biochim. Biophys. Acta.* (2001) **1499**: 180-190

Shoop, R.D., Chang, K.T., Ellisman, M.H. and Berg, D.K. Synaptically driven calcium transients via nicotinic receptors on somatic spines. *J. Neurosci.* (2001) **21**: 771-781

Sim, A.T.R., Baldwin, M.L., Rostas, J.A., Holst, J. and Ludowyke, R.I. The role of serine/threonine protein phosphatases in exocytosis. *Biochem. J.* (2003) **373**: 641-659

Slater, S.J., Ho, C. and Stubbs, C.D. The use of fluorescent phorbol esters in studies of protein kinase C-membrane interactions. *CPL* (2002) **116**: 75-91

Smith, C. A persistent activity-dependent facilitation in chromaffin cells is caused by Ca^{2+} activation of protein kinase C. *J. Neurosci.* (1999) **19**: 589-598

Soliakov, L. and Wonnacott, S. Involvement of protein kinase C in the presynaptic nicotinic modulation of [(3)H]-dopamine release from rat striatal synaptosomes. *Br. J. Pharmacol.* (2001) **132**: 785-791

Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J.E. SNAP receptors implicated in vesicle targeting and fusion. *Nature* (1993) **362**: 318-324

Sontag, J.M., Sanderson, P., Klepper, M., Aunis, D., Takeda, K. and Bader, M.F. Modulation of secretion by dopamine involves decreases in calcium and nicotinic currents in bovine chromaffin cells. *J. Physiol.* (1990) **427**: 495-517

Stallcup, W.B. Sodium and calcium fluxes in clonal nerve cell line. *J. Physiol. (Lond.)* (1979) **286**: 525-540

Stauderman, K.A. and Pruss, R.M. Different patterns of agonist-stimulated increases of 3H-inositol phosphate isomers and cytosolic Ca^{2+} in bovine adrenal chromaffin cells: comparison of the effects of histamine and angiotensin II. *J. Neurochem.* (1990) **54**: 946-953

Stauderman, K.A. and Murawsky, M.M. The inositol 1,4,5-trisphosphate-forming agonist histamine activates a ryanodine-sensitive Ca^{2+} release mechanism in bovine adrenal chromaffin cells. *J. Biol. Chem.* (1991) **266**: 19150-19153

Stauderman, K.A. and Pruss, R.M. Dissociation of Ca^{2+} entry and Ca^{2+} mobilization responses to angiotensin II in bovine adrenal chromaffin cells. *J. Biol. Chem.* (1989) **264**: 18349-18555

Stauderman, K.A., Murawsky, M.M. and Pruss, R.M. Agonist-dependent pattern of cytosolic Ca^{2+} changes in single bovine adrenal chromaffin cells: relationship to catecholamine release. *Cell. Reg.* (1990) **1**: 683-691

Stauffer, T.P., Ahn, S. and Meyer, T. Receptor-induced transient reduction in the plasma membrane PtdIns(4,5)P_2 concentration monitored in living cells. *Curr. Biol.* (1998) **8**: 343-346

Stenbeck, G., Harter, C., Brecht, A., Herrmann, D., Lottspeich, F., Orci, L. and Wieland, F.T. Betá-COP, a novel subunit of coatomer. *EMBO J.* (1993) **12**: 2841-2845

Stevens, C.F. and Sullivan, J.M. Regulation of the readily releasable vesicle pool by protein kinase C. *Neuron* (1998) **21**: 885-893

Stevens, C.F. and Williams, J.H. "Kiss and run" exocytosis at hippocampal synapses. *Proc. Natl. Acad. Sci. USA.* (2000) **97**: 12828-12833

Stevens, D.R., Wu, Z.X., Matti, U., Junge, H.J., Schirra, C., Becherer, U., Wojcik, S.M., Brose, N. and Rettig, J. Identification of minimal protein domain required for priming activity of Munc13-1. *Cur. Biol.* (2005) **15**: 1-6

Stoehr, S.J., Smolen, J.E., Holz, R.W. and Agranoff, B.W. Inositol trisphosphate mobilizes intracellular calcium in permeabilized adrenal chromaffin cells. *J. Neurochem.* (1986) **46**: 637-640

Stroud, R.M., McCarthy, M.P. and shuster, M. Nicotinic acetylcholine receptor superfamily of ligand-gated ion channels. *Biochemistry.* (1990) **29**: 11009-11023

Südhof, T.C. The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* (1995) **375**: 645-653

Suh, B.C. and Kim, K.T. inhibition by ethaverine of catecholamine secretion through blocking L-type channels in PC12 cells. *Biochem. Pharmacol.* (1994) **47**: 1262-1266

Surya, A., Stadel, J.M. and Knox, B.E. Evidence for multiple, biochemically distinguishable states in the G protein-coupled receptor, rhodopsin. *Trends Pharmacol. Sci.* (1998) **19**: 243-247

Suzuki, T. and Kachi, T. Similarities and differences in supporting and chromaffin cells in the mammalian adrenal medullae: an immunohistochemical study. *Anat. Res.* (1996) **244**: 358-65

Swartz, K.J. Modulation of Ca^{2+} channels by protein kinase C in rat central and peripheral neurons: disruption of G protein mediated inhibition. *Neuron* (1993) **11**: 305-320

Szallasi, Z., Bogi, K., Gohari, S., Biro, T., Acs, P. and Blumberg, P.M. Non-equivalent roles for the first and second zinc fingers of protein kinase Cdelta. Effect of their mutation on phorbol ester-induced translocation in NIH 3T3 cells. *J. Biol. Chem.* (1996) **271**: 18299-18301

Tachikawa, E., Mizuma, K., Kudo, K., Kashimoto, T., Yamato, S., Ohta, S. Characterization of the functional subunit combination of nicotinic acetylcholine receptors in bovine adrenal chromaffin cells. *Neurosci. Lett.* (2001) **312**: 161-164

Takahashi, T., Forsythe, I.D., Tsujimoto, T., Barnes-Davies, M. and Onodera, K. Presynaptic calcium current modulation by a metabotropic glutamate receptor. *Science* (1996) **274**: 594-597

Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum. *J. Biol. Chem.* (1977) **252**: 7603-7609

Takano, K., Stanfield, P.R., Nakajima, S. and Nakajima, Y. Protein kinase C-mediated inhibition of an inward rectifier potassium channel by substance P in nucleus basalis neurons. *Neuron.* (1995) **14**: 999-1008

Takiyyuddin, M.A., Cervenka, J.H., Pandian, M.R., et al. Neuroendocrine sources of chromogranin-A in normal man: clues from selective stimulation of endocrine glands. J. Clin. Endocrinol. Metab. (1990) **71**: 360-369

Taraska, J.W., Perrais, D., Ohara-Imaizumi, M., Nagamatsu, S. and Almers, W. Secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells. *Proc. Natl. Acad. Sci. USA.* (2003) **100**:2070-2075

Taylor, S.C. and Peers, C.C. Store-operated Ca^{2+} influx and voltage-gated Ca^{2+} channels coupled to exocytosis in pheochromocytoma (PC12) cells. *J. Neurochem.* (1999) **73**: 874-880

Taylor, S.C., Green, K.N., Carpenter, E. and Peers, C. Protein kinase C evokes quantal catecholamine release from PC12 cells via activation of L-type Ca^{2+} channels. *J. Biol. Chem.* (2000) **275**: 26786-26791

Tchakarov, L., Zhang, L., Rose, S.D., Tang, R. and Trifaro, J.M. Light and electron microscopic study of changes in the organization of the cortical actin cytoskeleton during chromaffin cell secretion. *J. Histochem. Cytochem.* (1998) **46**: 193-203

Terbush, D.R. and Holz, R.W. Activation of protein kinase C is not required for exocytosis from bovine adrenal chromaffin cells: the effects of protein kinase C (19-31), Ca^{2+}/ CaM kinase II (291-317), and staurosporine. *J. Biol. Chem.* (1990) **265**: 21179-21184

Terbush, D.R. and Holz, R.W. Effects of phorbol esters, diglyceride, and cholinergic agonists on subcellular distribution of protein kinase C in intact or digitonin-permeabilized adrenal chromaffin cells. *J. Biol. Chem.* (1986) **261**: 17099-17106

Terbush, D.R., Bittner, M.A. and Holz, R.W. Ca^{2+} influx causes rapid translocation of protein kinase C to membranes: studies on the effects of secretagogues in adrenal chromaffin cells. *J. Biol. Chem.* (1988) **263**: 18873-18879

Teschemacher, A.G. and Seward, E.P. Bidirectional modulation of exocytosis by angiotensin II involves multiple G-protein-regulated transduction pathways in chromaffin cells. *J. Neurosci.* (2000) **20**: 4776-4785

Thastrup, O., Cullen, P.J., Drøbak, B.K., Hanley, M.R. and Dawson, A.P. Thapsigargin, a tumor promoter, discharge intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. U.S.A.* (1990) **87**: 2466-2470

Tim, D.W., Christie, M.I., Dainty, I.A., Wilkinson, G.F. and Willars, G.B. Ca^{2+} signalling by recombinant human CXCR2 chemokine receptors is potentiated by P2Y nucleotide receptors in HEK cells. *Br. J. Pharmacol.* (2002) **135**: 1199-1208

Tischler, A.S. Chromaffin cells as models of endocrine cells and neurons. *Ann. N. Y. Acad. Sci.* (2002) **971**:366-370

Toker, A. Signaling through protein kinase C. *Front. Biosci.* (1998) **3**: D1134-D1147

Toker, A. and Newton, A.C. Cellular signalling: pivoting around PDK-1. *Cell* (2000) **103**: 185-188

Torrão, A.S and Britto, L.R.G. Neurotransmitter regulation of neural development: acetylcholine and nicotinic receptors. *An. Acad. Bras. Cienc.* (2002) **74**: 453-461

Tovey, S.C. and Willars, G.B. Single-cell imaging of intracellular Ca^{2+} and phospholipase C activity reveals that RGS 2, 3, and 4 differentially regulate signaling via the Galphaq/11-linked muscarinic M3 receptor. *Mol. Pharmacol.* (2004) **66**: 1453-1464

Trifaró, J.M. and Vitale, M.L. Cytoskeleton dynamics during neurotransmitter release. *Trends Neurosci.* (1993) **16**: 466-472

Trifaró, J.M. The 1989 Upjohn Award Lecture. Cellular and Molecular mechanisms in hormone and neurotransmitter secretion. *Can. J. Physiol. Pharmacol.* (1989) **68**: 1-16

Trifaró, J.M., Fournier, S. and Novas, M.J. The p65 protein is a calmodulin-binding protein present in several types of secretory vesicles. *Neuroscience* (1989) **29**: 1-8

Trifaró, J.M. Molecular motors involved in chromaffin cell secretion. *Ann. N. Y. Acad. Sci.* (2002) **971**: 222-231

Trifaró, J.M., Bader, M.F. and Doucet, J.P. Chromaffin cell cytoskeleton: its possible role in secretion. *Can. J. Biochem. Cell Biol.* (1985) **63**: 661-679

Trifaró, J.M., Kenigsberg, R.L., Colé, A., Lee, R.W. and Hikita, T. Adrenal paraneurone contractile proteins and stimulus-secretion coupling. *Can. J. Physiol. Pharmacol.* (1984) **62**: 493-501

Trifaro, J.M., Lejen, T., Rose, S.D., Dumitrescun Pene, T., Barkar, N.D. and Seward, E.P. Pathways that control cortical F-actin dynamics during secretion. Neurochem. Res. (2002) **27**: 1371-85

Trifaró, J.M., Rosē, S.D., Lejen, T. and Elzagallaai, B. Two pathways control chromaffin cell cortical F-actin dynamics during exocytosis. *Biochimie.* (2000) **82**: 339-352

Trifaró, J.M., Lee, R.W.H., Kenigsberg, R.L. and Côlé, A. Contractile proteins and chromaffin cell function. *Adv. Biosci.* (1982) **5**: 151-158

Trifaró, J.M. Common mechanisms of hormone secretion. *Annu. Rev. Pharmacol.* Toxicol. (1977) **17**: 27-47

Tsao, P.I. and Von Zastrow, M. Diversity and specificity in the regulated endocytic membrane trafficking of G-protein-coupled receptors. *Pharmacol. Ther.* (2001) **89**: 139-147

Tsien, R.Y. The green fluorescent protein. *Ann. Rev. Biochem.* (1998) **67**: 509-544

Tsuboi, T. and Rutter, G.A. Multiple forms of "kiss-and-run" exocytosis revealed by evanescent wave microscopy. *Curr. Biol.* (2003) **13**: 563-567

Tsutakawa, S.E., Medzihradszky, K.F., Flint, A.J., Burlingame, A.L., Koshland, D.E. and Jr, J. Determination of in vivo phosphorylation sites in protein kinase C. *J. Biol. Chem.* (1995) **270**: 26807-26812

Turner, K.M., Burgoyne, R.D. and Morgan, A. Protein phosphorylation and the regulation of synaptic membrane traffic. *Trends Neurosci.* (1999) **22**: 459-464

Turner, N.A., Walker, J.H., Ball, S.G. and Vaughan, P.F. Down-regulation or long-term inhibition of protein kinase C (PKC) reduce noradrenaline release

evoked via after either PKC-dependent or PKC-independent pathways in human SH-SY5Y neuroblastoma cells. *Neurosci. Lett.* (1996) **220**: 37-40

Udenfriend, S., Cooper, J.R., Clark, C.T. and Baer, J.E. Rate of turnover of epinephrine in the adrenal medulla. *Science.* (1953) **117**: 663-665

Ulloa-Aguirre, A., Stanislaus, D., Janovick, J.A. and Conn, P.M. Structure-activity relationships of G-protein-coupled receptors. *Arch. Med. Res.* (1999) **30**: 420-435

Vainio, P.J., Viluksela, M. and Tuominen, R.K. Nicotine-like effects of continine on protein kinase C activity and noradrenaline release in bovine adrenal chromaffin cells. *J. Aut. Pharmacol.* (1998) **18**: 245-250

Valtorta, F., Meldolesi, J. and Fesce, R. Synaptic vesicles: is kissing a matter of competence? *Trends. Cell. Biol.* (2001) **11**: 324-328

Vanderbeld, B. and Kelly, G.M. New thoughts on the role of the beta-gamma subunit in G-protein signal transduction. *Biochem. Cell. Biol.* (2000) **78**: 537-550

Varoqueaux, F., Sigler, A., Rhee, J.S., Brose, N., Enk, C., Reim, K. and Rosenmund, C. Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. *Proc. Natl. Acad. Sci. U.S.A.* (2002) **99**: 9037-9042

Vaughan, P.F.T., Walker, J.H. and Peers, C. The regulation of neurotransmitter secretion by protein kinase C. *Mol. Neurosci.* (1995) **18**: 125-155

Verhage, M., Maia, A.s., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., van de Berg, T.K., Missler, M., Geuze, H.J. and Sudhof, T.C. Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science.* (2000) **287**: 864-869

Vijayaraghavan, S., Pugh, P.C., Zhang, Z.W., Rathouz, M.M. and Berg, D.K. Nicotinic receptors that bind α-bungarotoxin on neurons raise intracellular free Ca^{2+}. *Neuron.* (1992) **8**: 353-362

Vinet, R. and Vargas, F.F. L- and T-type voltage-gated Ca^{2+} currents in adrenal medulla endothelial cells. *Am. J. Physiol.* (1999) **276**: H1313-H1322

Vitale, M.L., Rodríguez Del Castillo, A. and Trifaró, J.M. Protein kinase C activation by phorbol esters induces chromaffin cell cortical filamentous actin disassembly and increases the initial rate of exocytosis in response to nicotine receptor stimulation. *Neuroscience* (1992) **51**: 463-474

Vitale, M.L., Rodríguez Del Castillo, A., Tchakarov, L. and Trifaró, J.M. Cortical filamentous actin disassembly and Scinderin redistribution during chromaffin cell stimulation precede exocytosis, a phenomenon not exhibited by gelsolin. *J. Cell Biol.* (1991) **113**: 1057-1067

Vitale, M.L., Seward, E.P. and Trifaró, J.M. Chromaffin cell cortical actin network dynamics control the size of the release-ready vesicle pool and the initial rate of exocytosis. *Neuron* (1995) **14**: 353-363

Vitale, N., Gasman, S., Caumont, A.S., Gensse, M., Galas, M.C., Chasserot-Golaz, S. and Bader, M.F. Insight in the exocytotic process in chromaffin cells: regulation by trimeric and monomeric G proteins. *Biochemie.* (2000) **82**: 365-373

Viviani, B., Galli, C.L. and Marinevich, M. Is actin polymerization relevant to neurosecretion? A study on neuroblastoma cells. Biochem. Biophys. Res. Commun. (1996) **223**: 712-717

Von Euler, U. and Lishajko, F. Improved technique for fluorimeteric estimation of catecholamines. *Acta. Physiol. Scand.* (1961) **51**: 348-355

Von Rüden, L. and Neher, E. A Ca^{2+}-dependent early step in the release of catecholamines from adrenal chromaffin cells. *Science* (1993) **262**: 1061-1065

Wakade, A.R., Malhotra, R.K. and Wakade, T.D. Phorbol ester facilitates $^{45}Ca^{2+}$ accumulation and catecholamine secretion by nicotine and excess K^+ but not by muscarine in rat adrenal medulla. *Nature* (1986) **321**: 698-700

Wakelam, M.J. Diacylglycerol-when is it an intracellular messenger? *Biophys. Acta.* (1998) **1436**: 117-126

Walaas, S.I. and Sefland, I. Modulation of calcium-evoked [^{3}H] noradrenaline release from permeabilized cerebrocortical synaptosomes by the MARCKS protein, calmodulin and the actin cytoskeleton. *Neurochem. Int.* (2000) **36**: 581-593

Walent, J.H. Porter, B.W. and Martin, T.F.J. A novel 145 kD brain cytosolic protein reconstitutes Ca^{2+}-regulated secretion in permeable neuroendocrine cells. *Cell.* (1992) **70**: 765-775

Wallace, D.J., Chen, C. and Marley, P.D. Histamine promotes excitability in bovine adrenal chromaffin cells by inhibiting an M-current. *J. Physiol.* (2002) **540**: 921-939

Wang, C.T., Grishanin, R., Earles, C.A., Chang, P.Y., Martin, T.F.J., Chapman, E.R. and Jackson, M.B. Synaptotagmin modulation of fusion pore kinetics in regulated exocytosis of dense-core vesicle. *Science* (2001) **294**: 1111-1115

Wang, H., Yu, M., Ochani, M., Amella, C.A., Tanovic, M., susarla, S., Li, J.H., Wang, H., Yang, H., Nlloa, L., Al-Abed, Y., Czura, C.J. and Tracey, K.J. Nicotinic acetylcholine receptor $\alpha 7$ subunit is an essential regulator of inflammation. *Nature.* (2003) **421**: 384-388

Wang, W. and Creutz, C.E. Regulation of chromaffin granule aggregating activity of annexin 1 by phosphorylation. *Biochemistry* (1992) **31**: 9934-9939

Watanabe, T., Masuo, Y., Matsumoto, H., Suzuki, N., Ohtaki, T., Masuda, Y., Kitada, C., Tsuda, M. and Fujino, M. Pituitary adenylate cyclase activating polypeptide provokes cultured rat chromaffin cells to secrete adrenaline. *Biochem. Biophys. Res. Commun.* (1992) **182**: 403-411

Waters, J. and Smith, S. Phorbol esters potentiate evoked and spontaneous release by different presynaptic mechanisms. *J. Neurosci.* (2000) **20**: 7863-7870

Waymire, J.C., Bennett, W.F., Boecheme, R., Hankins, L., Gilmer-Waymire, K. and Hancock, J.W. Bovine adrenal chromaffin cells: high-yield purification and viability in suspension culture. *J. Neurosci. Methods* (1983) **7**: 329-351

Way, K.J., Chou, E. and King, G.L. Identification of PKC-isoform-specific biological actions using pharmacological approaches. *TiPS* (2000) **21**: 181-187

Ways, D.K., Cook, P.P., Webster, C. and Parker, P.J. Effect of phorbol esters on protein kinase C-ξ. *J. Biol. Chem.* (1992) **267**: 4799-4805

Wecker, L., Guo, X., Rycerz, A.M., Edwards, S.C. Cyclic AMP-dependent protein kinase (PKA) and protein kinase C phosphorylate sites in the amino acid sequence corresponding to the M3/M4 cytoplasmic domain of alpha4 neuronal nicotinic receptor subunits. *J. Neurochem.* (2001) **76**: 711-720

Weihe, E., Tao-Cheng, J., Schaefer, M.K. and Erickson, J.D. Visualization of the vesicular acetylcholine transporter in cholinergic nerve terminals and its targeting to a specific population of small synaptic vesicles. *Proc. Natl. Acad. Sci. U.S.A.* (1996) **93**: 3547-3552

Weiner, N. and Rabadjija, M. The regulation of norepinephrine synthesis. Effect of puromycin on the accelerated synthesis of norepinephrine associated with nerve stimulation. *J. Pharmacol. Exp. Ther.* (1968) **164**: 103-114

Weiss, C. and Atlas, D.C. The bradykinin receptor-a putative receptor-operated channel in PC12 cells: studies of neurotransmitter release and inositol phosphate accumulation. *Brain Res.* (1991) **543**: 102-110

Wenger, B.W., Bryant, D.L., Boyd, R.T. and McKay, D.B. Evidence for spare nicotinic acetylcholine receptors and a beta 4 subunit in bovine adrenal chromaffin cells: studies using bromoacetylcholine, epibatidine, cytisine and mAb35. *J. Pharmacol. Exp. Ther.* (1997) **281**: 905-913

Werry, T.D., Christie, M.I., Dainty, I.A., Wilkinson, G.F. and Willars, G.B. Ca^{2+} signalling by recombinant human CXCR2 chemokine receptors is potentiated by P2Y nucleotide receptors in HEK cells. *Br. J. Pharmacol.* (2002) **135**: 1199-1208

Werry, T.D., Wilkinson, G.F. and Willars, G.B. Mechanisms of cross-talk between G-protein-coupled receptors resulting in enhanced release of intracellular Ca^{2+}. *Biochem. J.* (2003) **374**: 281-296

Wess, J. Molecular basis of receptor/G-protein-coupling selectivity. *Pharmacol. Ther.* (1998) **80**: 231-264

Wetsel, W.C., Khan, W.A., Merchenthaler, I., Rivera, H., Halpern, A.E., Phung, H.M., Negro-Vilar, A. and Hannun, Y.A. Tissue and cellular distribution of the extended family of protein kinase C isoenzymes. *J. Cell Biol.* (1992) **117**: 121-133

Wheldon, L.M., Nahorski, S.R. and Willars, G.B. Inositol 1,4,5-trisphosphate-independent calcium signalling by platelet-derived growth factor in the human SH-SY5Y neuroblastoma cell. *Cell Calcium* (2001) **30**: 95-106

Wickman, K.D. and Clapham, D.E. G protein regulation of ion channels. *Curr. Opin. Neurobiol.* (1995) **5**: 278-285

Wilkinson, S.E., Parker, P.J. and Nixon, J.S. isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem. J.* (1993) **294**: 335-337

Willars, G.B. and Nahorski, S.R. Quantitative comparisons of muscarinic and bradykinin receptor-mediated Ins(1,4,5)P_3 accumulation and Ca^{2+} signalling in human neuroblastoma cells. *Br. J. Pharmacol.* (1995a) **114**: 1133-1142

Willars, G.B. and Nahorski, S.R. Heterologous desensitisation of both phosphoinositide and Ca^{2+} signalling in SH-SY5Y neuroblastoma cells: a role for intracellular Ca^{2+} store depletion? *Mol. Pharmacol.* (1995b) **47**: 509-516

Willars, G.B., McArdle, C.A. and Nahorski, S.R. Acute desensitization of phospholipase C-coupled muscarinic M3 receptors but not gonadotropin-releasing hormone receptors co-expressed in alphaT3-1 cells: implications for mechanisms of rapid desensitization. *Biochem. J.* (1998a) **333**: 301-308

Willars, G.B., Muller-Esterl, W. and Nahorski, S.R. Receptor phosphorylation does not mediate cross talk between muscarinic M(3) and bradykinin B(2) receptors. *Am. J. Physiol.* (1999) **277**:C859-C869

Willars, G.B., Challiss, R.A.J., Stuart, J.A. and Nahorski, S.R. Constrasting effects of phorbol ester and agonist-mediated activation of protein kinase C on phosphoinositide and Ca^{2+} signalling in a human neuroblastoma. *Biochem. J.* (1996) **316**: 905-913

Willars, G.B., Nahorski, S.R. and challiss, R.A.J. Differential regulation of muscarinic acetylcholine receptor-sensitive polyphosphoinositide pools and consequences for signaling in human neuroblastoma cells. *J. Biol. Chem.* (1998b) **273**: 5037-5046

Willars, G.B., Royall, J.E., Nahorski, S.R., El-Gehani, F., Everest, H. and McArdle, C.A. Rapid down-regulation of the type I inositol 1,4,5-trisphosphate receptor and desensitisation of gonadotropin-releasing hormone-mediated Ca^{2+} responses in alpha T3-1 gonadotropes. *J. Biol. Chem.* (2001) **276**: 3123-3129

Williams, T.P. and McGee, R. The effects of membrane fatty acid modification of clonal pheochromocytoma cells on depolarisation-dependent exocytosis. *J. Biol. Chem.* (1982) **257**: 3491-3500

Wilson, S.P. and Kirshner, N. The acetylcholine receptor of the adrenal medulla. *J. Neurochem.* (1977) **28**: 687-695

Wilson, S.P. Vasoactive intestinal peptide elevate cyclic AMP levels and potentiates secretion in bovine adrenal chromaffin cells. *Neuropeptides* (1988) **11**: 17-21

Winkler, H., Apps, D.K. and Fischer-Colbrie, R. The molecular function of adrenal chromaffin granules: established facts and unresolved topics. *Neuroscience* (1986) **18**: 261-290

Winkler, H. and Westhead, E. The molecular organization of adrenal chromaffin granules. *Neuroscience* (1980) **5**: 1803-1823

Wojcikiewicz, R.J.H. and Nahorski, S.R. Modulation of signalling initiated by phosphoinositidase-C-linked receptors. *J. Exp. Biol.* (1993) **184**: 145-159

Wolf, M., LeVine, H., May, W.S., Cautrecasas, P. and Sahyoun, N. A model for intracellular translocation of protein kinase C involving synergism between Ca^{2+} and phorbol esters. *Nature* (1985) **317**: 546-549

Wonnacott, S. Presynaptic nicotinic ACh receptors. Trends. *Neurosci.* (1997) **20**: 92-98

Yamaguchi, T., Shirataki, H., Kishida, S., Miyazaki, M., Nishikawa, J., Wada, K., Numata, S., Kaibuchi, K. and Takai, Y. Two functionally different domains of rabphilin-3A, Rab3A p25/smg p25A-binding and phospholipid- and Ca^{2+}-binding domains. *J. Biol. Chem.* (1993) **268**: 27164-27170

Yanagita, T., Kobayashi, H., Yamamoto, R., Kataoka, H., Yokoo, H., Shiraishi, S., Minami, S.I., Koono, M. and Wada, A. Protein kinase C-α and -ε down-regulate cell surface sodium channel via differential mechanisms in adrenal chromaffin cells. *J. Neurochem.* (2000) **74**: 1674-1684

Yang, C.F. and Kazanietz, M.G. Divergence and Complexities in DAG signalling: looking beyond PKC. *Trends Pharmacol. Sci.* (2003) **24**: 602-608

Yedovitzky, M., Mochly-Rosen, D., Johnson, J.A., Gray, M.O., Ron, D., Abramovitch, E., Cerasi, E. and Nesher, R. *J. Biol. Chem.* (1997) **272**: 1417-1420

Yin, H.L. and Stossel, T.P. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium dependent regulatory protein. *Nature (Lond.)* (1979) **281**: 583-586

Young, S., Parker, P.J., Ullrich, A. and Stabel, S. Down-regulation of protein kinase C is due to an increased rate of degradation. *Biochem. J.* (1987) **244**: 775-779

Zamponi, G.W., Bourinet, E., Nelson, D., Nargeot, J. and Snutch, T.P. Crosstalk between G proteins and protein kinase C mediated by the calcium channel $\alpha_1$subunit. *Nature* (1997) **385**: 442-446

Zerbes, M., Bunn, S.J. and Powis, D.A. Histamine causes Ca^{2+} entry via both a store-operated and a store-independent pathway in bovine adrenal chromaffin cells. *Cell. Calcium.* (1998) **23**: 379-86

Zhang, G., Kazanietz, M.G., Blumberg, P.M. and Hurley, J.H. Crystal structure of the cys2 activator-binding domain of protein kinase C delta in complex with phorbol ester. *Cell* (1995a) **81**: 917-924

Zhang, L., Marcu, M.G., Nau, S.K. and Trifaró, J.M. Recombinant scinderin in enhances exocytosis, an effect blocked by two scinderin-derived actin-binding peptides and PIP_2. *Neuron* (1996a) **17**: 287-296

Zhang, L., Rodríguez Del Castillo, A. and Trifaró, J.M. Histamine-evoked chromaffin cell scinderin redistribution, F-actin disassembly and secretion: in the absence of cortical F-actin disassembly, an increase in intracellular Ca^{2+} fails to trigger exocytosis. *J. Neurochem.* (1995b) **65**: 1297-1308

Zhang, Z.W., Coggan, J. and Berg, D.K. Synaptic currents generated by neuronal acetylcholine receptors sensitive to α-bungarotoxin. *Neuron* (1996b) **17**: 1231-1240

Zhang, J.Z., Davletov, B.A., Südhof, T.C. and Anderson, R.G.W. Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. *Cell* (1994) **78**: 751-760

Zhou, Z. and Neher, E. Calcium permeability of nicotinic acetylcholine receptor channels in bovine adrenal chromaffin cells. *Pflügers Arch* (1993) **425**: 551-557

Zimlichman, R., Goldstein, D.S., Zimlichman, S., Stull, R. and Keiser, H.R. Angiotensin II increases cytosolic calcium and stimulates catecholamine release in cultured bovine adrenomedullary cells. *Cell Calcium.* (1987) **8**: 315-325

Zucker, R.S. Exocytosis: a molecular and physiological perspective. *Neuron* (1996) **17**: 1049-1055