Ca^{2+} SIGNALLING IN BOVINE ADRENAL CHROMAFFIN CELLS.

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Cells possess two mechanisms, the inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and ryanodine receptors, by which Ca^{2+} in intracellular stores can be mobilised. There are also a number of pathways which can mediate Ca^{2+} entry across the cell plasma membrane. In this study bovine adrenal chromaffin cells were used to investigate the role of intracellular Ca^{2+} stores in Ca^{2+} signalling and the relationship between Ca^{2+} entry and store release.

The major part of the exocytotic process in chromaffin cells is due to Ca^{2+} entry across the plasma membrane. Bradykinin (an Ins(1,4,5)P_3 generating agonist) and nicotine (a depolarising stimulus) were found to evoke catecholamine secretion in the presence of extracellular Ca^{2+}. Nicotinic responses were abolished in the absence of extracellular Ca^{2+}, whereas bradykinin resulted in reduced catecholamine secretion, indicating that Ca^{2+} release from intracellular stores may activate secretion.

Studies in permeabilised chromaffin cells showed that both Ins(1,4,5)P_3 and caffeine induced Ca^{2+} mobilisation from intracellular Ca^{2+} stores. Challenge of the chromaffin cells with inositol 4,5-bisphosphorothioate resulted in depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores. However, subsequent addition of caffeine stimulated Ca^{2+} mobilisation, indicating that the caffeine releasable stores had not been emptied. Ins(1,4,5)P_3 and caffeine, when added simultaneously, resulted in a larger response than each of these agonists alone. Ryanodine pretreatment inhibited subsequent caffeine responses. Ins(1,4,5)P_3 was able to stimulate Ca^{2+} release after prior depletion of the ryanodine-sensitive Ca^{2+} stores, providing a pharmacological differentiation of these stores, suggesting that the Ins(1,4,5)P_3 receptor-expressing stores may be physically different from the ryanodine receptor-expressing Ca^{2+} stores in chromaffin cells.

Experiments using epifluorescence microscopy were carried out to investigate the relationship between Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores in fura-2 loaded intact chromaffin cells. Bradykinin evoked Ca^{2+} responses appeared to involve activation of ryanodine receptors, probably occuring secondary to Ca^{2+} release via Ins(1,4,5)P_3 receptors. Depletion of the Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores resulted in activation of Ca^{2+} entry indicating that the Ins(1,4,5)P_3- and ryanodine-sensitive Ca^{2+} stores are both linked to the promotion of Ca^{2+} entry.

Several aspects of Ca^{2+} signalling have been elucidated in this study, notably the possible expression of at least two different Ca^{2+} stores and the degree of physical or functional overlap between the Ins(1,4,5)P_3 receptor-expressing and ryanodine-receptor expressing Ca^{2+} stores. Ca^{2+} release from the ryanodine-sensitive Ca^{2+} stores was found to activate Ca^{2+} entry across the chromaffin cells plasma membrane. These findings may have important implications for our understanding of how Ca^{2+} signalling occurs in adrenal chromaffin and other excitable cells *in vivo*.

This thesis is dedicated, with thanks,
to my parents and Elizabeth
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CHAPTER 1:

INTRODUCTION

1.1 THE ADRENAL MEDULLA AND THE CHROMAFFIN CELL.

The adrenal medulla receives cholinergic innervation from the sympathetic nervous system via the splanchnic nerve. Stimulation of the splanchnic nerve leads to release of catecholamines and other substances, from their storage sites in the chromaffin granules of the adrenal medulla chromaffin cells. These chromaffin cells have been widely used by those interested in neuronal function as a source of information into how cytosolic Ca^{2+} regulation affects different important aspects of cellular function. In particular this cell type has been used as a model for the relationship between Ca^{2+} regulation and stimulus-secretion coupling.

Studies on this system up to 1970 were carried out on retrogradely perfused glands *in vitro*. It was studies on intact glands that postulated the role of Ca^{2+} in the stimulus-secretion coupling. Although these glands did produce some clear data their use was limited. In dose response studies and electrophysiological experiments problems arose due to the fact that responses may desensitize over time. Furthermore, it was not possible to study detailed intracellular events. The variety of cell types within intact adrenal glands (such as surrounding cortical cells, and perhaps other non-chromaffin cells) gives rise to problems of interpretation. The chromaffin cells needed to be purified and maintained in culture to produce clearer data.

Different techniques were developed to this end. Early attempts involved enzymatic digestion using pancreatic enzymes (Douglas et al., 1967; Livett et al., 1983). Subsequent developments included collagenase enzymatic digestion (Fenwick et al., 1980), gradient centrifugation, and differential plating (Kilpatrick et al., 1980; Livett et al., 1983; Waymire et al., 1983; Livett, 1984). Using these techniques investigators were able to isolate relatively pure chromaffin cells and culture them in monolayers or in suspension. The chromaffin cells maintained as monolayers are most amenable to experimental use when they are between 3 and 8 days old. The development of primary culture methods for adrenal chromaffin cells provide us with a large number of isolated cells which are relatively homogenous.

Chromaffin cells have proved to be a very useful model for the study of postsynaptic events and mechanisms involved in stimulus-secretion coupling in neurons. Chromaffin cells are derived, during embryogenesis, from the same precursors as sympathetic neurons so they have many common properties with neurons. They possess voltage dependent Ca^{2+}- and Na^{+}- channels, a large range of receptors linked to stimulation of exocytosis, and a variety of neuronal-specific proteins (for an early review see Livett, 1984 and Burgoyne, 1984). Receptors linked to Ins(1,4,5)P_3 (inositol-1,4,5-trisphosphate) production were later found (for reviews see Berridge, 1987; Berridge and Irvine, 1989; Burgoyne, 1991) and Ins(1,4,5)P_3-induced Ca^{2+} release in bovine adrenal chromaffin cells was shown by Stoehr et al., 1986. Since then numerous studies have been carried out in order to further characterise Ca^{2+} regulation in chromaffin cells.

Catecholamine secretion in response to acetylcholine in chromaffin cells was first shown by Feldberg et al., in 1934. Subsequent research lead Douglas to introduce the term "stimulus-secretion coupling" in 1968. This term referred to the events leading from the stimulation of a cell to catecholamine release in the extracellular environment. The earliest evidence that calcium has an important role in stimulus secretion coupling was work done by Douglas and Rubin, 1961. Their results on retrogradely perfused adrenal glands indicated that catecholamine release in response to nicotinic stimulation was dependent on the presence of Ca^{2+} in the medium. In the absence of calcium no catecholamine release was detected. In the work that followed evidence for Ca^{2+} entry in catecholamine release was found and an increase in intracellular calcium concentration was suggested as the intracellular signal leading to secretion (Douglas and Poisner, 1962; Poisner and Douglas, 1962; Poisner and Douglas, 1966).

The secretory vesicle of the chromaffin cell is the chromaffin granule. Chromaffin cells can be classified according to the content of the chromaffin granule. There are two types, one that contains adrenaline secretory granules and one that contains noradrenaline secretory granules. The presence or absence of the enzyme

phenylethanolamine N-methyltransferase (PNMT), which is responsible for the biosynthesis of adrenaline from noradrenaline, can be used as a criterion to characterise chromaffin cells as adrenergic or noradrenergic (Goldstein et al., 1971; Schultzberg et al., 1989; Moro et al., 1991). Other than the catecholamines the chromaffin cell granules also contain ATP (adenosine 5'-triphosphate), enkephalins, chromogranins (the soluble proteins of chromaffin cells termed by Blaschko et al., 1967) and dopamine beta-hydroxylase (Viveros and Wilson, 1983).

Despite the development of purification techniques described above it is as yet impossible to produce 100% pure chromaffin cells. This is due to the presence in the culture of residual non-chromaffin cells, perhaps mainly vascular endothelial and smooth muscle cells. Furthermore, heterogeneity appears to exist even between chromaffin cells, for example in the aspect of granular contents (Viveros and Wilson, 1983) as previously discussed. Hence, results may differ between experiments performed on different culture cells. We must always bear this in mind when we analyse chromaffin cell data. This may therefore explain variations in data presented here.

The purpose of this Thesis was to contribute to our understanding of the role of Ca^{2+} in catecholamine secretion by characterising the intracellular Ca^{2+} stores and Ca^{2+} signalling pathways in populations and single bovine adrenal chromaffin cells. The effect of Ca^{2+} mobilisation from intracellular Ca^{2+} stores in stimulating Ca^{2+} entry was also studied.

1.2 STIMULUS-SECRETION COUPLING.

Chromaffin cells have been widely used by those interested in the mechanisms involved in secretion. The term 'stimulus-secretion coupling' was first introduced by Douglas, in 1968. This term referred to the events mediated between stimulation of a cell and the release of the catecholamine product in the extracellular environment.

In early experiments retrogradly perfused intact adrenal glands were used and co-secretion of molecular granular contents e.g. ATP and proteins was discovered (Douglas and Poisner, 1966). The stages which were involved in secretion were described as: a, stimulation of a chromaffin cell causes: b, Ca^{2+} entry; c, granule translocation; d, fusion of granule and plasma membranes (exocytosis); e, plasma membrane recovery (endocytosis). For an early review on mechanisms of secretion from chromaffin cells see Burgoyne, 1984. Most of the early studies on secretion were using isolated granule preparations and it was later proposed that the isolated granules differ significantly from their counterparts *in vivo* (Ornberg et al., 1988).

In the studies that followed "leaky" chromaffin cells were used. This technique, introduced by Baker and Knight in 1979, used electrically permeabilised cells which were permeable to small ions but not to larger proteins and enzymes (Knight and Baker, 1982). Later other detergents were used to produce chemically permeabilised cells ("skinned" cells as they were called at that time) e.g. saponin (Brooks and Treml, 1983), digitonin (Dunn and Holz, 1983). For a review on early techniques involved in secretion experiments see Livett, 1984. Later staphylococcal alpha toxin (Bader et al., 1986), and streptolysin-O were used to permeabilise chromaffin cells (Sontag et al., 1990).

There is evidence that cytosolic proteins are involved in secretion. Early experiments, in isolated granules, indicated that calmodulin had a role in exocytosis which was related to the levels of intracellular Ca^{2+} since a rise in intracellular calcium levels resulted in increased calmodulin binding to the granule membranes (Hikita et al., 1984). Although calmodulin was not able to stimulate exocytosis it was found to be

required to reconstitute secretion in long term digitonin-permeabilised cells (Sarafin et al., 1987). The role of calmodulin in secretion is not clear yet since others have found that calmodulin had no effect in saponin- (Brooks and Treml, 1984) and streptolysin O-permeabilised chromaffin cells (Ahnert-Hilger et al., 1989).

Another protein proposed to be involved in secretion is calpactin (the 36 kDa component) since it has been shown to restore secretion in permeabilised cells (Ali et al., 1989). Calpactin is present on the plasma membrane of chromaffin cells and belongs to the annexin family : a family consisting of widely distributed Ca^{2+}- and phospholipid binding proteins which contain a sequence of 70 amino acids repeated four times, and may mediate calcium- dependent membrane- membrane interactions (Creutz, 1992). Calpactin has a very similar Ca^{2+} affinity to that of exocytosis (Drust and Creutz, 1988). Chromaffin cells have been shown to possess lipocortin (annexin I), calpactin (annexin II), annexin VI and synexin (annexin VII). Synexin was also proposed to have a role in secretion but this is still a controversial issue. For a review on the role of annexins in exocytosis see Burgoyne, 1991. Recent evidence indicates that two other factors, Exo1 and Exo2, are capable of stimulating exocytosis in a Ca^{2+} and MgATP- dependent manner (Morgan and Burgoyne, 1992; Morgan et al., 1993; Burgoyne and Morgan, 1993).

Protein kinase C (PKC) which is activated after diacylglycerol (DAG) generation is also been involved in secretion. Phorbol esters which are known to act as analogues of DAG and therefore activate PKC have been shown to enhance the calcium-dependent catecholamine release in permeabilised chromaffin cells (Knight and Baker, 1983 and 1985). Use of protein kinase inhibitors, such as staurosporine or sphingosine, resulted in partial inhibition of the Ca^{2+}- dependent response (Burgoyne et al., 1988), suggesting a role for PKC in exocytosis. The role of PKC in secretion still remains controversial and the suggested possible roles can be summarised as follows: a) the mediation of calcium entry b) the mediation of exocytotic events c) receptor- effector linkage or d) the modulation of the fusion of granule and plasma membrane (for a review of the role of PKC in secretion see Burgoyne, 1991).

Early work indicated that synapsin I, a nerve terminal specific protein, is associated with the cross link of synaptic vesicles within a cytoskeletal network (Südhof et al., 1989). A similar role has been proposed for the cortical actin network (cytoskeleton) in exocytosis. The organisation of the cytoskeleton in chromaffin cells has been suggested to prevent granules from reaching exocytotic sites on the plasma membrane. Nicotinic stimulation in chromaffin cells has been shown to result in Ca^{2+}-independent actin depolymerisation (Cheek and Burgoyne, 1986; 1987). In the experiments that followed evidence was obtained that nicotine, high potassium, or phorbol ester PMA stimulation resulted in a decrease in cytoskeletal actin (Burgoyne et al., 1989a). This evidence has led Cheek and Burgoyne, 1991 to suggest that the cytoskeleton acts as a barrier in exocytosis in chromaffin cells.

Recent work has identified a number of cytosolic and membrane proteins involved in exocytosis such as synaptotagmin, synaptobrevin, and synaptophysin. Synaptotagmin (p65) was the first membrane protein detected in both synaptic vesicles and secretory granules. Synaptotagmin binds Ca^{2+} (Brose et al., 1992) and interacts with syntaxin (p35), a synaptic protein implicated in docking synaptic vesicles to the plasma membrane (Bennett et al., 1992; Bennett and Scheller, 1993). They proposed the name syntaxin from the Greek συνταξις meaning "putting together in order" because it was found associated with N-type voltage-sensitive Ca^{2+} channels. Therefore, syntaxin may function in docking synaptic vesicles near Ca^{2+} channels at presynaptic active zones. Furthermore, the interaction of synaptotagmin with syntaxin and Ca^{2+} channels at the plasma membrane would place these complexes into a favourable position at the site of Ca^{2+} influx and triggers exocytosis (see Fig. 1.1).

Synaptophysin (p38) is an abundant glycoprotein on the synaptic vesicles which may have a role in the structural organisation of the synaptic vesicles and may also have an essential mediatory role in exocytosis since antibodies to synaptophysin interfere with transmitter secretion in neuromuscular synapses (Alder et al., 1992a and b).

Synaptobrevins, which are also called vesicle-associated membrane proteins (VAMPs), have been proposed to have a role in vesicular transport between the endoplasmic reticulum and the Golgi (Lian and Ferro-Novick, 1993) and between the Golgi and the plasma membrane (Protopopov et al., 1993). It has been suggested that synaptobrevins interact with the synaptic plasma membrane proteins syntaxin and SNAP-25 (Söllner et al., 1993). There is also evidence that two soluble factors N-ethylmaleimide-sensitive fusion protein (NSF) and α/β/γ/-SNAP (SNAP: Soluble-NSF-attachment-proteins) are involved in the process of vesicle docking and fusion (Rothman and Ocri, 1992). Recent data on digitonin-permeabilised chromaffin cells provided strong evidence for a role for NSF and SNAPs in regulated exocytosis (Morgan and Burgoyne, 1995).

In order to explain the specificity of vesicular membrane fusion a comprehensive model, the SNARE hypothesis, has been proposed (Söllner et al., 1993). This hypothesis predicts that some of the proteins that have been implicated in vesicle fusion form a complex, the 20S particle, that is a key mediator of membrane fusion processes. According to this model NSF and SNAP proteins form a complex (NSF/SNAP) when the SNAPs are attached to membranes. This association requires a set of membrane-associated proteins, the SNAP receptors (SNAREs). The SNAREs, NSF, and SNAPs proteins together constitute the 20S particle. This model predicts that SNAREs contribute to the specificity of vesicle/target membrane interactions. It appears that the essential components involved in synaptic vesicle or secretory granule exocytosis have been identified. However, the precise function of the proteins involved in secretion has not yet been established.

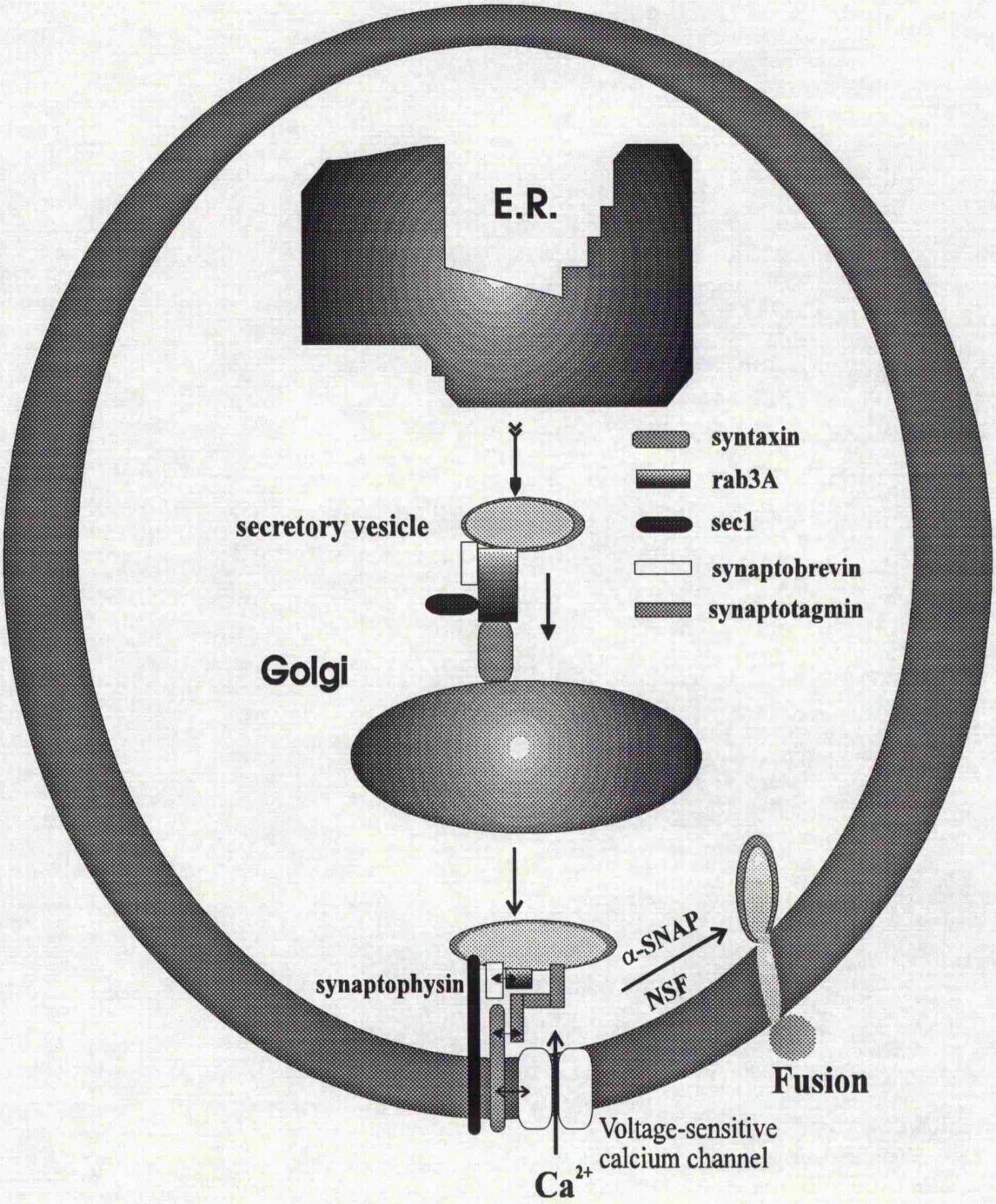


Fig 1.1. Proposed model for the molecular machinery involved in the secretory pathway. This Fig. also demonstrates possible interactions between proteins involved in synaptic vesicle docking and fusion.

1.2.1 Receptors Involved in Catecholamine Secretion.

The first receptor linked with catecholamine secretion in chromaffin cells was the nicotinic cholinergic receptor. Early experiments on intact adrenal glands provided evidence that nicotine stimulation resulted in secretion (Douglas et al., 1967). It was Wilson and Kirschner, in 1977 who presented evidence for the existence of a nicotinic acetylcholine receptor on the plasma membrane of the adrenal medulla. Nicotinic stimulation causes Ca^{2+} entry through the nicotinic receptor channel, and through voltage-sensitive Ca^{2+} channels. The rise in Ca^{2+} due to the calcium entry is the signal for the secretion. Nicotinic stimulation results in the biggest response. Nicotinic stimulation and high potassium depolarisation stimulates Ins(1,4,5)P_3 production through Ca^{2+}- dependent activation of phospolipase C and can be blocked by calcium channels blockers (Eberhard and Holz, 1987, 1988; Sasakawa et al., 1987). There is also evidence that nicotine stimulated a rapid increase in the levels of inositol pentakisphosphate, but the role of this inositol phosphate in stimulus secretion coupling remains unclear (Sasakawa et al., 1989; 1990).

Experiments in digitonin permeabilised cells showed that inositol phospholipid hydrolysis may have a role in secretion in response to nicotine (Eberhard and Holz, 1987 and 1988). Further, they also demonstrated that release in response to Ca^{2+} was separated into two stages; a rapid initial MgATP- independent followed by a slower MgATP- dependent (Holz et al., 1989; Bittner and Holz, 1992 a and b). They suggested that there is a requirement for inositol phospholipids during the MgATP-dependent phase of the release response and that ATP is responsible for maintaining the levels of these lipids (Eberhard et al., 1990). Moreover, they also proposed that the effect of the phospholipids was not related to Ins(1,4,5)P_3 production since introduction of Ins(1,4,5)P_3 to the experiment did not reverse the inhibition. Further work needs to be done to investigate where endogenous levels of ATP are sufficient to sustain the ATP- independent phase, the role of the phospholipid turnover, and Ins(1,4,5)P_3- induced Ca^{2+} release from intracellular stores in secretion.

Activation of a large number of receptors linked to Ins(1,4,5)P_3 production e.g. angiotensin II, bradykinin, histamine, muscarinic, prostaglandin E_2, has been shown to stimulate secretion (for a review see Burgoyne, 1991). However, they also mobilised Ca^{2+} from different intracellular stores. Histamine has been shown to stimulate Ca^{2+} release from both Ins(1,4,5)P_3-sensitive and caffeine-sensitive stores (Stauderman and Murawsky, 1991), whereas angiotensin II (Zimlichman et al, 1987), bradykinin (Plevin and Boarder, 1988; Plevin et al., 1990) and muscarinic receptors (Cheek and Burgoyne, 1985; Kao and Schneider, 1985) are linked to production of inositol phosphates. In chromaffin cells receptor activation in general either results in depolarisation and subsequent opening of voltage-sensitive Ca^{2+} channels, or direct stimulation of phospholipase C and Ins(1,4,5)P_3 production.

1.2.2 Role of Ca^{2+} in Secretion.

The first evidence that Ca^{2+} had an important role in catecholamine release in response to nicotinic stimulation came from work done by Douglas and Rubin, in 1961. They showed that in the absence of calcium in the perfusing medium no response could be seen. In work that followed evidence indicated the involvement of Ca^{2+} in secretion and demonstrated calcium entry in response to stimuli (Douglas and Poisner, 1962; Poisner and Douglas, 1962). Based on this evidence Douglas, in 1968 suggested that an increase in the intracellular ionised Ca^{2+} concentration was the intracellular signal for secretion.

In bovine adrenal chromaffin cells $^{45}CaCl_2$ influx was triggered in response to nicotinic and high extracellular potassium (Holz et al., 1982; Kilpatrick et al., 1982). The importance of cytosolic calcium levels can be observed in permeabilised or "leaky" chromaffin cells where addition of micromolar levels of calcium, when MgATP was present, could stimulate secretion, in the absence of external stimulus. Calcium was found to evoke half maximal secretion at a concentration of 1 μM (Knight and Baker, 1982; Dunn and Holz, 1983).

So generally secretion is a exocytotic process requiring an increase in cytoplasmic Ca^{2+} as a signal. Membrane depolarisation causes Ca^{2+} entry through the voltage-sensitive channels (VSCCs). Recent data proposed that these channels are clustered near release sites (Smith and Augustine, 1988; Robitaille et al., 1990). It has been suggested that when these channels are open they generate 'microdomains' of Ca^{2+} where the $[Ca^{2+}]_i$ can reach concentrations of 200-300 μM (Llinas et al., 1992).

Although depolarisation is the major trigger for exocytosis there is recent evidence suggesting that Ins(1,4,5)P_3 induced Ca^{2+} release from intracellular stores can stimulate regulated exocytosis. In chromaffin cells (Augustine and Neher, 1992), and SH-SY5Y cells (Vaughan et al., 1993; Atcheson et al., 1994; McDonald et al., 1994) activation of G-protein linked receptors has been shown to stimulate exocytosis which was largely independent of extracellular calcium. These results indicated that intracellular Ca^{2+} may be involved in the secretion process.

1.3 INTRODUCTION TO CALCIUM SIGNALLING.

Calcium is known to mediate a plethora of cellular functions including secretion, excitation and contraction, gene expression and conductivity changes. The major problem with Ca^{2+} studies is that it has always been difficult to measure intracellular $[Ca^{2+}]_i$ levels. So although it is more than a century since the first indication of the role of Ca^{2+} in muscle contraction, and therefore as an intracellular messenger (Ringer, 1883), it is only recently that Ca^{2+} studies became a major area of research.

Early attempts at the study of cytosolic Ca^{2+} were made with micro-injection of calcium sensitive photoproteins such as aequorin (Ridgway and Ashley, 1967) or with the Ca^{2+} -selective electrodes and micro-electrodes (Ashley and Campell, 1979). These methods made a restricted contribution to our understanding of Ca^{2+} regulation because they were used for Ca^{2+} measurements in giant cells; both of these methods were of limited use in small cells. These methods required microinjection or permeability of cells and were mostly only of use in single cell experiments.

Various techniques were developed during the last decade in order to better examine the levels of intracellular $[Ca^{2+}]_i$ in populations of intact cells. A breakthrough came with the development of a new generation of permeable dyes (for review see Cobbold and Rink, 1987; Tsien, 1989). The first to be introduced was the Quin-2 acetoxymethylester (Tsien et al., 1982) which made it possible to measure the concentration of intracellular calcium ($[Ca^{2+}]_i$) ions in populations of any size cell (Tsien, 1981). This method was simple enough and required only basic laboratory instrumentation in contrast with the previous ones that require advanced optical or electronic technologies. Quin- 2 binds Ca^{2+} with a 1:1 stoichiometry, has a K_d of 114 nM (pH 7.05, 37^0C) and shows a 6- fold increase in fluorescence signal at fairly short excitation wavelengths (339 nm) with a maximum absorption coefficient of 6 $M^{-1} \cdot cm^{-1}$ (Grynkiewicz et al., 1985). The short wavelength, small absorption coefficient, and

the fact that Quin-2 does not show a useful Ca^{2+}- induced wavelength shift to produce a ratio mode are the limitations in the use of Quin- 2.

Superior dyes like fura-2 (Grynkiewicz et al., 1985) and Indo-1 were developed from the novel Ca^{2+} chelator BAPTA (Tsien, 1980) and the study of intracellular Ca^{2+} became much easier. Fura -2, which forms a 1:1 complex with calcium, has a K_d of 224 nM, a higher quantum efficiency (compared with Quin -2) resulting in about 30- fold increase in the fluorescence signal and a maximum absorption coefficient of 33 $M^{-1} \cdot cm^{-1}$ (Grynkiewicz et al., 1985). Fura-2 was found to be more resistant to photodestruction than Quin -2, being able to produce adequate signals for minutes to hours of observations in single cells (Becker and Fay, 1987). Fura-2 provides a useful basis for ratio mode with a wide range of ratio signals since when the signal at 340 nm excitation increases 3- fold with Ca^{2+} saturation, the 380 nm signal decreases 10- fold. All of the above characteristics make Fura-2 the most commonly used Ca^{2+} indicator.

Resting levels of intracellular Ca^{2+} were found to be between 50-200 nM (Carafoli, 1987; Exton, 1988; Tsien, 1989). Levels of the extracellular Ca^{2+} are known to be at about 1.8 mM (Carafoli, 1987). It is obvious then that Ca^{2+} in the extracellular milieu is 10,000- fold higher than that of cytoplasm. It is also known that cells have a negative plasma membrane potential at about -60 mV. Due to this electrochemical gradient there is an electrical driving force for Ca^{2+} entry.

Ca^{2+} inside the cells is either bound to various ligands like Ca^{2+} -binding proteins (CaBPs, such as: calmodulin, calsequestrin and calreticulin, see Heizmann, 1991) and inorganic anions, or stored in the mitochondria and other non-mitochondrial intracellular pools. These non-mitochondrial stores like the endoplasmic reticulum and a hypothetical Ca^{2+} specialised organelle, the calciosome (Volpe et al., 1988; Hashimoto et al., 1988; Pozzan et al., 1988), can also release Ca^{2+} via the action of intracellular messengers. The name calciosome was given to a population of small, membrane bound structures distributed throughout the cytoplasm. Cells have a large Ca^{2+} buffering capacity and even small rises in intracellular free calcium ($[Ca^{2+}]_i$)

levels require the movement of large amounts of Ca^{2+}. Different Ca^{2+}-dependent kinases and various Ca^{2+}-calmodulin dependent kinases are involved in Ca^{2+} regulation within the cell.

Cytosolic free ionized calcium, $[Ca^{2+}]_i$, is generally a very small proportion (< 1%) of total cellular calcium content (Hodgkin and Keynes, 1957), normally rigorously controlled. Elevated $[Ca^{2+}]_i$ may be toxic for the cells (Chalfie and Wolinsky, 1990, Meldrum and Garthwaite, 1990). The other major mechanism, including those mentioned above, of controlling Ca^{2+} levels within the cell is Ca^{2+} extrusion. Given the extreme electrochemical gradient across the plasma membrane it is clear that the operation of Ca^{2+}-extrusion mechanisms maintain the low levels of $[Ca^{2+}]_i$. There are two main pathways of Ca^{2+} -extrusion, via plasma membrane Ca^{2+} ATPase and/or via a Na^+/Ca^{2+} exchanger.

Ca^{2+} homeostasis is a very sophisticated and complex phenomenon. The basic mechanisms of Ca^{2+} regulation in a typical mammalian cell are outlined in Fig. 1.2.

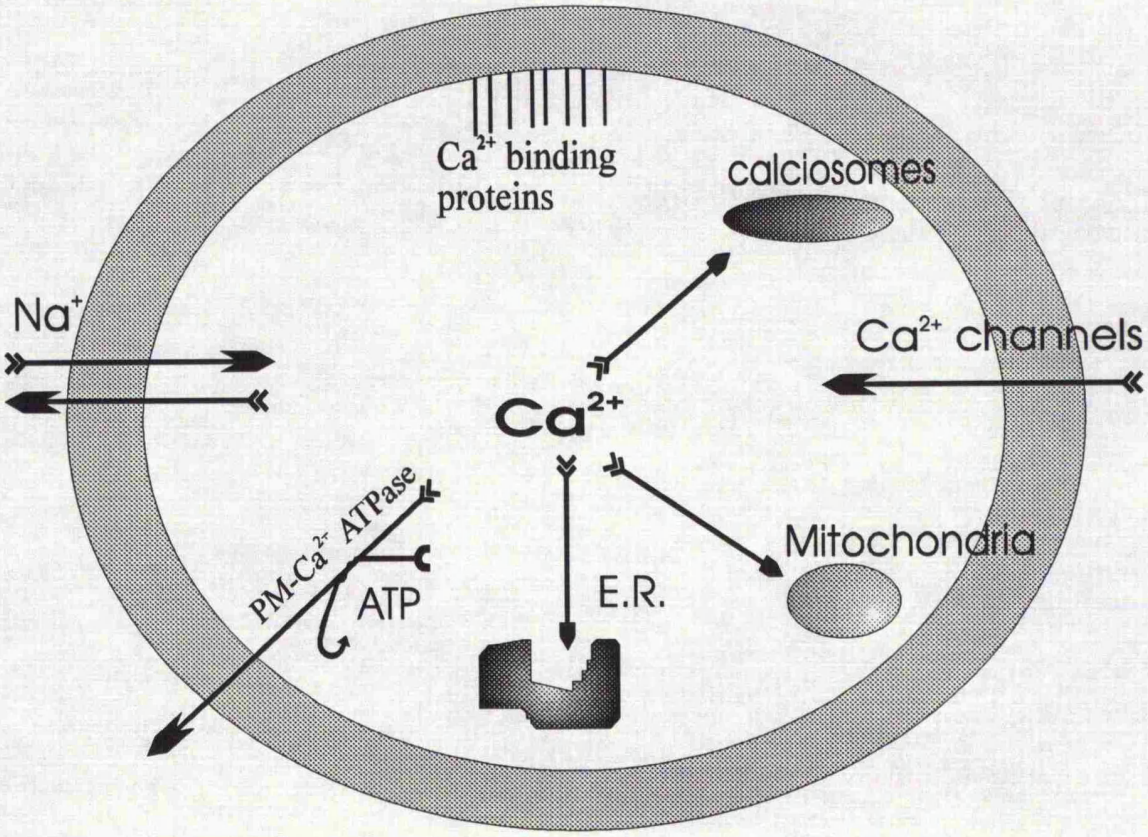


Fig. 1.2. Basic mechanisms of calcium regulation.

Ca^{2+} influx into the cell through various Ca^{2+} channels.

Ca^{2+} can be either :

(i) stored in intracellular stores like the E.R. or the calciosomes from which it can be released again.

(ii) stored in to the mitochondria.

(iii) bound to proteins or inorganic ions.

Calcium extrusion also occurs via :

(i) Plasma membrane Ca^{2+}- ATPase (PM-Ca^{2+}- ATPase).

(ii) Na^{+} /Ca^{2+} exchanger.

1.4 THE PLASMA MEMBRANE.

The plasma membrane is one of major regulators for Ca^{2+} homeostasis but although it acts as a barrier it does allow some communication between the cytoplasm and the extracellular milieu. The plasma membrane has a crucial role in the regulation of intracellular Ca^{2+}. Two main mechanisms are present in the plasma membrane for this purpose : one to remove Ca^{2+} from the cells and the other to allow passive Ca^{2+} influx across the membrane.

1.4.1 Mechanisms of Ca^{2+} Extrusion.

1.4.1.i The Plasma Membrane Ca^{2+}-ATPase Extrusion Mechanism.

Evidence for the existence of Ca^{2+} -pumping activity in the plasma membrane of erythrocytes was first presented by Schatzmann, in 1966. Similar Ca^{2+} -pumping activity was later discovered in isolated plasma membrane from bovine adrenal medulla (Leslie and Borowitz 1975; Wilson and Kirschner 1976), although the possibility of contamination from the pumping activity of the ER was not taken into consideration. The plasma membrane Ca^{2+}-ATPases belong to the class of P- type- ion- motive ATPases that characteristically form a phosphorylated intermediate during the reaction cycle (for review see Carafoli, 1987). The enzyme responsible for this plasma membrane activity was later found in all eukaryotic cells investigated (Carafoli, 1987). Purification and isolation of the enzyme showed it to be a monomeric enzyme with a molecular weight of between 130-140 kDa (Niggli et al., 1981a and b; Shull and Greeb, 1988) and with a stoichiometry close to 1 Ca^{2+} per ATP hydrolysed. Both the N- and C- termini of this enzyme are believed to be located on the cytoplasmic side of the plasma membrane. The number of the transmembrane domains is not yet clear but it may be as many as ten (Shull and Greeb, 1988).

Two ATP-binding sites with different affinity for ATP have been proposed to be present (Richards et al., 1978; Muallem and Karlish, 1980) but it is still not clear

whether these two sites are separate or just the same site changing between two different affinities for ATP. One of the sites is the catalytic one with high affinity for ATP- binding (Km ~ 1 μM) and the other one is with low affinity for ATP (Km ~100 μM).

The presence of a typical calmodulin- binding domain near the C- terminus has been proposed by Shull and Greeb in 1988 following first evidence that calmodulin stimulates Ca^{2+}-ATPase activities in erythrocytes (Schatzmann, 1983). Binding of the calmodulin to the enzyme modulates the enzyme to a high Ca^{2+} affinity state (Carafoli, 1987). This direct interaction of calmodulin (not calmodulin-dependent phosphorylation) with the enzyme increases enormously the affinity of the enzyme for ATP (Rossi et al., 1985; Missiaen et al., 1991).

When the levels of cytosolic Ca^{2+} are in the physiological range the calmodulin remains dissociated from the Ca^{2+}-ATPase. A rise in the concentration of cytosolic Ca^{2+} initiates the association of calmodulin with the plasma membrane Ca^{2+}-ATPase (in the absence of added Mg^{2+}) which gives the onset for pumping activity to extrude Ca^{2+} from the cells (Scharf and Foder, 1982). It appears that in the presence of Mg^{2+} the high Mg^{2+}-dependent ATPase activity obscures the Ca^{2+}-ATPase activity so it is difficult to measure. The association of calmodulin with the Ca^{2+}-ATPase proceeds slowly and therefore the responding pump- activity increases slowly.

High levels of Ca^{2+} and association with calmodulin are not the only activators of the plasma membrane Ca^{2+}-ATPases. Acidic lipids and long-chain polyunsaturated fatty acids can also activate the Ca^{2+}-ATPases in the absence of calmodulin. Most of the phosphatidylinositol metabolites have been tested as to whether they can activate the plasma membrane Ca^{2+}-ATPase and positive evidence has been found for the following: PtdIns, PtdIns4P, PtdIns4,5P_2, phosphatidic acid, and phosphatidylserine (Niggli et al., 1981a and b; Choquete et al., 1984; Enyedi et al., 1987; Missiaen et al., 1989).

Another factor which has been shown to activate the Ca^{2+}-ATPase pump extrusion is protein kinase C (Caroni and Carafoli, 1981; Rickard and Sheterline,

1987). The only evidence that phosphorylation activates the plasma membrane Ca^{2+}-ATPases derives from the cAMP-dependent kinase which has been shown to activate the plasma membrane Ca^{2+}-ATPase (Carafoli, 1988). In cardiac (Neyses et al., 1985) and skeletal (Mickelson et al., 1985) muscle the plasma membrane Ca^{2+}-ATPase is regulated by cAMP-dependent protein kinase where in vascular smooth muscle it is regulated indirectly by cGMP-dependent protein kinase (Baltensperger et al., 1988; Vrolix et al., 1988). Not all the isoforms of the plasma membrane Ca^{2+}-ATPases appear to contain the sequence needed for the cAMP-dependent phosphorylation. The amino acid sequence of PMCA2 and PMCA3b (Plasma Membrane Ca^{2+}- ATPase) does not include a sequence encoding a potential cAMP-dependent phosphorylation site (Greeb and Shull, 1989).

Recently, four genes have been identified that are able to encode different plasma membrane Ca^{2+}- pumps, the PMCA family (Shull and Greeb, 1988; Strehler et al., 1989). The members of this family are PMCA1 (PMCA1a: refers to the 1176-amino acid variant and PMCA1b: refers to the 1220- amino acid variant), PMCA2, PMCA3 (PMCA3a: refers to the 1159- amino acid variant and PMCA3b: refers to the 1206- amino acid variant) and PMCA4. Evidence for other splicing variants such as PMCA1c and PMCA1d, PMCA2f, PMCA2g, PMCA2h, PMCA4a and PMCA4b has been demonstrated, showing that plasma membrane Ca^{2+}-ATPases are more diverse than they were originally believed to be (for review see Carafoli, 1992). A protein, detected in bovine brains (Brandt et al., 1988), which contains only 71 residues is thought to be member of this family (PMCA5), but the evidence is still inconclusive. There are structural and functional differences between these isoforms but little is known yet. The PMCA1 isoform is widely expressed in tissues where the PMCA2 and PMCA3 appear to have a high degree of tissue-specificity. Splicing variants with different regulatory properties and tissue-specific distribution may reflect differences in the steady-state for Ca^{2+}, a role that PMCA pumps are believed to maintain.

1.4.1.ii The Na^+/Ca^{2+} Exchanger.

The other major mechanism of Ca^{2+} efflux from the cells is the Na^+/Ca^{2+} exchange which was first described in cardiac muscle and invertebrate nerve (Reuter and Seitz, 1968; Blaustein and Hodgkin, 1969). This mechanism is present in the plasma membrane of excitable cells but its existence in all the non-excitable cells is not yet established. It is well documented that the level of activity of this mechanism changes from tissue to tissue and in excitable cells is more active than in non-excitable cells. This explains why the importance of the Na^+/Ca^{2+} exchanger in non excitable cells is debated.

The Na^+/Ca^{2+} exchanger is an electrogenic, voltage sensitive mechanism which drives Na^+ into the cells and removes Ca^{2+} from them without any requirement for ATP (Caroni and Carafoli, 1983). The stoichiometry ratio of Na^+/Ca^{2+} has been found to be 3:1 in heart sarcolemma vesicles (Reuter, 1985). Therefore, due to the electrogenity of the exchanger, depolarisation of the cells would lead to Ca^{2+} influx, and hyperpolarisation of the cell would cause Ca^{2+} efflux having the opposite effects on Na^+.

The Na^+/Ca^{2+} exchanger is similar to the Ca^{2+}-ATPase and has two affinity sites for intracellular Ca^{2+} and extracellular Na^+. The high affinity site is achieved when the exchanger is phosphorylated by normal ATP concentrations (Reinlib et al., 1983). The Na^+/Ca^{2+} exchanger appears to have much higher Vmax (~10-fold) and Kmax (~ 2-10 μM) than the plasma membrane Ca^{2+}-ATPase (Reeves and Sutko, 1979). It becomes clear that the Na^+/Ca^{2+} exchanger is a high capacity, low affinity system for elevated intracellular Ca^{2+} concentrations while the Ca^{2+}-ATPase is a high affinity, low capacity mechanism. The Na^+/Ca^{2+} exchanger system is proposed to be responsible for the removal of high amounts of Ca^{2+} where the Ca^{2+}-ATPase produces a slow passive efflux of Ca^{2+}.

Evidence for the presence of Na^+/Ca^{2+} exchange activity in bovine adrenal chromaffin cells has been controversial. No evidence for Na^+/Ca^{2+} exchange was

found in bovine adrenal medulla slices (Rink, 1977). Later, some claimed to have found evidence in bovine adrenal chromaffin cells for this exchange by using ouabain to inhibit the Na^+/Ca^{2+} pumping activity (Garcia et al., 1980, 1981; Pocock, 1983a) confirming data obtained from other experiments using Na^+ -free sucrose solution (Nishimura et al., 1981). At the same time Pocock, 1983b argued that the effects of ouabain were not due to the inhibition of the Na^+/Ca^{2+} exchanger but due to the inhibition of Ca^{2+} pump. In the work that followed (Sorimachi et al., 1981; Kao and Cheung, 1990; Chern et al., 1992) the presence of the Na^+/Ca^{2+} exchanger in isolated plasma membrane was reported and its activity was compared to that of the plasma membrane Ca^{2+}-ATPase (Powis et al., 1991).

Based on evidences that Ca^{2+} efflux was increased after stimulating exocytosis and decreased in the absence of exocytosis Jan and Schneider (1992) proposed that the Na^+/Ca^{2+} extrusion mechanism may have a negative feedback role to modulate the phenomenon of exocytosis in chromaffin cells. Rises in the level of cytosolic Ca^{2+} in response to stimuli such as caffeine and DMPP (1,1- dimethyl -4- phenylpiperazinium; a nicotinic receptor agonist) were higher in the absence of extracellular Na^+ (Chern et al., 1992) suggesting another role for the Na^+/Ca^{2+} exchanger in resetting $[Ca^{2+}]_i$ levels following stimulation of the cells. Their data showed that Na^+/Ca^{2+} exchanger plays a crucial role in Ca^{2+} regulation in the resting and stimulated states, but it becomes the major pathway at higher calcium levels where the Ca^{2+} pump is more significant at lower calcium levels. These evidences have led to the suggestion that the Na^+/Ca^{2+} exchange mechanism contributes to the regulation of resting $[Ca^{2+}]_i$ levels in chromaffin cells although the role of the Na^+/Ca^{2+} exchange system is still controversial in chromaffin cells.

1.4.2 Ca^{2+} ENTRY.

1.4.2.i Plasma Membrane Ca^{2+} Channels.

Plasma membrane of the cells possesses calcium channels that allow passive influx of calcium across the membrane. These channels are basically membrane spanning proteins and their conformation in open state causes Ca^{2+} influx.

The major mechanism in excitable cells which allows Ca^{2+} influx is the voltage operated Ca^{2+} channels (Voltage-sensitive- Ca^{2+} -channels, VSCCs). The channels which belong to this family (VSCCs) are among the most heterogeneous of ion channels. The other main mechanism consists of those channels which require direct ligand binding to operate and are described as Receptor- Operated- Ca^{2+} -Channels (ROCs). When the ligand, bound to the receptor, is an internal diffusible second messenger the channels are described as Second- Messenger- Operated- Channels (SMOCs- Meldolesi and Pozzan, 1987).

Studies on exocytosis gave the first evidence for the presence of VSCCs in chromaffin cells (Douglas and Rubin, 1961; Douglas and Poisner; 1962). Later Poisner and Douglas (1966) showed that all the stimuli which were known to stimulate secretion (i.e. histamine, bradykinin, angiotensin II) required the presence of extracellular Ca^{2+}. Studies using fluorescent dyes gave further evidence that the rise in intracellular Ca^{2+} in response to stimuli was dependent on the presence of extracellular Ca^{2+} (Kao and Schneider, 1986).

Stimulation of the nicotinic receptor in $^{45}Ca^{2+}$ studies has been shown to stimulate Ca^{2+} entry (Holz et al., 1982; Kilpatrick et al., 1982) which may be sufficient to stimulate secretion (Boarder et al., 1987). The nicotinic receptor, containing an intrinsic ion channel (Huganin and Racker, 1982) permeable to Na^{+}/Ca^{2+} (Kilpatrick et al., 1982), was the first identified ROCs channel. Histamine and angiotensin II stimulation in chromaffin cells causes Ca^{2+} entry but not via their receptors since they

do not contain intrinsic ion channels. The most likely explanation is that these receptors are indirectly linked to a Ca^{2+} channel via the mediation of a G protein.

The stimulation of receptors coupled to phosphatidylinositol- 4,5 -bisphosphate (PtdIns-4,5P_2) could activate the SMOC Ca^{2+} entry (Meldolesi and Pozzan, 1987). Various proposals have been offered in an effort to explain how direct or indirect action of Ca^{2+}, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 alone, or combined, could stimulate the SMOC Ca^{2+} entry (see below).

1.4.2.ii VSCCs.

Three types of voltage sensitive channels, i.e. the L, N and T type, have been described initially in neurons (Nowycky et al., 1985) which differ in their electrophysiological properties. Since then evidence has been found for many other types of VSCCs e.g. the P- type channel (Llinas et al., 1989; Plummer et al., 1989) and the Q- type channel (Adams et al., 1993; Wheeler et al., 1994). Voltage-sensitive Ca^{2+} channels in neurons differ in location, biophysical and pharmacological properties, and modulation. Evidence from purified N-type (McEnery et al., 1991) and L-type (Campell et al., 1988) channels showed that they both are multisubunit protein complexes. The α_1 subunit has been proposed to determine the functional properties of the Ca^{2+} channel (for review see Tsien et al., 1991; Olivera et al., 1994).

The L- type channels (25pS single channel conductance with 110mM Ba^{2+}) have been shown to be blocked by dihydropyridine channel blockers in skeletal muscle (Fleckenstein, 1977). L-type channels are activated by high voltage (HVA). The L-type channels are pharmacologically defined by their ability to bind dihydropyridines, benzothiazepines and phenylalkylamines (Fosset et al., 1983; Kokubun et al., 1986; Glossman et al., 1987). There is evidence that as well as modulation by voltage the activity of Ca^{2+} channels can be altered by receptor-mediated events. Analysis of single-channel current strongly supports the hypothesis that c-AMP or c-AMP-

dependent protein kinase phosphorylation increases the probability of channel opening (Reuter et al., 1986).

Various subtypes of the high voltage- activated Ca^{2+} channels described in neurons (Tsien et al., 1988; Tsien et al., 1991; Swandulla et al., 1991) are present in bovine adrenal chromaffin cells. Dihydropyridines antagonists were shown to inhibit depolarisation stimulated calcium entry whilst agonists enhanced the release response giving evidence that chromaffin cells possess L-type VSCCs (Fenwick et al., 1982; Garcia et al., 1984; Boarder et al., 1987; Fonteriz et al., 1987). Dihydropyridine antagonists do not completely inhibit release or Ca^{2+} entry responses to high extracellular potassium (Boarder et al., 1987; Owen et al., 1989a and b) suggesting that an alternative channel may be responsible for some of the Ca^{2+} entry in response to potassium. Recent data suggested that L-type channels are also sensitive to ω-AgatoxinIIIA (ω-AgaIIIA : Mintz et al., 1991).

There is evidence indicating that chromaffin cells possess other types of VSCCs and the number of VSCCs on chromaffin cells is still a controversial matter. Some authors suggest the presence of only L-type channels in chromaffin cells, while others postulate the presence of both L- and N- type channels (Pun et al., 1988; Ballesta et al., 1989; Jan et al., 1990; Artalejo et al., 1992; Lopez et al., 1994). The N- type VSCCs activate over a similar range to the L- type (HVA) but can be distinguished at the single channel; firstly by their lower single-channel conductance (13pS with Ba^{2+}), secondly by their insensitivity to dihydropyridine, and thirdly their almost complete inactivation at holding potentials more positive than -30 to -20 mV. The conductivity of these channels is irreversibly blocked by ω-conotoxin (Plummer et al., 1989; Aosaki and Kasai 1989). High sensitivity to ω-conotoxin GVIA (CTx-GVIA: a N- type channel blocker as shown by Hirning et al., 1988) is a definitive pharmacological criterion for the classification of VSCCs as of the N-type.

Other evidence for the existence of a third type of VSCCs on the chromaffin cells has been demonstrated by Artalejo et al., 1991. This has characteristics close to those described for the P- type channel (Llinas et al., 1989). The P- type Ca^{2+} channel

was first identified in cerebellar Purkinje neurons and later in other neurons (Regan et al., 1991). These channels are HVA, activated at potentials less negative than -50mV and have a single channel conductance between 9-20pS. They can be distinguished by their insensitivity to dihydropyridines and ω- conotoxin and characterised by their sensitivity to FTX, a toxin fraction partially purified from the venom of the funnel web spider *'Agenelopsis aperta'* (Llinas et al., 1989). P-type Ca^{2+} channels in neurons are selectively blocked by ω-AgatoxinIVA (ω-Aga-IVA): a purified peptide from the funnel web spider *'Agenelopsis Aperta'* as described by Mintz et al., 1992.

More recent reports have demonstrated the presence of a P- type channels in bovine adrenal chromaffin cells (Gandia et al., 1993; Albillos et al., 1993). Studies looking at whole-cell Ba^{2+} currents and membrane capacitance concluded that chromaffin cells only possess L-, N-, and P- type Ca^{2+} channels (Artalejo et al., 1992; Artalejo et al., 1994). In contrast with these findings Albillos et al., 1993 demonstrated that furnidipine (a novel L-type Ca^{2+} channel blocker), ω-conotoxin GVIA and ω-agatoxin IVA did not completely block the whole-cell Ba^{2+} current in whole-cell patch-clamped chromaffin cells. Their data suggested that those three types of Ca^{2+} channels contribute 88% of the Ba^{2+} current.

The picture became more complicated with the use of a new toxin, ω-conotoxin (CTx-MVIIC) from the marine snail *'Conus magus'* (Hillyard et al., 1992) which recognises the Q- type, as well as the N- and P- type Ca^{2+} channels. The high-affinity target of this conotoxin has been proposed in cerebellar granule cells to represent the O-type channel with the Q-type having lower affinity (Adams et al., 1993). The Q-type Ca^{2+} channels in cerebellar granule neurons are resistant to inhibition by ω-agatoxin IVA at concentrations sufficient to block the P- type Ca^{2+} channel.

There is evidence now that a Q-type-like Ca^{2+} channels is present in bovine chromaffin cells (Lopez et al., 1994). Their results show that furnidipine with ω-conotoxin GVIA and ω-agatoxin IVA (Aga-IVA) blocked 87% of Ca^{2+} uptake and 50% of the potassium stimulated catecholamine release. When furnidipine and CTx-

MVIIC were combined they completely inhibited catecholamine secretion and Ca^{2+} entry indicating that a Q-type channel is present in chromaffin cells. Their data also suggested that Q- and L- type Ca^{2+} channels contribute to the control of secretion to a greater degree than N- and P- type channels. They also make suggestions for the geographic localisation of the Ca^{2+} channels proposing that Q- and L- types are located at the secretory active site where the N- and P- type channels are located away from the secretory site.

1.4.2.iii ROCs.

The nicotinic receptor/channel, which transports mainly Na^+ and K^+ and is permeable to Ca^{2+}, is the best characterised ROC channel in the adrenal chromaffin cell. Nicotinic stimulation was first demonstrated in intact adrenal glands (Douglas et al., 1967). In the studies that followed the presence of a nicotinic acetylcholine receptor was indicated at the adrenal medulla plasma membrane (Wilson and Kirschner, 1977). The nicotinic stimulation of catecholamine release (when maximally effective concentrations of nicotine were applied) was not inhibited in a stereospecific manner by the dihydropyridine nicardipine (Boarder et al., 1987), while high K^+ stimulation was. This suggested that Ca^{2+} entry through an alternative channel (i.e. not a L- channel, not a dihydropyridine sensitive channel) may occur in the chromaffin cells. Catecholamine release in response to nicotinic stimulation was found not to be tetrodotoxin (TTX: a voltage-sensitive sodium channel blocker) sensitive (Boarder et al., 1987). There is enough evidence to support the case that high nicotinic stimulation can produce sufficient calcium entry through the nicotinic acetylcholine receptor to elicit secretion (Boarder et al., 1987; Kilpatrick et al., 1982). In the absence of the calcium channel blockers it is probable that Ca^{2+} enters the cell mainly through the VSCCs following stimulation with agonists of the acetylcholine receptor.

1.4.2.iv SMOCs.

It has been shown that activation of receptors coupled to the hydrolysis of phosphatidylinositol- 4,5-bisphosphate [PtdIns(4,5)P_2] causes Ca^{2+} influx as well as Ca^{2+} release from internal stores (Meldolesi and Pozzan, 1987). Agonist stimulation (linked to Ins(1,4,5)P_3 generation) mediates Ca^{2+} release in two phases. The initial Ca^{2+} response is transient and thought to represent Ca^{2+} mobilisation from intracellular Ca^{2+} stores. This is followed by a prolonged phase of sustained Ca^{2+} release which is dependent on the presence of extracellular calcium (Lambert and Nahorski, 1990). Although evidence is still lacking, this Ca^{2+} entry is thought to be through the SMOCs channels. These channels do not display characteristics of typical channels and the amount of Ca^{2+} flowing through these channels is small (Penner et al., 1988). Various second messengers have been proposed as candidates for activation of the Ca^{2+} SMOCs channels, such as: Ca^{2+}, Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 alone or combined.

It has been suggested that $[Ca^{2+}]_i$ itself could activate Ca^{2+} permeable channels in the plasma membrane; evidence however is scarce (Petersen and Maruyama, 1983; Tscharner et al., 1986). This was the first hypothesis to proclaim that $[Ca^{2+}]_i$ could serve as a second messenger to regulate these channels (Partridge and Swandulla, 1988).

Later evidence has shown that inositol phosphates may have direct effects on SMOC channels (Fig. 1.3a). It has further been shown that these channels can be regulated by Ins(1,4,5)P_3 in lymphocyte plasma membranes by Kuno and Gardner (1987), and in mast cells by Penner et al., (1988). Later results demonstrated evidence for a new Ins(1,4,5)P_3 receptor localised in the plasma membrane of lymphocytes which appear to cause Ca^{2+} entry (Khan et al., 1992a, b). Similar results have been obtained in olfactory cells where Ins(1,4,5)P_3-sensitive calcium channels appear to exist (Restrepo et al., 1990; Fadool and Ache, 1992). Recent evidence reported the presence of Ins(1,4,5)P_3- receptor activity and Ins(1,4,5)P_3-like immunoreactivity on

the plasma membrane of other cells (Sharp et al., 1992; Fujimoto et al., 1992; McDonald et al., 1993). This evidence suggests that increased intracellular Ins(1,4,5)P_3 concentration could cause Ca^{2+} entry via the mediation of plasma membrane Ins(1,4,5)P_3 receptors. It is still not known which types of the Ins(1,4,5)P_3-receptors are expressed into the plasma membrane but it is possible that it is a new type of the Ins(1,4,5)P_3- receptor family, the plasma membrane- Ins(1,4,5)P_3- receptor type.

Ins(1,3,4,5)P_4, first identified by Batty et al., in 1985, was assigned to have a significant role in the regulation of Ca^{2+} entry by Irvine (1990 and 1992). It has been proposed (Irvine, 1991 and 1992; Irvine and Cullen; 1993) that Ins(1,3,4,5)P_4 receptor Ca^{2+} channels may directly interact with Ins(1,4,5)P_3 receptors to stimulate Ca^{2+} entry. Ins(1,3,4,5)P_4 has been reported to stimulate Ca^{2+} mobilisation from intracellular stores in adrenal microsomes (Ely et al., 1990) and SH-SY5Y cells (Gawler et al., 1990; and 1991; Wilcox et al., 1993a, b and c). Studies on pig cerebellar and SH-SY5Y membranes proposed that Ins(1,3,4,5)P_4 may also be a weak agonist of the Ins(1,4,5)P_3 receptor (Wilcox et al., 1993a, b and c), indicating that the effect of Ins(1,3,4,5)P_4 was not mediated via an Ins(1,3,4,5)P_4 receptor but via the Ins(1,4,5)P_3 receptor. Although plasma membrane Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 receptors may stimulate Ca^{2+} entry it seems unlikely that they represent the major mechanism of Ca^{2+} influx.

Extracellular Ca^{2+}, it was suggested, entered straight to the cytosol, or first to the ER, and then to the cytosol (Putney, 1986 and 1990). According to Putney's proposed mechanism Ca^{2+} entry can be produced by an indirect effect of Ins(1,4,5)P_3 (capacitative entry theory).

1.4.3 Capacitative Calcium Entry.

According to this model the Ca^{2+} content of the E.R. regulates the Ca^{2+} entry into the cell (Putney, 1986 and 1990). Later it was confirmed in parotid acinar cells

that emptying of the intracellular pool was giving the signal for Ca^{2+} entry. The original model proposed that external calcium was directly refilling the Ins(1,4,5)P_3-sensitive store (see Fig. 1.2b). This mechanism was later revised by Putney (1990). The main principles remain the same except that depletion of the Ca^{2+} content of the intracellular store caused, by an unknown mechanism, the opening of a plasma membrane channel which allows Ca^{2+} entry to the cytosol and not directly to the Ins(1,4,5)P_3-sensitive store (see Fig. 1.3c).

This model agrees with recent data showing that the emptying of intracellular Ca^{2+} stores is sufficient to activate Ca^{2+} entry in many cells (Hoth and Penner, 1992; Robinson et al., 1992). However, this theory does not explain how the Ca^{2+} content of the E.R. controls the rate of the Ca^{2+} entry. Although there is enough evidence to show that the Ca^{2+} stores communicate with the plasma membrane to control Ca^{2+} entry, the nature of the signal is still unknown. Different mechanisms have been proposed to explain this communication between the endoplasmic reticulum and the plasma membrane. For instance, one early model (Merritt and Rink, 1987c) proposed a gap-junction like connection between the Ca^{2+} store and the plasma membrane. According to this model Ca^{2+} flows directly into the store and then to the cytoplasm provided that the Ins(1,4,5)P_3 receptor channel is open.

Other models propose that a soluble second messenger, which is released when the intracellular pool is depleted, is responsible for this communication. The following have been proposed as candidates for this second messenger : Ca^{2+} (Tscharner et al., 1986), metabolites of cytochrome P-450 (Garcia et al., 1992; Alvarez et al., 1992), tyrosine kinase (Vostal et al., 1991; Sargeant et al., 1993: Lee et al., 1993c), and Ins(1,3,4,5)P_4 acting together with Ins(1,4,5)P_3 (Irvine 1989, 1992). Even more recently evidence has been shown for a novel diffusible messenger (CIF: calcium influx factor) that may link depletion of Ins(1,4,5)P_3-sensitive-calcium stores to Ca^{2+} entry (Randriamampita and Tsien 1993). Studies on *Xenopus* oocytes demonstrated this CIF factor is generated when the cell calcium stores have been depleted (Parekh et al., 1993). Formation of this CIF factor has been linked to the opening of ion channels

causing Ca^{2+} influx (Clapham, 1993). The ion channel proposed for this action is the calcium release-activated current (Icrac) which has been recently characterised (Hoth and Penner, 1992; Zweifach and Lewis, 1993). This current is not inhibited by heparin and is highly selective for calcium (Hoth and Penner, 1992).

Another possible mechanism is that instead of a second messenger, conformational or structural changes in intracellular stores, if close to the plasma membrane, might themselves be the signal transmitted to proteins in the plasma membrane (for review see Putney and Bird, 1993a and b). The possible role of cytoskeleton in transmitting this signal, if the intracellular organelles are not close to the plasma membrane, has been examined (Guillemette et al., 1988; Rossier et al., 1991; Terasaki and Jaffe, 1991; Jaffe and Terasaki, 1993). At present the mechanism by which the intracellular compartments communicate with the plasma membrane is uncertain.

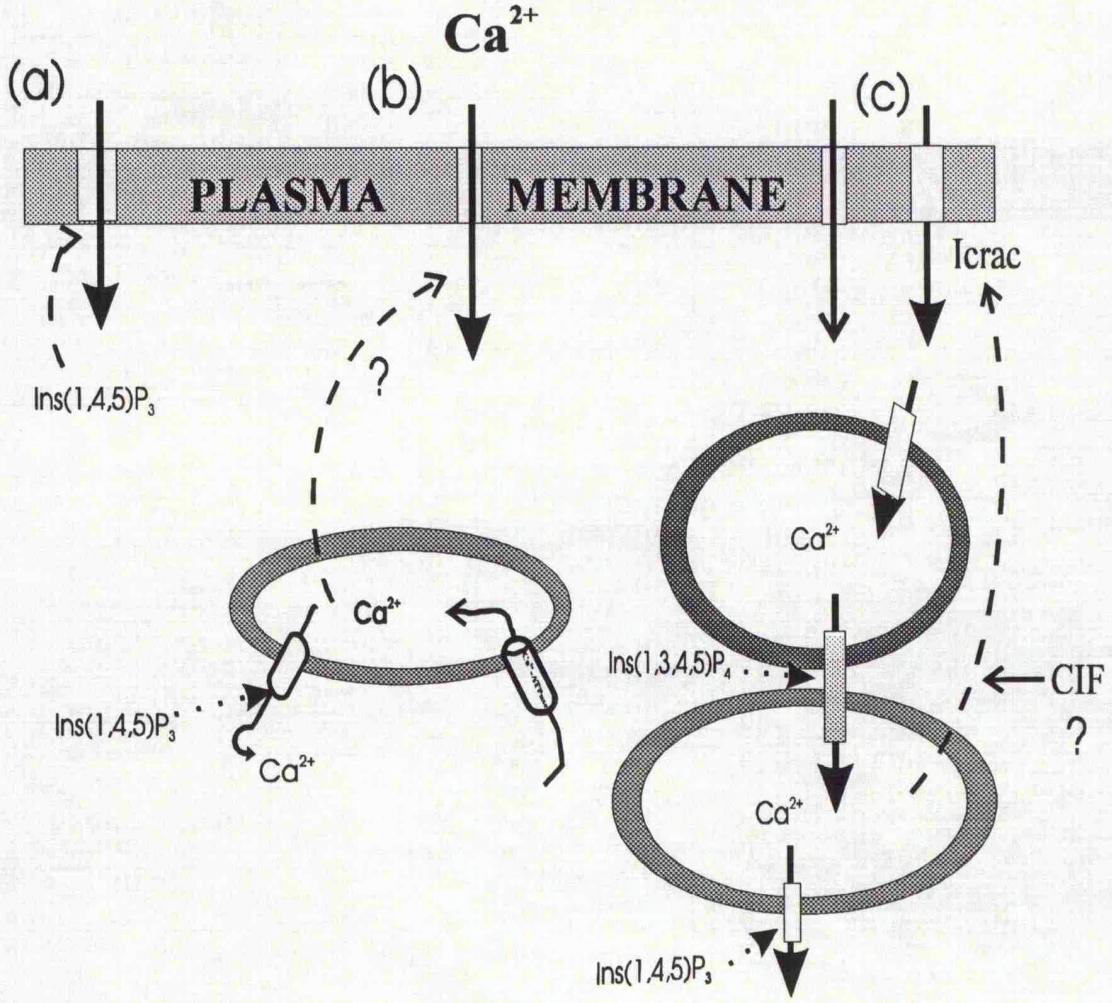


Fig. 1.3. This diagram outlines the proposed mechanisms of Ca^{2+} entry into the cells and the possible role of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. (a) Ins(1,4,5)P_3- directly controls the Ca^{2+} entry through a Ca^{2+} channel in the plasma membrane (plasma membrane-Ins(1,4,5)P_3- receptor ?).

(b) Ins(1,4,5)P_3- indirectly controls Ca^{2+} entry due to depletion of the intracellular stores, thus activating direct Ca^{2+} entry into the store (capacitative entry theory).

(c) Extended version of (b). Indirect Ca^{2+} entry (revised capacitative entry theory) into the store and a possible role for Ins(1,3,4,5)P_4. The possible role of CIF factor and the involvement of the Icrac channel are also outlined in the above figure.

1.5 THE Ins(1,4,5)P_3 RECEPTOR AND THE Ins(1,4,5)P_3-SENSITIVE Ca^{2+} STORES.

It was in 1953 when the inositol lipid cellular signalling system was first reported in pancreatic slices (Hokin and Hokin, 1953) as a ^{32}P incorporation into phospholipids. Thirty years later the first data showing a second messenger function of Ins(1,4,5)P_3 was demonstrated in permeabilised pancreatic acinar cells (Streb et al., 1983). Since then this function of Ins(1,4,5)P_3 has been seen in a variety of cells including bovine adrenal chromaffin cells (Stoehr et al., 1986; Kao, 1988) and many aspects of this signalling system have become clear.

Stimulation of cell-surface receptors initiates the formation of at least two second messengers, Ins(1,4,5)P_3 and diacylglycerol (DAG: a protein kinase C activator). Both of these second messengers are formed by the hydrolysis of an inositol lipid precursor placed in the plasma membrane. The procedure of this formation is as follows:

Binding of an agonist to its cell-surface receptor causes conformational changes in the receptor which activate, via a guanine nucleotide binding protein (G-protein), a polyphosphoinositide-specific phospholipase C (PLC). PLC then catalyses the hydrolysis of phosphatidylinositol 4-5 bisphosphate [PtdIns(4,5)P_2] into Ins(1,4,5)P_3 and DAG. DAG can be formed by the hydrolysis of other phospholipids but that is not the case for Ins(1,4,5)P_3 which can be formed only from the hydrolysis of PtdIns(4,5)P_2. Recent evidence indicates wider diversity on the components (G proteins, PLC, Ins(1,4,5)P_3 and PKC) of this signalling pathway.

G- proteins (heterotrimeric structure, consisting of α, β, γ subunits) are dissociated after agonist binding to its receptor into G_α and $G_{\beta,\gamma}$ subunits (Wu et al., 1993). This dissociation is achieved by the exchange of GDP for GTP. There is a whole family of G- proteins: the pertussis toxin-sensitive G_i and G_o, and pertussis toxin-insensitive Gq (G_{aq}, G_{a11}, G_{a14}, $G_{\alpha15}$, G_{a16}) and G_s. For review on G- proteins see Gilman, 1991; Bourne et al., 1991; Simon et al., 1991; Hepler and Gilman, 1992,

and for discussion of role of $\beta\gamma$ subunits in signal transaction see Sternweis, 1994). The α subunit of Gq- protein activates phospholipase C ($PLC_{\beta 1}$).

There are three major family members in the PLC family: the β, which couple to G proteins; γ which interacts with tyrosine kinase- linked receptors and δ the role of which is still unknown (Rhee and Choi, 1992; Cockcroft and Thomas, 1992). Each family has other members, for example the β family has β_1, β_2, β_3 and possibly the β_4 (Lee et al., 1993b), the γ family: γ_1, γ_2, and the δ family has the δ_1, δ_2 and δ_3 members. There is evidence for a PLC_α (Bennett et al., 1988) but others suggest that it is a thiol-protein disulphide oxidoreductase and hence not a PLC (Srivastava et al., 1991). Another proposed member of the PLC family is the PLC_ε but there is no sequence data on this yet (Cockcroft and Thomas, 1992).

When Ins(1,4,5)P_3 is formed it is rapidly metabolised by two enzymes. The first enzyme which produces dephosphorylation is a 5-phosphatase. The other enzyme is a 3-kinase which phosphorylates inositol phospholipids. The existence of these two enzymes initiates a very complex pathway of inositol phosphate metabolism. The actual purpose of such a complicated pathway is still unclear since most of the metabolites are inactive in mobilising Ca^{2+} except the Ins(1,3,4,5)P_4 and the cyclic (1:2,4,5)InsP_3 (first discovered by Wilson et al., 1985a) in some studies (Irvine et al., 1986: Wilson et al., 1985b) but not all (Willcocks et al., 1989). However, the metabolism of Ins(1,4,5)P_3 regulates important aspects of cell signalling, firstly the recycling of inositol essential for the synthesis and maintenance of the inositol phospholipids, and secondly the control of the cellular Ins(1,4,5)P_3 concentration and hence the response level of cells to receptor stimulation.

It is well documented that Ins(1,4,5)P_3 can mobilise Ca^{2+} from non-mitochondrial intracellular stores. It has been suggested that Ins(1,4,5)P_3-sensitive intracellular calcium channels are responsible for the Ca^{2+} mobilising function of Ins(1,4,5)P_3. It was in 1988 when a protein Ins(1,4,5)P_3- receptor (Receptor- R_I) was reported in membranes of rat cerebellum (Supattapone et al., 1988b). In their studies the receptor was initially solubilised with Triton X-100 and then purified in multiple

chromatography steps using DEAE- cellulose, heparin- agarose and concanavalin A-sepharose. This proposed purified protein has a relative molecular mass of 260 kDa (Furuichi et al., 1989; Mignery et al., 1989) and within Purkinje cells is localised on parts of the endoplasmic reticulum (E.R.) particularly near the nucleus (Ross et al., 1989). However, Furuichi et al., 1989 indicated that this protein (P400) was present at the plasma membrane, the postsynaptic density and the E.R. The primary sequence of this receptor proposed an idea of the structural basis of the Ins(1,4,5)P_3- receptor and indicated sequence homology with the ryanodine receptor as described in the sarcoplasmic reticulum of skeletal muscle (Furuichi et al., 1989; Mignery et al., 1989; Mignery et al., 1990).

Another member of the Ins(1,4,5)P_3- receptor family, the Ins(1,4,5)P_3-R_{II}, was later described (Südhof et al., 1991; Ross et al., 1992). Their data suggested 70% homology between the two receptors and gave evidence for the existence of two other members of the family: the Ins(1,4,5)P_3-R_{III} and Ins(1,4,5)P_3-R_{IV}. Their data also demonstrated partial sequences for the C- terminal domains of the Ins(1,4,5)P_3-R_{III} and Ins(1,4,5)P_3-R_{IV}. Others argued that the Ins(1,4,5)P_3-R_{IV}, which shares sequence identity with the Ins(1,4,5)P_3-R_{III}, represents a splice variant of the type Ins(1,4,5)P_3-R_{III} receptor or a cloning artefact (Mignery and Südhof, 1993). The Ins(1,4,5)P_3-R_{III} cDNAs have now been isolated from rat and human cell lines demonstrating a 62% and 65% overall sequence homology with the mammalian Ins(1,4,5)P_3-R_I and Ins(1,4,5)P_3-R_{II} respectively (Blondel et al., 1993; Yamamoto et al., 1994).

In bovine adrenal glands three subtypes of the Ins(1,4,5)P_3-R_I are expressed (Nakagawa et al., 1991). They differ in two small segments, one located within the Ins(1,4,5)P_3- binding site (S_I segment) and the other near to phosphorylation and ATP binding sites (S_{II} segment). Proportion analysis of the S_I- and S_{I-}- subtypes was somewhat similar to that reported for the cerebellum but only one (the Ins(1,4,5)P_3-RS_{IIABC-}) of the four S_{II} subtypes expressed in cerebellum was detected (Nakagawa et al., 1991). The subtypes carrying the S_{II} segments are expressed only in the nervous

system, whereas the Ins(1,4,5)P_3-RS_{IIABC-} isoform is expressed ubiquitously (Nakagawa et al., 1991).

The location of the Ins(1,4,5)P_3-sensitive stores had always been an enigma. The endoplasmic reticulum is a complicated membrane complex and has been proposed as being the site of the Ins(1,4,5)P_3-sensitive pools. The ER can be separated into the rough endoplasmic reticulum (RER) which binds with ribosomes and smooth endoplasmic reticulum (SER) which is a large, mostly continuous structure consisting of undefined vesicles and cisternae. The Ca^{2+} storage capacity is generally associated with the SER although not exclusively. Whatever the case it is well accepted that the intracellular Ca^{2+} stores are compartmentalised.

The other suggested site for the Ins(1,4,5)P_3-sensitive store is a novel organelle, the 'calciosome', that appears as small, smooth vesicles distributed throughout the cytoplasm having some properties characteristic of the sarcoplasmic reticulum (Volpe et al., 1988; Hashimoto et al., 1988; Pozzan et al., 1988). Calciosome contains the calcium binding protein calsequestrin. Recent studies on Purkinjie neurons suggest that this vesicle may be heterogeneous as some may be sensitive to ryanodine (Volpe et al., 1991), indicating that Ins(1,4,5)P_3-sensitive stores and calciosome may not necessarily be synonymous.

The whole family of Ins(1,4,5)P_3 receptors share same common characteristics. They are all large proteins containing typical transmembrane -spanning domains close to the C- terminus which is localised within the cytoplasmic site. Four of the subunits appeared to combine to form the Ins(1,4,5)P_3-sensitive calcium channel. The N-terminus is exposed to the cytoplasm containing the Ins(1,4,5)P_3 binding site. There is a large hydrophobic and cytosolic coupling domain between the two termini on which regulatory sites for phosphorylation and ATP binding are located (Ehrlich and Watras, 1988; Ferris et al., 1990). ATP has been shown to increase Ins(1,4,5)P_3-induced Ca^{2+} release (Ehrlich and Watras, 1988). The Ins(1,4,5)P_3- receptor has now been reported to phosphorylated by cAMP-dependent protein kinase (protein kinase A: PKA) at the modulatory domain-P-sites (Yamamoto et al., 1989; Nakade et al., 1994). Functional

sites for protein kinase C (PKC), calmodulin (CaM) and Ca^{2+}/CaM- dependent protein kinase II (CaMKII) have been proposed within the modulatory domain (Ferris et al., 1990; Ferris et al., 1991; Ferris and Snyder, 1992). Recent data has also suggested autophosphorylation of the Ins(1,4,5)P_3- receptor (Ferris et al., 1992).

Because the Ins(1,4,5)P_3 binding site is on the N- terminus (Mignery and Südhof, 1990; Miyawaki et al., 1991), distant from the Ca^{2+} channel at the C-terminus (Furuichi et al., 1989; Mignery et al., 1989, and 1990) it has been concluded that Ins(1,4,5)P_3 binding leading to channel opening must cause a large number of conformational changes over a span of virtually 1400 amino acid residues (for review see Ferris and Snyder, 1992).

The properties of the Ins(1,4,5)P_3 receptors reflect their physiological function. Normal cytosolic Ca^{2+} levels reported to inhibit Ins(1,4,5)P_3 binding to its receptor with IC_{50} of about 300 nM (Danoff et al., 1988) showing that Ca^{2+} released by the Ins(1,4,5)P_3 could have a feed-back effect to inhibit further Ins(1,4,5)P_3 induced Ca^{2+} mobilisation. It has been demonstrated that the Ins(1,4,5)P_3 receptor displays a bell shaped response to calcium (Bezprozvanny et al., 1991) so the Ca^{2+} has a function as a coagonist with Ins(1,4,5)P_3 to release Ca^{2+}. According to that Ins(1,4,5)P_3 has little effect on its receptor in the absence of calcium, but becomes increasingly efficient as the Ca^{2+} concentration increases, reaching a maximum at about 300 nM. After this Ca^{2+} concentration Ca^{2+} begin to have an inhibitory effect on Ins(1,4,5)P_3-induced Ca^{2+} release. Evidence for a Ca^{2+} binding site on the Ins(1,4,5)P_3- receptor was recently demonstrated (Mignery et al., 1992). Heparin was also found to inhibit the binding of Ins(1,4,5)P_3 to its purified receptor (Supattapone et al., 1988b).

It is not only cytosolic calcium that regulates the Ins(1,4,5)P_3 receptor. Luminal calcium concentration can also modulate the Ins(1,4,5)P_3 receptor channels. It has been suggested that the amount of Ca^{2+} accumulated in the Ins(1,4,5)P_3-sensitive Ca^{2+} intracellular stores regulates Ins(1,4,5)P_3-gated Ca^{2+} channels sensitivity, with increasing sensitivity when the stores are filled with Ca^{2+} (Missiaen et al., 1991; and 1992).

The mechanisms by which luminal Ca^{2+} regulates Ins(1,4,5)P_3 receptor is still not known although various have been proposed (Irvine, 1990; Missiaen et al., 1992). This sensitivity of the Ins(1,4,5)P_3 receptor to luminal $[Ca^{2+}]_i$ could contribute to the phenomenon termed as 'quantal' release (Muallem et al., 1989; see also Bootman, 1994). According to 'quantal' release submaximal doses of Ins(1,4,5)P_3 stimulate a rapid release of a fraction of the content of the Ins(1,4,5)P_3-sensitive Ca^{2+} store (Muallem et al., 1989; Meyer and Stryer, 1991; Bootman et al., 1992a and b). Addition of the same dose even after an interval would not result in a further Ca^{2+} mobilisation and only higher doses can stimulate a further Ca^{2+} release (for a review see Irvine, 1990). 'Quantal' release has been described in artificial lipid vesicles (liposomes) when they are reconstituted with cerebellar purified Ins(1,4,5)P_3 receptor (Ferris et al., 1992), indicating that it is an intrinsic property of the Ins(1,4,5)P_3 receptor unrelated to luminal $[Ca^{2+}]_i$ and not dependent on the presence of other proteins such as calsequestrin. However, studies on singe permeabilised hepatocytes suggested that quantal release is an artefact caused by permeabilisation procedures which disrupt the continuity of the Ins(1,4,5)P_3-sensitive stores (Renard-Rooney et al., 1993). Recent evidence in carefully permeabilised smooth muscle cells and intact HeLa cells demonstrated that quantal release is a phenomenon independent of fragmentation due to permeabilisation (Short et al., 1993; Bootman et al., 1994).

Another characteristic of the Ins(1,4,5)P_3 receptor is that it does not desensitize so any decrease in the Ca^{2+} release is due to the rapid metabolism of Ins(1,4,5)P_3. However, there is evidence that chronic muscarinic stimulation reduced Ins(1,4,5)P_3-receptor concentration in SH-SY5Y human neuroblastoma cells (Wojcikiewicz et al., 1992 and 1994). Prolonged muscarinic receptor activation resulted type 1 Ins(1,4,5)P_3 receptor degradation. Their data also showed that Ins(1,4,5)P_3-induced Ca^{2+} mobilization, and Ins(1,4,5)P_3 binding was inhibited by this long term muscarinic stimulation, and the physiological importance of these findings remains obscure.

1.6 THE Ca^{2+} -INDUCED Ca^{2+} RELEASE MECHANISM AND STORE.

In 1965 Weiss and Bianchi suggested that Ca^{2+} induced Ca^{2+} release from intracellular stores of the sarcoplasmic reticulum. Before then caffeine and ryanodine were known to generate muscle contraction in muscle fibres (Frank 1960; Bianchi 1961; 1963) and many investigators suspected that this action was dependent on the ability of caffeine and ryanodine to induce Ca^{2+} release from internal stores. The first demonstration of the Ca^{2+}- induced Ca^{2+} release (CICR) mechanism was in skinned muscle cells (Ford and Podolsky 1968; 1970; Endo et al., 1968; 1970). The first report of a CICR mechanism in cardiac cells was in 1975 by Fabiato and Fabiato. The caffeine induced Ca^{2+} release can be used as an indicator of the CICR mechanism. Caffeine releasable intracellular Ca^{2+} stores have been found in neurons (Lipscombe et al., 1988; Thayer et al., 1988a) and in bovine adrenal chromaffin cells (Burgoyne et al., 1989b; Cheek et al., 1990 and 1991; Liu et al., 1991) as well as other cell types.

The receptor associated with the CICR mechanism is the ryanodine receptor. The ryanodine receptor (Ryan- R_1) in skeletal muscle is a protein with an apparent molecular weight of about 500 kDa (Inui et al., 1987; Smith et al., 1988; Takeshima et al., 1989; Meissner, 1994). At the same time cardiac isoforms of the ryanodine receptor (Ryan- R_2) was reported in rabbit cardiac muscle sarcoplasmic reticulum (Otsu et al., 1990a). The cardiac muscle and skeletal muscle ryanodine receptor share only 66% overall amino acid sequence identity and differ in other regulatory properties (Meissner and Henderson 1987; Otsu et al., 1990a). The mammalian cardiac form of the ryanodine receptor varies in molecular sequence from that of the skeletal muscle but the secondary structures of the two proteins are predicted not to significantly differ (Otsu et al., 1990a). There are slight differences in the cardiac and neuronal form which have lower molecular weights than the skeletal form (for review see McPherson and Campbell, 1993). The ryanodine receptor reported in neurons (Walton et al., 1991) and sea urchin eggs (McPherson et al., 1992) seem to be analogous to the cardiac ryanodine receptor (Ryan-R_2). Recent evidence identified the existence of a

gene (β4) responsible for the encoding of another ryanodine receptor (Ryan-R_3) which is similar to, but distinct from, the muscle ryanodine receptors (Giannini et al., 1992; Hakamata et al., 1992). The sequence of proteins encoded by the cDNA clone b4 in mink lung epithelial cells (Mv1Lu) demonstrated that the brain type Ryan-R_3 is significant shorter but shares an overall sequence identity of 57% and 61% with the skeletal and cardiac muscle ryanodine receptor, respectively (Giannini et al., 1992). The amino acid sequence of Ryan-R_3 cloned from rabbit cDNA library shares an overall sequence identity of 67% and 70% with the Ryan-R_1 and Ryan-R_2 respectively (Hakamata et al., 1992).

The ryanodine receptor contains an intrinsic Ca^{2+} channel with a single channel conductance, when activated, of about 100 ps (in ~ 54 mM Ca^{2+}) being highly permeable to Ca^{2+}. The conductance of the channel can be elevated by adding ATP (Smith et al., 1985), or caffeine (Sitsapesan and Williams, 1990) to the cytoplasmic side of the receptor. Addition of Mg^{2+} (Smith et al., 1985), H^+ ions, ruthenium red (Smith et al., 1988), calmodulin (Smith et al., 1989) decreases the channel conductance.

However, differences exist between the skeletal and cardiac ryanodine receptors. The cardiac form of the ryanodine receptor is more sensitive to activation by Ca^{2+} and caffeine and inhibition by Mg^{2+} than the skeletal form (Zimanyi and Pessah, 1991). It has also been demonstrated to be a better substrate for phosphorylation by Ca^{2+}-calmodulin dependent protein kinase II (CaMKII) and cAMP-dependent protein kinase (Witcher et al., 1991). The plant alkaloid ryanodine opens the channels at low (nanomolar) concentrations but closes them at higher concentrations (micromolar).

The ryanodine receptor has a structural and molecular homology with the Ins(1,4,5)P_3 receptor. The ryanodine receptor is larger than the Ins(1,4,5)P_3 receptor and the homology is considerable in the C- terminus and negligible in the N- terminus. They are both proposed to be homotetramers composed of monomers with a large N-terminus. These monomers have been predicted to have at least four transmembrane domains near the C- terminus with some of the regions co-operating to form the Ca^{2+}

channel. Both the N- and C- terminal of each monomer are predicted to reside on the cytoplasmic side of the membrane (Takeshima et al., 1989; Otsu et al., 1990b). They also show limited DNA sequence homology (Furuichi et al., 1989; Mignery et al., 1989). The structural and molecular homology has given rise to the idea that the two proteins are members of a distinctive family of Ca^{2+} permeable receptors (Gill, 1989) which may account for many of the functional similarities that exist between the ryanodine and the Ins(1,4,5)P_3 receptor.

There is now sufficient evidence that chromaffin cells possess ryanodine-sensitive Ca^{2+} stores (Burgoyne et al., 1989b; Cheek et al., 1990). While a large variety of cell types have both ryanodine-sensitive stores and Ins(1,4,5)P_3-sensitive Ca^{2+} stores (neurons, chromaffin cells, smooth muscle, sea urchin eggs) others possess only ryanodine-sensitive stores (skeletal muscle) or Ins(1,4,5)P_3-sensitive pools as in *Xenopus* oocytes (Parys et al., 1992).

Very recent data demonstrated 'quantal' release from ryanodine-sensitive Ca^{2+} stores in chromaffin cells (Cheek et al., 1993b) suggesting that this phenomenon is not a characteristic only of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores. Their data also suggest that $[Ca^{2+}]_i$ increases the sensitivity of the caffeine-sensitive Ca^{2+} store. They proposed that heterogeneity between caffeine-sensitive Ca^{2+} stores may reflect differences in the filling state of these stores, indicating that luminal Ca^{2+} provides a mechanism in controlling quantal release from caffeine-sensitive Ca^{2+} stores.

A major unanswered question about the caffeine-sensitive Ca^{2+} stores is their location. There is evidence for both co-localisation with the Ins(1,4,5)P_3-releasable Ca^{2+} stores in PC12 cells (Clementi et al., 1992) and distinct localisation in sea urchin eggs (Galione et al., 1991). This issue is still controversial in chromaffin cells. Techniques using spatial imaging of Ca^{2+} indicate that caffeine and Ins(1,4,5)P_3 generate Ca^{2+} transients with different localisation's (Burgoyne et al., 1989b). Experiments in populations of chromaffin cells showed that part of the Ins(1,4,5)P_3-sensitive store is also caffeine-sensitive (Liu et al., 1991). Whatever the story is, the data suggests that these two stores may link to produce calcium signals.

There are reports demonstrating that Ca^{2+} released from Ins(1,4,5)P_3-sensitive pools may be able to stimulate Ca^{2+} release from the ryanodine-sensitive stores (Kasai and Augustine, 1990; Neylon et al., 1990). There is also evidence that the channel opening of the brain ryanodine receptor can be modulated by Ins(1,4,5)P_3. For instance in the presence of low but suprathreshold Ca^{2+}, Ins(1,4,5)P_3 increases the channel opening probability (Vassilev et al., 1987; Ashley, 1989; Valdivia et al., 1990). This issue is controversial since others have not been able to pinpoint any alteration in the ryanodine channel, or the mediated Ca^{2+} release, by Ins(1,4,5)P_3 (Ehrlich and Watras, 1988; Palade et al., 1989).

There is, however, another second messenger which is able to stimulate Ca^{2+} release as was demonstrated recently. This second messenger is cyclic- adenosine 5'-diphosphate ribose (cyclic ADP-ribose) a low molecular weight metabolite of the pyridine nucleotide, nicotinamide adenine dinucleotide (NAD^+). It was first identified by Lee and his colleagues in 1990 when studying Ca^{2+} mobilisation by microsomes derived from the endoplasmic reticulum of sea urchin eggs (Dargie et al., 1990). Cyclic ADP-ribose has been found to be present in many cells types (Rusinko and Lee 1989; Galione, 1992). Their results demonstrated that the enzymes responsible for the cyclic ADP-ribose synthesis were present in brain extracts. This NAD metabolite has been reported to stimulate Ca^{2+} mobilisation from the ryanodine-sensitive Ca^{2+} stores in sea urchin eggs (Galione et al., 1991). Further, cyclic ADP-ribose-induced Ca^{2+} release is apparently distinct from the Ins(1,4,5)P_3-sensitive Ca^{2+} stores (Lee, 1993; Lee et al., 1993a; Meszaros et al., 1993; Morrissete et al., 1993; Galione, 1993). Studies on rat brain microsomes revealed that the cyclic ADP-ribose induced- Ca^{2+} release was inhibited by ryanodine (White et al., 1993). There are possibilities that cyclic ADP-ribose is an endogenous activator of the ryanodine receptor channel and therefore has a significant role in Ca^{2+} homeostasis and signalling.

The physiological role of the ryanodine-sensitive Ca^{2+} stores remain to be defined. It has been demonstrated that in heart the Ca^{2+} entering through the voltage-sensitive channels can be amplified by the CICR mechanism. This might be the case in

neurons as well (Friel et al., 1992a and b). This Ca^{2+} amplification can lead to formation of repetitive Ca^{2+} spikes (Friel et al., 1992b). There is evidence that this may also be the case in mouse eggs (Swann, 1992) and in pancreatic acinar cells (Nathanson et al., 1992). The physiological role of the CICR mechanism may therefore be to amplify and propagate intracellular Ca^{2+} signals including those produced from the Ins(1,4,5)P_3-releasable Ca^{2+} stores (Endo, 1977; Lipscombe et al., 1988; Malgaroli et al., 1990).

1.7 AIMS OF THIS STUDY.

As discussed above bovine adrenal chromaffin cells have been widely used by those interest in stimulus secretion coupling. These cells also possess a variety of mechanisms which cause $[Ca^{2+}]_i$ elevation making them a very useful model for those interested in Ca^{2+} signalling and Ca^{2+} homeostasis. The object of this study was to further characterise the intracellular Ca^{2+} stores in chromaffin cells in order to increase our knowledge of this subject. The major aims were as follows :

(i) To investigate the possible involvement of intracellular Ca^{2+} stores in stimulating secretion in chromaffin cells by examining the Ca^{2+} requirements for catecholamine production evoked by depolarising stimuli and agonists linked to $Ins(1,4,5)P_3$ production.

(ii) To study the characteristics and the mechanisms involved in Ca^{2+} release from intracellular Ca^{2+} stores. The relationship of the intracellular stores was examined in permeabilised populations of chromaffin cells using a Ca^{2+}- selective electrode. Synthetic analogues of $Ins(1,4,5)P_3$ which are poorly metabolised were used to examine if more than one store is present in chromaffin cells. These studies showed that chromaffin cells possess two intracellular Ca^{2+} stores these being $Ins(1,4,5)P_3$-sensitive and caffeine-sensitive, respectively. These experiments also provided evidence that the two stores may partially overlap.

(iii) To further examine the relationship between the intracellular Ca^{2+} stores in populations of permeabilised plated chromaffin cells loaded with $^{45}Ca^{2+}$. Store depleting agents were used to investigate the role of the intracellular Ca^{2+} stores and examine their relationship. The characteristics and mechanisms involved in Ca^{2+} mobilisation from these stores were also studied.

(iv) To study the effect of store depletion on Ca^{2+} entry across the chromaffin cells plasma membrane by using intact populations of cells loaded with the fluorescent dye fura 2-AM e.g. by examining the effect of a Ca^{2+}-ATPase inhibitor (thapsigargin) mediated store depletion and caffeine induced Ca^{2+} release from intracellular stores on Ca^{2+} entry.

(v) To examine the relationship between Ins(1,4,5)P_3-sensitive and caffeine-sensitive Ca^{2+} stores in single chromaffin cells by using epifluorescence microscopy. This was done by investigating the effect of agonist evoked Ca^{2+} release on the caffeine response and the effect of caffeine evoked Ca^{2+} release on the agonist response.

CHAPTER 2 : METHODS

2.1 INITIAL PREPARATION.

Chromaffin cells were isolated from bovine adrenal medullae by enzymatic digestion using an adaptation of the methods of Livett (1984) and Waymire et al., (1983). Ten to twelve adrenal glands obtained from the local abattoir were transferred to the laboratory within 30-60 min. From these eight were selected and dissected free of fat, leaving a small collar of tissue around the central lobular vein. Next a piece of plastic tube was inserted and secured with thread at the neck to facilitate the injection of Lockes solution (154 mM NaCl, 5.6 mM KCl, 5.6 mM glucose, 5.0 mM HEPES, pH 7.4 with 5 mM NaOH) containing 28 mg/ml of heparin (from porcine intestinal mucosa). This solution was prepared on the same day, incubated for 30 minutes, and sterilized (filtered through a sterile 0.45 μM acrodisk) as were all the solutions used in this cell preparation.

Lockes solution and heparin were injected into the glands until they were full. The cortex of each swollen gland was then repeatedly pierced with an 18g needle. The plastic tubes were connected to a Gilson Multipuls peristaltic perfusion system pump which was set up to run at 1ml/min. The glands were then retrogradely perfused for 30 minutes at room temperature with Lockes solution containing heparin in order to remove red blood cells.

The medulla tissue was then removed from the cortex. This was achieved by a further 30 minutes perfusion at 37^0C in a water bath with 100 ml of Lockes solution containing 1g/l collagenase (Sigma type II), 0.3 g/l protease (Sigma type IV) and 25 mg/l DNAase (Sigma type I). This collagenase digestion enables the removal of the medulla tissue and also initiates the process of dissociating the chromaffin cells.

The glands were then removed from the collagenase solution and transferred to a tissue culture cabinet. Here they were washed with ethanol, and all ensuing procedures were carried out under sterile conditions. The glands were bisected and the medulla removed using a scraping action with a scalpel. The medulla was placed in a small sterile beaker containing 25 ml of Lockes solution where it was carefully

chopped with scissors. More Lockes solution was then added to make the final volume up to 70 ml. The supernatant was poured off and filtered through a 100 μM mesh into 50 ml of sterile Lockes. A small sample of cells were diluted with 1/20 trypan blue and counted using a heamocytometer. The supernatant was then spun for ten minutes at 200 x g to remove the collagenase from the cells. The remaining pellet containing the chromaffin cells was resuspended in 25 ml of Lockes (S1). The remaining tissue in the beaker was digested twice more in 50 ml of collagenase solution for 30 minutes at 37^0C with a Camlab Tri-Stir variable speed stirrer. The supernatant was treated as above. These two collagenase digestion's gave S2 and S3 pellets. The number of cells found in each preparation varied from 500 - 1000 million cells. Such variations could be due to differences in the batches of collagenase but are usual when using this method.

2.1.1 Purification Techniques.

It is necessary to remove tissue debris and any remaining blood cells. This was achieved by spinning the cells at various speeds for 10 minutes at room temperature using a Sarstedt LC-1K centrifuge. The pellets from the three enzymatic digestion's (S1, S2 and S3) with 25 ml of sterile Lockes were spun at 160 x g and then resuspended with an 18g needle into 80 ml of sterile Lockes. They were then filtered through a 60 μm mesh and spun at 120 x g to remove connective tissue debris. Lastly, the cell pellets were resuspended into BSS (without Ca^{2+} and Mg^{2+} added) and spun at 80 x g for ten minutes to remove red blood cells. They were then placed into complete medium, ready for culture. The total number of cells was decreased by 20-30% due to the centrifugation procedures. The culture medium used was Dulbecco's modified medium (DMEM) supplemented with 10% foetal calf serum (FCS), 1 ml/100ml of 100 x non-essential amino acids (NEAA), 27 mg/ml of glutamine, 250 μg/ml of fungizone, 25 I.U./ml of penicillin and streptomycin, 50 μg/100ml of gentamycin, 5 μM fluorodeoxyuridine (FDU) and 5 μM of cytosine arabinoside (Ara-C).

2.2 DIFFERENTIAL PLATING AND CULTURING.

Centrifugation was the first step in the purification process. To further purify the chromaffin cells a differential plating technique adapted from Waymire et al., (1983) was employed. Cells in culture medium were placed in 175 cm^2 tissue culture flasks at 50-60 ml/ flask and incubated at 37^0C in 5% CO_2/95% humidified air for 3.5 hours. Over this period the contaminating endothelial cells separate and cling to the bottom of the flask allowing the pouring of the relatively pure chromaffin cell fraction into a large beaker. Using an 18g needle (to ensure the cells did not clump) the cells were then transferred to another beaker and plated onto "Primaria" 24 well plates at a

density of $1x10^6$ cells/well in 1 ml of complete medium. After two or three days of incubation the cells were ready for use.

The final cell preparation contains 85-90% pure chromaffin cells as seen by visual inspection. This standard treatment requires slight modification depending upon the nature of planned experiment. In the case of catecholamine secretion and $^{45}Ca^{2+}$ release experiments the chromaffin cells were plated onto "Primaria" 24 well plates. Whereas for Ca^{2+} measurements in population of permeabilised cells the chromaffin cells were plated onto 60 mm diameter tissue culture petri dishes or in NUNC 175 cm^2 tissue culture flasks at a density of $1x10^6$ cells/1 ml of complete medium. However, for single cell studies the chromaffin cells were plated onto 22mm diameter glass coverslips at a density of $1x\ 10^5$ cells/ml of culture medium.

In all the cases chromaffin cells were incubated at 37^0C in 5% CO_2/95% humidified air and used for experiments after 2-3 days in culture but never later than 7 days after the initial preparation. Progressive changes in both the number and the appearance of the secretory granules have been reported in chromaffin cells when maintained in culture for long periods. Chromaffin cells also appear to lose their adrenergic nature in favour of noradrenergic characteristics (Unsicker et al., 1980). No significant changes have been reported when chromaffin cells are used, as in this study, between 3-4 days in culture (Livett, 1984).

2.3 BOVINE CHROMAFFIN GRANULE PREPARATION.

Fresh bovine adrenal glands obtained from a local abattoir were placed on ice and 10-20 mg of medullae were dissected. The medullae were chopped with a pair of scissors, transferred in 50 ml of 0.3 M sucrose, and homogenised by using an Ultra-Turrax at low speed until a fine suspension was formed. Another 50 ml of 0.3 M sucrose was added and the medullae were further homogenised on ice by using a Teflon/ glass homogeniser.

The homogenates were centrifuged at 1,000 x g for 10 minutes, the supernatant removed and recentrifuged at 9,000 rpm (spin 2) for another 10 minutes. In order to examine Ins(1,4,5)P_3 release from bovine adrenal medullary secretory vesicles two different protocols(i and ii) were carried out after this spin to obtain highly purified vesicles.

(i) The pellet (P1) produced by the second spin was diluted in 0.3 M sucrose solution (~3 ml/pellet) and placed carefully on top of 20 ml of 1.6 M sucrose in tubes and centrifuged at 117,000 x g for 90 minutes. The pellet (P2), containing highly purified vesicles, was separated from the supernatant. The P2 pellet was added to buffer (A) (120 mM KCl, 20 mM HEPES, 6 mM $MgCl_2$, 5 mM Na-succinate, 5 mM ATP, 2 mM KH_2PO_4, pH 7.2 with 5 M KOH) then homogenised by using an Ultra-Turrax system, and was then ready for use.

(ii) The P1 pellet was homogenised by using an Ultra-Turrax system and placed on top of 25 ml of buffer (B) (0.32 M sucrose, 140 mM KCl, 10 mM HEPES/ KOH, 5 μM EGTA, pH 7.2 with 5 M KOH). At this stage we were not able to see any gradient so we repeated the S2 spin at 10,000 x g for 25 minutes. The pellet obtained (P2ii) was diluted with buffer (C) (0.3 M sucrose, 20 mM MOPS/KOH, 5 mM EDTA, pH 7.3) at about 3 ml/pellet. The P2ii pellet was homogenised with an Ultra-Turrax system and layered on top of 25 ml of buffer (D) (buffer D: 1.6 M sucrose, 140 mM KCl, 10 mM HEPES/ KOH, 5 μM EGTA, pH 7.2 with 5 M KOH). The next step was to spin it at 117,000 x g for 90 minutes in order to obtain the pellet (P3ii) containing

highly purified vesicles. Lastly, it was homogenised by using an Ultra-Turrax system before use.

In order to carry out Ca^{2+} measurements in the bovine granule prepared with the first protocol (i) the granule was loaded with 30 μM $CaCl_2$ for 30 minutes at 37^0C and then centrifuged twice at 13,000 x g for 10 minutes at room temperature. The pellet was washed with 1 ml of buffer A after each centrifugation. The bovine vesicles were then ready to be placed at a Ca^{2+}-selective electrode.

For Ca^{2+} studies in the bovine vesicles prepared with the second protocol (ii) the vesicles were loaded with the second 30 μM $CaCl_2$ for 30 minutes at 37^0C and then centrifuged twice at 11,000 x g for 12 minutes. The pellet was washed with 1 ml of buffer D after each centrifugation. The bovine chromaffin granules produced by each method were used the same day for Ca^{2+} measurements. The protein content of the bovine vesicles was found to be at about 1 mg/ml by using Bradford assay (see Chapter 2, section 11).

The existence of chromaffin secretory vesicles in adrenal medulla is well known. These chromaffin granules are high Ca^{2+}- buffering capacity vesicles and contain 30-40 mM Ca^{2+} (Bulenda and Gratzl 1985; Winkler and Westhead, 1980). The average size of the secretory vesicles is 280 nm (Winkler and Westhead 1980), they represent about 10% of the chromaffin cell volume, and they do not possess a 100- kDa Ca^{2+}- ATPase protein. These chromaffin granules contain a Na^+ /Ca^{2+} exchange mechanism (Krieger-Brauer and Gratzl, 1982) which may be responsible for Ca^{2+} reuptake in these vesicles. It has been reported that Ins(1,4,5)P_3 can release Ca^{2+} from adrenal medullary secretory vesicles (Yoo and Albanesi, 1990; Yoo, 1991). In their hands 10 μM of Ins(1,4,5)P_3 was able to stimulate release of 3.5 nmol of Ca^{2+} /mg of protein. This response was blocked by prior addition of heparin (an established Ins(1,4,5)P_3- receptor antagonist: Berridge and Irvine, 1989; Nahorski and Potter, 1989), indicating a clear Ins(1,4,5)P_3 effect. These vesicles contain the high capacity low affinity Ca^{2+}- binding protein chromogranin which Yoo and Albanesi believe shares characteristics with calsequestrin and represents 40 % of the soluble proteins in

the granules. Their data also suggested that chromaffin granules accumulate Ca^{2+} in an ATP- independent manner.

This very interesting effect of Ins(1,4,5)P_3 on chromaffin secretory vesicles suggests an improbable but very attractive theory that these granules represent the Ins(1,4,5)P_3-sensitive or caffeine-sensitive Ca^{2+} stores in chromaffin cells. Experiments were carried out in order to reproduce their findings. Although two different protocols of preparing the chromaffin granules were used Ins(1,4,5)P_3 was not able to stimulate Ca^{2+} release. This possibility does not appear to be the case in chromaffin cells since ATP did not seem to be necessary for refilling these granules (Kostron et al., 1977; Krieger-Brauer and Gratzl, 1982; Yoo and Albanesi 1990) - a premise which experiments performed in this study support.

2.4 CATECHOLAMINE RELEASE.

Chromaffin cells between 3-7 days old were used for catecholamine secretion experiments. All the procedures were carried out in a water bath at 37^0C and all solutions used were prewarmed to 37^0C. The cells were washed twice with 0.5 ml of HEPES buffered Balanced Salt Solution (BSS containing : 125 mM NaCl, 5.4 mM KCl, 16.2 mM $NaHCO_3$, 5.5 mM glucose, 30 mM HEPES, 0.8 mM $MgSO_4$, 1.0 mM NaH_2P0_4, pH to 7.4 with 5M NaOH). The BSS solution was then removed and the cells were stimulated by agonists added in 0.5 ml of BSS and left to stand for the relevant time (depending on the nature of the experiment).

The reaction was terminated by removing the supernatant from the well and transferring it to an eppendorf microfuge tube on ice. Each well, now containing only cell content, had 0.25 ml of ice-cold HCl added to it. The plates were then placed in a freezer until frozen. Separate procedures were then carried out on the supernatant and the cell contents to determine catecholamine release in each.

2.4.1 Preparing the Supernatant for Analysis.

The supernatant was spun at 200 x g for 2-3 minutes in order to pellet any remaining cells. Then, 0.45 ml of the supernatant was transferred to new eppendorf microfuge tubes and 50 µl of HCl was added to bring the final concentration of HCl to 0.1 M. The supernatant was now ready to be analyzed by an HPLC with electrochemical detection (Mefford, 1981).

2.4.2 Preparing the Cell Contents for Analysis.

The plates were removed from the freezer and allowed to defrost; 250 µl of 0.1 M HCl was added; the cells were then scraped from the plates and transferred to eppendorf microfuge tubes. This procedure was repeated with another 500 µl of 0.1 M

HCl to ensure that all the cells had been removed. The contents of the eppendorf tubes were then centrifuged at 2000 x g for 3 minutes and 0.9 ml of the supernatant was dispensed into new eppendorf tubes. The cell contents were now ready to be analyzed by HPLC with electrochemical detection as before.

2.5 THE PRINCIPLES OF HPLC.

2.5.1 Standardisation and General.

The system used here to measure catecholamines was a high pressure reverse phase ion pair chromatography followed by electrochemical detection.

The HPLC comprised of a stationery phase of a 5μm octadecylsilyl Ultrasphere column, 4.6 mm by 25 cm with a mobile phase of : 6.8 g/l of sodium acetate, 5.7 g/l of citric acid, 2.4 g/l of sodium hydroxide, 0.3 g/l of sodium octyl sulphonate (ion pairing agent) and 0.37 g/l of EDTA, dissolved in a solution of methanol/water (1:9 v/v). The pH was adjusted with concentrated acetic acid, filtered through a 0.2 μm mesh, and degassed with helium before use.

The next stage was to inject the samples onto the column using a Magnus 1170 Autosampler. The electrochemical detector used was a Bioanalytical System, LC-4 amphoteric detector connected to a Shimadzu CR-1A integrator which after standardization converts the peaks observed into levels of catecholamines in the sample. Before each experiment the system was standardised by using 50 pmoles of noradrenaline and 50 pmoles of adrenaline (see Fig. 2.1a). A trace from an extract of chromaffin cells is shown in Fig. 2.1b and the identical extract spiked with 50 pmoles of standard noradrenaline and adrenaline is shown in Fig.2.1c. It can be seen that the spikes overlap completely verifying that the detected products are noradrenaline and adrenaline.

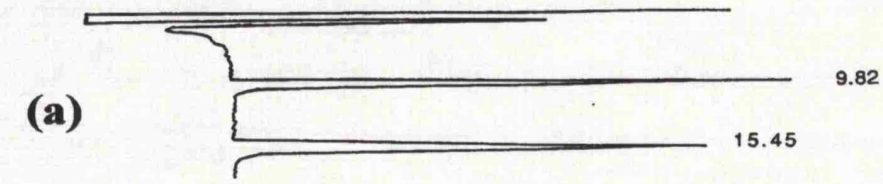
2.5.2 Sample Analysis.

After the system was calibrated, 220 μl of the supernatant (prepared as described above) were transferred into sample bottles on the autoinjector. A 100 μl sample of the cell contents (prepared as described above) was mixed with 900 μl of 0.1 M HCl, and transferred to sample bottles on the autoinjector.

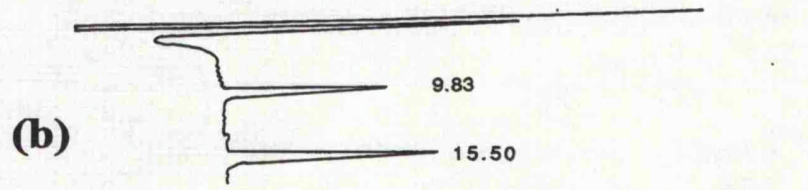
The data produced allowed catecholamine release to be expressed as a percentage by dividing the amount of catecholamines in the supernatant by those present in both the supernatant and cell contents.

$$\%\ \text{Noradrenaline or Adrenaline} = \frac{\text{Noradrenaline or Adrenaline in Supernatant}}{\text{Noradrenaline or adrenaline in Supernatant and Cell Content}} \times 100$$

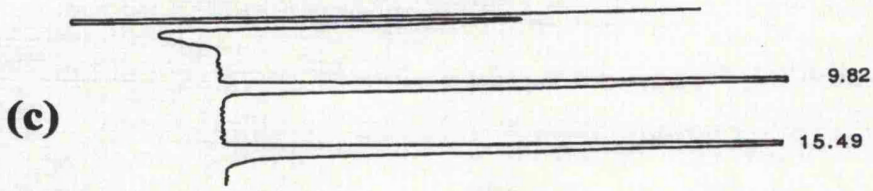
Results were expressed as mean ± S.E.M. and all experiments were performed in quadruplicate from a single cell preparation. Data was analysed statistically by the use of an unpaired Student's t-test, where $p<0.05$ was the accepted level of significance.



	NAME	TIME	CONC	HEIGHT
		6.29		131
1.	Noradrenaline	9.82	51.94	14522
2.	Adrenaline	15.45	52.10	12214



	NAME	TIME	CONC	HEIGHT
		6.76		83
1.	Noradrenaline	9.83	15.57	4353
		13.76		176
2.	Adrenaline	15.50	24.06	5646



	NAME	TIME	CONC	HEIGHT
		8.32		127
1.	Noradrenaline	9.82	64.00	17894
2.	Adrenaline	15.49	71.65	16797

Fig. 1.2. Standardisation of HPLC with electrochemical detection procedure.

(a) This trace is the result of the injection using standard of 50 pmoles of both noradrenaline and adrenaline onto the column. The legends show the retention times on the column and the calculation of the amount of standard added based on the height of the peak obtained. (b) This trace is the result of the addition of 100 μl of a 1/10 dilution of the chromaffin cell extract. (c) This trace is the result of the addition of 100 μl of 1/10 diluted chromaffin cell extract when spiked with 50 pmoles of standard noradrenaline and adrenaline.

2.6 MEASUREMENT OF $[Ca^{2+}]_i$.

2.6.1 Ca^{2+} -Selective Electrode.

Chromaffin cells were cultured in complete medium in 50 ml flasks or in petri dishes for 2-4 days. The method used here is that described in Wojcikiewicz et al., 1990. Cells from one flask or two petri dishes were harvested in 10 mM HEPES, 0.9% NaCl, 0.02% EDTA, pH 7.4 and then gently scraped off. Chromaffin cells were washed twice by centrifugation at 500 x g for 2 minutes and resuspended in 0.8 ml of ice cold "cytosol-like" medium (buffer A : 120 mM KCl, 20 mM HEPES, 6 mM $MgCl_2$, 5 mM Na-succinate, 2-5 mM ATP, 2mM KH_2PO_4, pH 7.2). The Ca^{2+} concentration of "the cytosol-like" medium was buffered to 500 nM by addition of 10 μM quin-2 (free acid). The cells were then exposed to ten discharges of a 3 μF capacitor. The electroporated permeabilised cells were diluted to 5 ml with buffer A and then centrifuged at 600 x g for 2 minutes and finally resuspended in 0.6-0.8 ml of buffer A. The amount of protein in the cells was found to be between 1-2 mg of cell protein/ml by using the Bradford Assay (see later 2.11).

Chromaffin cells were then placed in suspension with a magnetic stirrer in a small plastic beaker. The Ca^{2+}-selective electrode was calibrated by using quin 2 fluorescence and standard Ca^{2+} solutions every day. All experiments were carried out at a temperature of 20^0C to slow the formation and metabolism of $Ins(1,4,5)P_3$. Changes in Ca^{2+} in the stirred suspension were monitored with a Ca^{2+}-selective electrode (Clapper and Lee, 1985). The voltage changes occuring due to alterations in the relative $[Ca^{2+}]_i$ concentration, in response to stimuli, were logged by a chart recorder. After each experiment the relationship between the Ca^{2+}-electrode and changes in Ca^{2+} concentration was determined by sequential additions of known amounts of Ca^{2+} (for example 250 nM) to an aliquot containing buffer, quin-2 (5 μM), and chromaffin cells. The $[Ca^{2+}]$ levels in the buffer were calibrated with a fluorescence spectrophotometer. Similarly, additions of the same amounts of Ca^{2+}

were made to chromaffin cells placed at the Ca^{2+}-selective electrode. The voltage changes were logged and correlated to Ca^{2+} changes already determined by the spectrophotometer.

2.7 OTHER POPULATION MEASUREMENTS.

2.7.1 $^{45}CaCl_2$ Method for Permeabilised Cells in Suspension.

Chromaffin cells were cultured in petri dishes or 50 ml flasks for between 3 and 7 days before use. The method used was essentially a modification of Gershengorn et al., 1984, as described in Strupish et al., 1988. Chromaffin cells were harvested in HBS buffer (HBS : 155 mM NaCl, 10 mM HEPES, 0.02 % EDTA, pH 7.4) for 5 minutes and gently removed from their container. The cells were then washed twice in HBS, centrifuged twice at 500 x g for 2 minutes and finally resuspended in 0.8 ml of buffer B (buffer B : 120 mM KCl, 20 mM HEPES, 6 mM $MgCl_2$, 5 mM Na-succinate, 2-5 mM ATP, 2mM KH_2PO_4, pH 6.9).

The Ca^{2+} concentration in buffer B was adjusted to 200 nM $[Ca^{2+}]_i$ by adding an appropriate amount of EGTA. In order to permeabilise the cells they were exposed to 10 discharges of a 3 μM capacitor. The cells were then diluted in 5 ml of buffer B, centrifuged at 500 x g for 2 minutes, and finally resuspended in 4 ml of buffer B containing 2 μCi/ml of $^{45}CaCl_2$. Protein content as determined by Bradford assay was 1-2 mg/ml. The chromaffin cells were left for 15 minutes at room temperature to take up $^{45}CaCl_2$. When the cells were loaded with $^{45}CaCl_2$, 100 μl of cell suspension was added to 100 μl of buffer B which contained the stimuli. Simultaneously 0.5 ml of silicone oil (Dow Corning 550/556, 9:11 v/v) was added at room temperature and the mixture was centrifuged at 16,000 x g for 3 minutes to terminate the reaction. This spin separated the chromaffin cells from the buffer B. The water/oil layer was removed without disturbing the pellet (cells) and the tubes were drained for 15 minutes. When the cells were drained $^{45}Ca^{2+}$ radioactivity was determined. $^{45}Ca^{2+}$ release was expressed as a percentage by comparing the amount of $^{45}Ca^{2+}$ remaining in the stimulated cells with that contained in the unstimulated control cells (see equation).

% Calcium Release = 100 - (Value/total x 100)

Data are generally expressed as mean $\pm$ S.E.M. followed by the number of determinations (n). Data was analysed statistically by the use of an unpaired Student's t-test, where $p<0.05$ was the accepted level of significance.

Permeabilised chromaffin cells in suspension prepared as described sequestered $^{45}CaCl_2$ in a time- dependent manner in the presence of 5 mM ATP as can be seen in Fig. 2.2. Chromaffin cells accumulate $^{45}CaCl_2$ slower than SH-SY5Y cells ($P<0.05$ for the first 10 minutes). After 10 minutes $^{45}CaCl_2$ accumulation was not significantly different. Maximal accumulation was obtained at 20 minutes for both cell types; at 10 minutes more than 80% of the $^{45}CaCl_2$ was sequestered into the intracellular stores of the cells. The problem was that less $^{45}Ca^{2+}$ sequestered in the chromaffin cells (4-10 fold less) than that in SH-SY5Y cells. Although it was easy to obtain dose responses to Ins(1,4,5)P_3 in SH-SY5Y (Fig. 2.3) cells this was not the case in chromaffin cells. Since the amount of Ca^{2+} sequestered in the intracellular Ca^{2+} stores of chromaffin cells was not adequate under these conditions, this method was abandoned.

In SH-SY5Y cells maximal release was obtained with 3 μM Ins(1,4,5)P_3 which was able to mobilise 55% of the Ca^{2+} sequestered in the intracellular stores, with an EC_{50} of 350 $\pm$ 23 nM (n=4). Ionomycin (1 μM) stimulation resulted in release of 75% of total Ca^{2+} sequestered in intracellular stores. Maximally effective concentrations of Ins(1,4,5)P_3 access 80% of the total mobilisable intracellular Ca^{2+} stores in permeabilised SH-SY5Y cells. These findings are to the top of the range obtained in previous studies (Safrany et al., 1990 and 1991a and b; Wojcikiewicz and Nahorski, 1991; Wilcox et al., 1993a) which showed that Ins(1,4,5)P_3 evoked 80% of the total Ca^{2+} releasable with an EC_{50} of between 50- 320 nM.

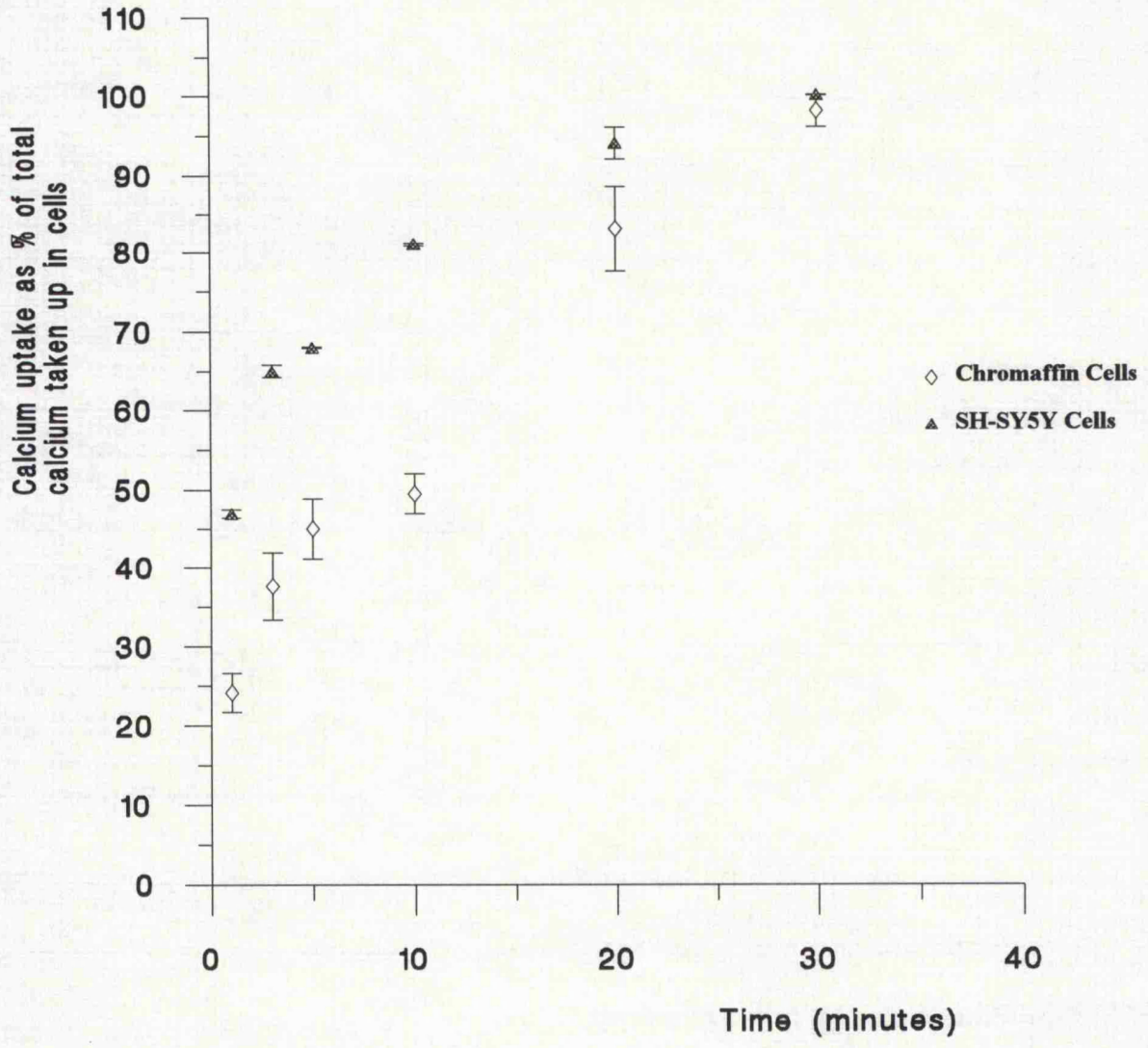


Fig. 2.2. $^{45}Ca^{2+}$ uptake in electroporated permeabilised chromaffin and SH-SH5Y cells in suspension. This is a time- course of $^{45}Ca^{2+}$ uptake in these cells. $^{45}Ca^{2+}$ uptake is expressed as percentage of total $^{45}Ca^{2+}$ taken up in cells. ATP was present at 5 mM. Results are mean values $\pm$ S.E.M. of three experiments each performed in duplicate.

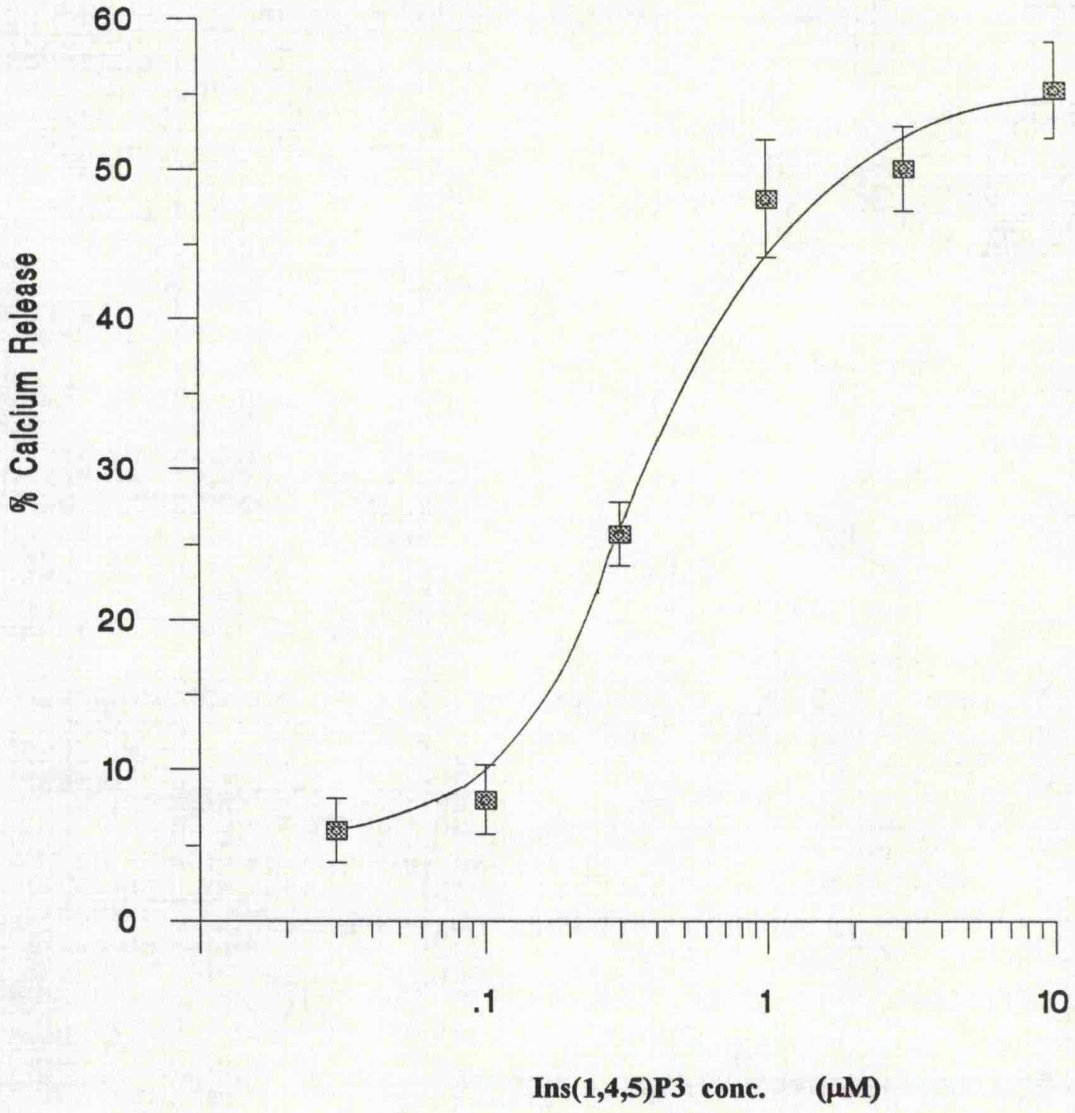


Fig. 2.3. Ins(1,4,5)P_3 dose response in electrically permeabilised SH-SY5Y cells. $[Ca^{2+}]_i$ levels were buffered at 200 nM. ATP was present at 2 mM. SH-SY5Y cells were loaded with $^{45}Ca^{2+}$ at 20^0C and then challenged with agonist. Results are mean values $\pm$ S.E.M. of four experiments.

2.7.2 $[Ca^{2+}]_i$ Measurement in Populations of Intact Chromaffin Cells (Fura 2-AM Method).

Chromaffin cells were cultured in complete medium for 2-5 days in petri dishes or 50 ml flasks before use. The cells from one flask or two petri dishes were harvested in 20 ml of HBS buffer (HBS : 155 mM NaCl, 10 mM HEPES, 0.02 % EDTA, pH 7.4) for 5 minutes and gently removed from their container. They were then washed twice in Krebs/Henseleit buffer (KHB : 118 mM NaCl, 11.7 mM glucose, 10.0 mM HEPES (free acid), 4.7 mM KCl, 4.2 mM $NaHCO_3$, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, pH 7.4), centrifuged at 500 x g for 2 minutes, and finally resuspended in 5 ml of KHB buffer.

The cells were centrifuged at 500 x g and resuspended in 2 ml of KHB buffer containing 5 μM of Fura 2-AM (acetoxymethylester). The cells were incubated with Fura 2-AM for 30 minutes at room temperature and then washed twice by centrifugation at 500 x g for 2 minutes and finally resuspended in 4 ml of KHB buffer. The next step was to transfer 1 ml of the KHB solution containing the chromaffin cells to a cuvette which was placed in a water bath. A further 2 ml of KHB buffer, which was prewarmed to 37^0C, was added. The cuvette contained a magnetic flea to allow mixing. Having left the cells for 5 minutes to warm up to 37^0C the fluorescence was measured by using a Perkin Elmer luminescence spectrophotometer. Excitation wavelength was set at 340 nm and 380 nm, and emission at 510 nm. The temperature was a constant 37^0C. The $[Ca^{2+}]_i$ was calibrated according to the Fura 2-AM dependent fluorescence signal as shown in the following equation :

$$[Ca^{2+}]_i\,(nM) = K_d \times (R - R_{min}) / (R_{max} - R) \times S_{fb}$$

where R = the fluorescence at the point of measurement, R_{min} the minimum fluorescence obtained by addition of 30 mM EGTA, R_{max} the maximum fluorescence obtained by addition of 1 μM of ionomycin, and S_{fb} the ratio of free/bound Ca^{2+} at

380 nm. The K_d for Fura 2 at 37^0C is 224 nM (Grynkiewicz et al., 1985). Before each experiment the background fluorescence was determined. The background value in autofluorescence for unloaded cells was automatically subtracted from the fluorescent signal by the P.C. connected to the spectrophotometer. Data are expressed as mean $\pm$ S.E.M. followed by the number of determinations (n). Data was analysed statistically by the use of an unpaired Student's t-test, where $p<0.05$ was the accepted level of significance.

2.8 $^{45}CaCl_2$ METHOD FOR PERMEABILISED PLATED CELLS.

Chromaffin cells were cultured on 24 well multiwell plates for 3-7 days before use. All procedures were carried out in a water bath and all solutions used were prewarmed at 37^0C unless otherwise indicated. The culture medium was aspirated and the cells were washed three times with 1 ml of BSS without Ca^{2+} (BSS containing : 125 mM NaCl, 5.4 mM KCl, 16.2 mM $NaHCO_3$, 5.5 mM glucose, 30 mM HEPES, 0.8 mM $MgSO_4$, 1.0 mM NaH_2P0_4, pH to 7.4 with 5M NaOH). The BSS solution was removed and the cells were incubated for 8 minutes with 0.25 ml of "cytosol-like" medium (I.C.B.- IntraCellular like Buffer: 120 mM KCl, 20 mM HEPES, 6 mM $MgCl_2$, 5 mM Na-succinate, 2-5 mM ATP, 2mM KH_2PO_4, pH 7.2) containing 0.1 mg/ml of saponin and 1 μCi/ml of $^{45}CaCl_2$. The Ca^{2+} concentration in the I.C.B. buffer was adjusted to the level required for each experiment with the appropriate amount of EGTA. Exposure of cells to 0.1 mg/ml of saponin resulted in permeabilisation of about 90% of the cells according to our tests (see later Table 5.1).

The I.C.B. buffer was then removed and a further 0.5 ml of I.C.B. buffer containing the stimuli was added and left for the appropriate time depending on the nature of the experiment. The supernatant was then removed and the reaction was terminated with addition of 0.5 ml of ice-cold 0.1 mM NaOH. The cells were removed and transferred to scintillation vials and were ready for $^{45}CaCl_2$ radioactivity assay. The $^{45}CaCl_2$ release was expressed as a percentage by comparing the amount of $^{45}CaCl_2$ remaining in the stimulated cells with that contained in the unstimulated cells (control). Data are expressed as mean $\pm$ S.E.M. followed by the number of determinations (n). Data was analysed statistically by the use of an unpaired Student's t-test, where $p<0.05$ was the accepted level of significance.

2.9 SINGLE CELL $[Ca^{2+}]_i$ MEASUREMENT.

Chromaffin cells were cultured on sterile glass 11 x 22 mm coverslips in six- or eight well multiwell plates for 3-7 days. One coverslip for each experiment was removed from the multiwell plates and placed in Krebs/Henseleit buffer (KHB : 118 mM NaCl, 11.7 mM glucose, 10.0 mM HEPES (free acid), 4.7 mM KCl, 4.2 mM $NaHCO_3$, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, pH 7.4). The glass coverslip containing the chromaffin cells was broken into small pieces. Some of the fragments of the coverslip remained in KHB buffer in order to be used for experimental determinations of the background values of autofluorescence. The rest of the cells were incubated with 2 μM Fura-2AM in KHB buffer for 20 minutes at room temperature. The KHB buffer containing the Fura- 2AM was removed and the cells were washed with new KHB buffer. The chromaffin cells remained in the KHB buffer for 30 minutes to ensure that deesterification was completed. The $[Ca^{2+}]_i$ measurements were carried out on individual cells by using standard epifluorescence microscopy (Photon Technology International (P.T.I.) Deltascan system).

2.9.1 The P.T.I. Deltascan System : General Principles.

Cells were optically isolated by means of manual x,y shutters fitted to the photomultiplier housing. Chromaffin cells were constantly perfused (1.0 ml/minute) with KHB buffer prewarmed to 37^0C. Test drugs were added directly onto the cells in the photomultiplier housing area via a multiline perfusion pipette. The excitation wavelengths were set at 340 and 380 nm alternately (frequency of cycling =1 per second). The emitted light was filtered at 510 nm (all the wavelengths were filtered except the 510 nm) so the emission at 510 nm was determined by estimating the ratio of the fluorescence at 340 nm excitation to the fluorescence at 380 nm excitation. The relative changes in the ratio of the 510 nm were monitored continuously by using a Photon Technology International Deltascan system and the signal was hosted by an IBM- compatible computer. Before each experiment the background fluorescence was determined. Several groups (more than eight) of unloaded cells were measured for this purpose and averaged for autosubtraction. The background value in autofluorescence for unloaded cells was subtracted from the fluorescent signal. The Fura- 2AM dependent fluorescence signal can be calibrated in terms of $[Ca^{2+}]_i$ but varies depending upon the calibration method used. Using the fluorescence ratios does not involve those uncertainties. The results obtained with this method were expressed as mean $\pm$ S.E.M. values of at least three experiments performed on cells from different preparations. Data was analysed statistically by the use of an unpaired Student's t-test, where $p<0.05$ was the accepted level of significance.

2.10 MEASUREMENT OF TOTAL INOSITOL PHOSPHATES.

2.10.1 Loading of Chromaffin Cells with [^{3}H]-*Myo*-Inositol.

Chromaffin cells were cultured for 3 days onto Primaria 24 well tissue culture plates. The cells were washed twice with sterile EBSS solution and labeled with 4 μCi/well of [^{3}H]-*myo*-inositol in 0.5 ml of EBSS containing 0.25 mg/100 ml of fungizone, 1 ml/100 ml of 100 x non essential amino acid, 25 mg/100 ml of glutamine, 5 μM of cytosine arabinoside and 5 μM of fluorodeoxyuridine for 48 hours. This time has been shown to be adequate for cellular labeling to reach equilibrium (Plevin and Boarder, 1988).

2.10.2 Experimentation

The experiments were carried out in a water bath and all the solutions used were prewarmed to 37^{0}C unless otherwise indicated. Chromaffin cells were labeled with [^{3}H]-*myo*-inositol and washed twice with 0.5 ml of BSS (w/o Ca^{2+} and Mg^{2+}) which contained 10 mM lithium chloride and 60 μM EGTA. Chromaffin cells were then permeabilised with 0.5 ml of "cytosol-like" buffer (I.C.B) containing 100 μg/ml of saponin for 1 minute. The permeabilised cells were stimulated for 5 or 10 minutes with 0.25 ml of I.C.B containing the test drug. To terminate the reaction 0.5 ml of ice cold methanol (MeOH) was added and from now on all ensuing procedures were carried out on ice.

The method used here is the adaptation (by Rooney and Nahorski, 1986) of the method used by Berridge et al., 1982. According to this method 0.25 ml of H_20 was added to the wells and the contents were scraped off and inserted into scintillation vials. A further 0.5 ml of MeOH was added to the wells to remove any remaining cells and transferred to the scintillation vials. Then, 0.3 ml of H_20 and 1 ml of chloroform was added and the samples were vortexed. From the aqueous upper layer of the

sample (containing the water soluble inositol phosphates) 2 x 0.75 ml were removed into new scintillation vials and 1 ml of an anion exchange Dowex-1 (50:50 mixture, chloride form) and 3 ml of H_20 were added. The mixture produced was vortexed and spun at 3,000 x g for 3 minutes. The supernatant containing the glycerophosphoinositols was poured off and 4 ml of H_2O was added to the remaining Dowex-1 containing the free inositol. The mixture was then vortexed and spun at 3,000 x g for 3 minutes. The supernatant was poured off as before and 1 ml of 0.1 M HCl was added. The new mixture was vortexed and spun at 3,000 x g for 3 minutes. The supernatant obtained containing the inositol phosphates was removed and 0.4 ml of this was transferred along with 5 ml of scintillation fluid to new scintillation vials. The results obtained after the samples were counted were expressed as mean $\pm$ S.E.M of d.p.m in inositol phosphates. Triplicate determinations were used for each representative experiment.

2.11 PROTEIN DETERMINATION.

2.11.1 Bradford Method.

The method described by Bradford (1976) was used to determine protein concentration. Firstly Bradford reagent was prepared by dissolving 100 mg of Coomasie Brill Blue G-250 in 50 ml of 95% ethanol and 100 ml of 85% (w/v) phosphoric acid. The final volume was made up to 1 l (litre) with H_2O and then filtered through a 42 ashless filter.

A 20 μl of sample was diluted with H_2O to make up a final volume of 1 ml. From this 0.25 ml was removed and 1.75 ml of H_2O and 1 ml of Bradford Reagent was added to make up a final volume of 2 ml. A set of BSA standard solutions (i.e. 0, 2.5, 5, 10, 15, 20, 25 mg/ml of protein) was prepared and protein concentrations determined spectrophotometrically at 595 nm. The calibration curve produced by the standard set of BSA solutions enabled us to determine the protein content of our own solution.

CHAPTER 3:

STIMULUS-SECRETION COUPLING IN BOVINE ADRENAL CHROMAFFIN CELLS.

3.1 INTRODUCTION

First evidence for the role of calcium in stimulus-secretion coupling in the adrenal medulla came from work done by Douglas and Rubin in 1961, where they showed that nicotinic stimulation of secretion was dependent on the presence of extracellular calcium. The presence of a nicotinic receptor was later indicated on the adrenal medulla plasma membranes (Wilson and Kirschner, 1977). Stimulation of the nicotinic acetylcholine receptor in chromaffin cells results in the opening of the voltage sensitive calcium channels and calcium entry through the nicotinic receptor channel and the VSCCs (Holz et al., 1982; Kilpatrick et al., 1982). The involvement of calcium was shown in permeabilised chromaffin cells where elevated cytosolic calcium (micromolar levels) stimulated secretion in the absence of external stimuli (Knight and Baker, 1982; Dunn and Holz, 1983).

As previously discussed activation of a large number of receptors linked to Ins(1,4,5)P_3 production e.g. angiotensin II, bradykinin, histamine, muscarinic and prostaglandin E_2, have been shown to stimulate secretion in chromaffin cells. They also mobilise Ca^{2+} from different intracellular stores. Histamine has been also shown to stimulate Ca^{2+} release from both Ins(1,4,5)P_3-sensitive and caffeine-sensitive stores (Stauderman and Murawsky, 1991), whereas angiotensin II (Zimlichman et al, 1987), bradykinin (Plevin and Boarder, 1988; Plevin et al., 1990) and muscarinic receptors (Cheek and Burgoyne, 1985; Kao and Schneider, 1985; Xu et al., 1992) are linked to production of inositol phosphates. These agonists resulted in variable catecholamine response with histamine the most potent among them.

It has also been reported that depletion of the Ins(1,4,5)P_3-sensitive store using the Ca^{2+}-ATPase inhibitor thapsigargin could evoke a small secretory response in chromaffin cells (Cheek and Thastrup, 1989). Other reports have shown that caffeine elicited a small catecholamine release from chromaffin cells (Cheek et al., 1990) which was inhibited by ryanodine (Teraoka et al., 1991), indicating a role for the intracellular calcium stores in secretion. Furthermore, caffeine evoked catecholamine secretion

from other species of chromaffin cells (Nakazato et al., 1992; Sorimachi et al., 1992; Xu and Forsberg, 1993). Ryanodine receptors mediate depolarisation activation (CICR) and a large number of reports have proposed that the ryanodine-sensitive Ca^{2+} store can act as a Ca^{2+} source (Lipscombe et al., 1988; Friel and Tsien, 1992a and b) or sink (Neher and Augustine, 1992; Barry and Cheek, 1994) after depolarisation-induced Ca^{2+} entry. The role of the ryanodine-sensitive Ca^{2+} store appears to be depended on the filling state of the store (Friel and Tsien, 1992a).

Previous studies in chromaffin cells (Plevin and Owen, 1988) on the effects of various bradykinin analogues such as Met-Lys- bradykinin (this analogue has a higher affinity than bradykinin at B_1 receptors and lower one for B_2), Des-arg^9-bradykinin (a B_1 receptor specific agonist), and Thi^{5,8},D-phen^7- bradykinin (a B_2 receptor specific antagonist) suggested that bradykinin evoked catecholamine secretion via the mediation of the B_2 subtype receptor (the properties of these analogues were described by Regoli and Barabe, 1980; Vavrek and Stewart, 1985). This was concluded because a) Des-arg^9- bradykinin was not able to elicit catecholamine release, b) Thi^{5,8},D-phen^7-bradykinin inhibited bradykinin induced catecholamine secretion and c) the EC_{50} for Met-Lys- bradykinin was higher than that of bradykinin. Evidence was also found suggesting that the B_2 receptor is coupled to phospholipase C (PLC).

Bradykinin stimulated secretion is dependent on the presence of extracellular calcium (O' Sullivan and Burgoyne, 1989). Studies on single chromaffin cells loaded with fura-2AM demonstrated that bradykinin stimulation of chromaffin cells resulted in a $[Ca^{2+}]_i$ elevation which was localised within an internal region of the store, where nicotinic or high K^+ challenge resulted in a rapid and transient $[Ca^{2+}]_i$ rise beneath the plasma membrane (O'Sullivan et al., 1989). This data suggested that the initial rise in $[Ca^{2+}]_i$ levels in response to bradykinin was due to calcium mobilisation from an internal store, and calcium elevation in response to nicotine or K^+ was due to calcium entry through the VSCCs. Their evidence also indicated that this calcium entry caused Ca^{2+} mobilisation from internal stores. However, at the same time, other reports (Kim

and Westhead, 1989) indicated that bradykinin evoked catecholamine secretion was abolished in the absence of extracellular calcium (less than 100 nM).

Depolarisation is the major trigger for exocytosis. However, there are recent reports suggesting that Ins(1,4,5)P_3 induced Ca^{2+} release from intracellular stores can stimulate regulated exocytosis. In chromaffin cells (Augustine and Neher, 1992), and SH-SY5Y cells (Vaughan et al., 1993; McDonald et al., 1994; Atcheson et al., 1994) activation of G-protein linked receptors has been shown to stimulate exocytosis which is partially independent of extracellular Ca^{2+}, implying a role for intracellular stored Ca^{2+} in the catecholamine secretion process.

This Chapter will deal with the role of Ca^{2+} in secretion. Two different stimuli which evoke catecholamines secretion were used for this purpose. Nicotine, a depolarising stimuli which is known to stimulate Ca^{2+} entry through the voltage sensitive channel, and bradykinin, which is linked to the inositol phosphate cycle (for a review see Miller, 1987) and stimulates Ca^{2+} release from internal stores. $[Ca^{2+}]_i$ buffered solutions were involved in order to assess the effect of extracellular and cytosolic $[Ca^{2+}]_i$ levels on the bradykinin and nicotine evoked secretion.

3.2 RESULTS.

Experiments in this Chapter were performed on chromaffin cells prepared as described in Chapter 2, section 4. Catecholamine release was measured as described in Chapter 2 section 5. In the experiments where the effect of calcium was studied the solutions were buffered in the appropriate calcium concentrations. Solutions were prepared with BSS without any added calcium. In order to prevent any calcium leaching from glass, plastic beakers were used. In this study 5 μM Quin-2 and up to 10 μM of EGTA were used to avoid problems with high EGTA containing buffers which could cause calcium leaching from the cells and thus depletion of intracellular stores. The calcium concentrations of the buffers was determined on a Perkin Elmer fluorescence spectrophotometer. Additions of EGTA (5-10 μM) or $CaCl_2$ (1 μM) were made to achieve the desirable concentration.

Previous reports from this laboratory have demonstrated that bradykinin, histamine and angiotensin II stimulate inositol phospholipid breakdown in chromaffin cells (Plevin and Boarder, 1988). Bradykinin was also shown to stimulate catecholamine release from bovine adrenal chromaffin cells in a dose dependent manner (Owen et al., 1989b). Maximal release was obtained with 100 nM bradykinin with an EC_{50} of 1.86 ± 0.02 nM. Time course of the bradykinin response, in the presence of 1.8 mM extracellular calcium, indicated that bradykinin was able to stimulate noradrenaline release within the first 10 seconds which increases rapidly for the first 3 minutes and results in maximal response at about 3 minutes, followed by a much slower prolonged rise.

Noradrenaline and adrenaline release, in the presence of 1.8 mM calcium, in response to nicotine and bradykinin is shown in Fig. 3.1. Release was measured over a three minute period and a maximally effective concentration of 30 μM nicotine evoked a large increase in catecholamine release up to 10-11 fold of the controls levels (25.36% ± 0.93 and 17.49% ± 1.00 of noradrenaline and adrenaline, respectively, expressed as a percentage of cell content). Maximally effective concentration of 100

nM bradykinin elicited a 4-5 fold increase in noradrenaline over control levels and a smaller but significant increase in adrenaline (9.94% $\pm$ 0.27 and 4.06% $\pm$ 0.24, respectively). The bradykinin response was 39.19% $\pm$ 1.74 (mean $\pm$ S.E.M., n=5) of the noradrenaline response obtained with nicotine (see table 3.1).

As discussed earlier catecholamine release in response to agonists is dependent on extracellular calcium entry. The effect of different Ca^{2+} concentrations on the bradykinin and nicotinic responses were examined. Fig. 3.2 shows the effect of various Ca^{2+} levels on adrenaline and noradrenaline release evoked by nicotine (30 μM). Nicotine evoked catecholamine release at 30 μM calcium. Maximal response was obtained in the presence of 500 μM calcium. In the presence of 10 μM $[Ca^{2+}]_i$ nicotine was not able to evoke catecholamine release.

The dependency of catecholamine release in response to bradykinin in the presence of extracellular Ca^{2+} was also examined. The effects of various Ca^{2+} concentrations on 100 nM bradykinin elicited noradrenaline release is illustrated in Fig. 3.3. Calcium concentration of 500 μM resulted in 69.97% $\pm$ 1.47 (mean $\pm$ S.E.M., n=4) of maximum response. Basal noradrenaline and adrenaline release was measured at various Ca^{2+} concentrations and was not found to significantly change (n=8).

The effect of different $[Ca^{2+}]_i$ levels on bradykinin and nicotine stimulated noradrenaline and adrenaline release is compared in Fig. 3.4 which demonstrates that both nicotinic and bradykinin response were dependent on the presence of extracellular calcium. Nicotinic response was abolished in the absence of external calcium (less than 100 nM), whereas that of bradykinin was diminished, although a small but significant part of the response was independent of calcium. Removal of extracellular calcium completely inhibited the nicotine response indicating that the Ca^{2+} gradient across the membrane had been removed. In the same conditions bradykinin evoked 3.04% $\pm$ 0.22 of the total cell content or 26.29% $\pm$ 0.85 (or 17.82% $\pm$ 1.73 when control release was subtracted, mean $\pm$ S.E.M., n=4) of the total catecholamine release evoked in the presence of 1.8 mM Ca^{2+}.

The use of permeabilised chromaffin cells was also involved in this study in order to further examine the role of extracellular calcium. Chromaffin cells were permeabilised with Pneumolysin (a thiol-activated toxin of *Streptococcus pneumoniae*, Saunders et al., 1989) for 20 minutes and the effect of low calcium levels in stimulating catecholamines release was examined. Pneumolysin (500 ng/ml) resulted in time dependent release (see Fig. 3.5) and maximal response was obtained in the presence of 1.8 mM $[Ca^{2+}]_i$. Addition of 1.8 mM of Ca^{2+} on chromaffin cells, which had been permeabilised with Pneumolysin for twenty minutes, evoked release of 25% of total cell catecholamine content.

Fig. 3.6 illustrates that low calcium levels can stimulate catecholamine release in permeabilised cells. There was no significant difference between secretion stimulated by 50 nM (n=1) or 150 nM (n=3) when compared with control (1mM EGTA). Catecholamine release was detected when calcium concentration was 500 nM and 1 μ M (n=3). At 500 nM and 1 μM calcium noradrenaline release was 2.54% $\pm$ 0.38 and 3.36% $\pm$ 0.42, respectively (basal release subtracted, mean $\pm$ S.E.M., n=3). The release was small (9.15% $\pm$ 0.42 and 9.97% $\pm$ 0.48 of cell content compared with 25% in the presence of 1.8 mM Ca^{2+}, see Fig. 3.5), but significantly above basal. It is surprising that there is no increase between 500 nM and 1 μM $[Ca^{2+}]_i$, when 1.8 mM $[Ca^{2+}]_i$ resulted in a much larger release.

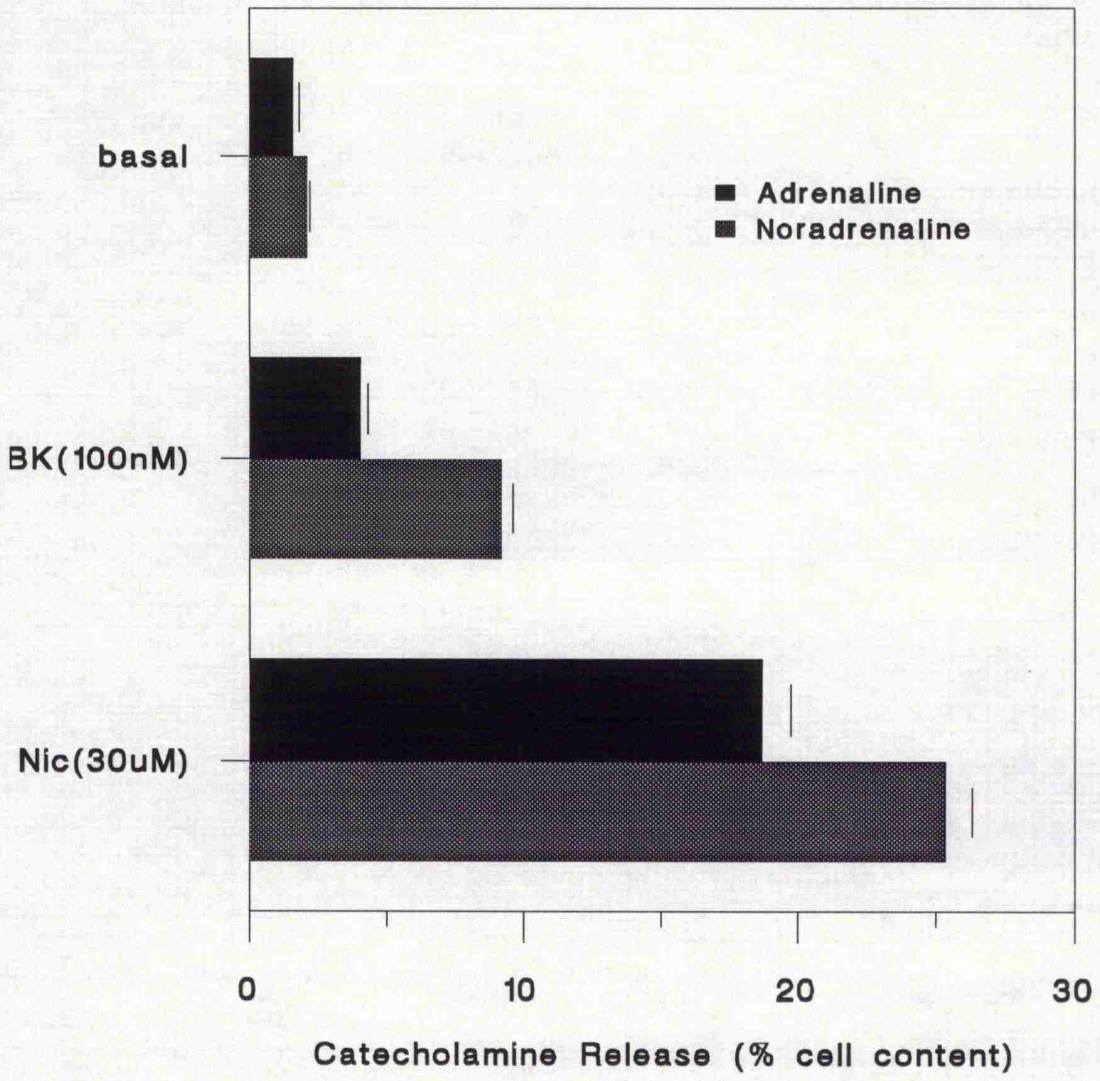


Fig. 3.1. Bradykinin (BK) and nicotine (Nic) stimulated catecholamine release. Maximally effective concentrations of BK (100 nM) and nicotine (30 μM) were used. Release of noradrenaline and adrenaline in response to three minutes stimulation in the presence of 1.8 mM extracellular Ca^{2+}. Data are mean ± S.E.M. (n=5). Release was significantly different from control ($P<0.001$).

Table 3.1. Effect of Bradykinin and nicotine on catecholamine release from bovine adrenal chromaffin cells.

Agonist	**Noradrenaline Release**	**Adrenaline Release**
	% of cell content	**% of cell content**
Bradykinin (100 nM)	9.94 ± 0.27	4.06 ± 0.24
Nicotine (30 μM)	25.36 ± 0.93	17.49 ± 1.00
	% of nicotinic response	% of nicotinic response
Bradykinin	39.19 ± 1.74	23.21 ± 0.24
	control subtracted	control subtracted
Bradykinin (100 nM)	7.95 ± 0.29	2.01 ± 0.35
Nicotine (30 μM)	23.15 ± 0.93	15.89 ± 1.01
	% of nicotinic response	% of nicotinic response
Bradykinin	34.34 ± 1.26	12.64 ± 0.34

Results were measured as adrenaline/ noradrenaline release of total cell content and as percentage release when controls subtracted. Average control release was for adrenaline 2.06 ± 0.23 and 1.58 ± 0.17 and for noradrenaline 1.99 ± 0.11 and 1.59 ± 0.21 for bradykinin and nicotine respectively. Calcium was present at 1.8 mM and the cells were stimulated for 3 minutes. Results are mean ± S.E.M. ($P<0.001$ significantly different from control) of five experiments each determined in quadruplicates or triplicates (n=5).

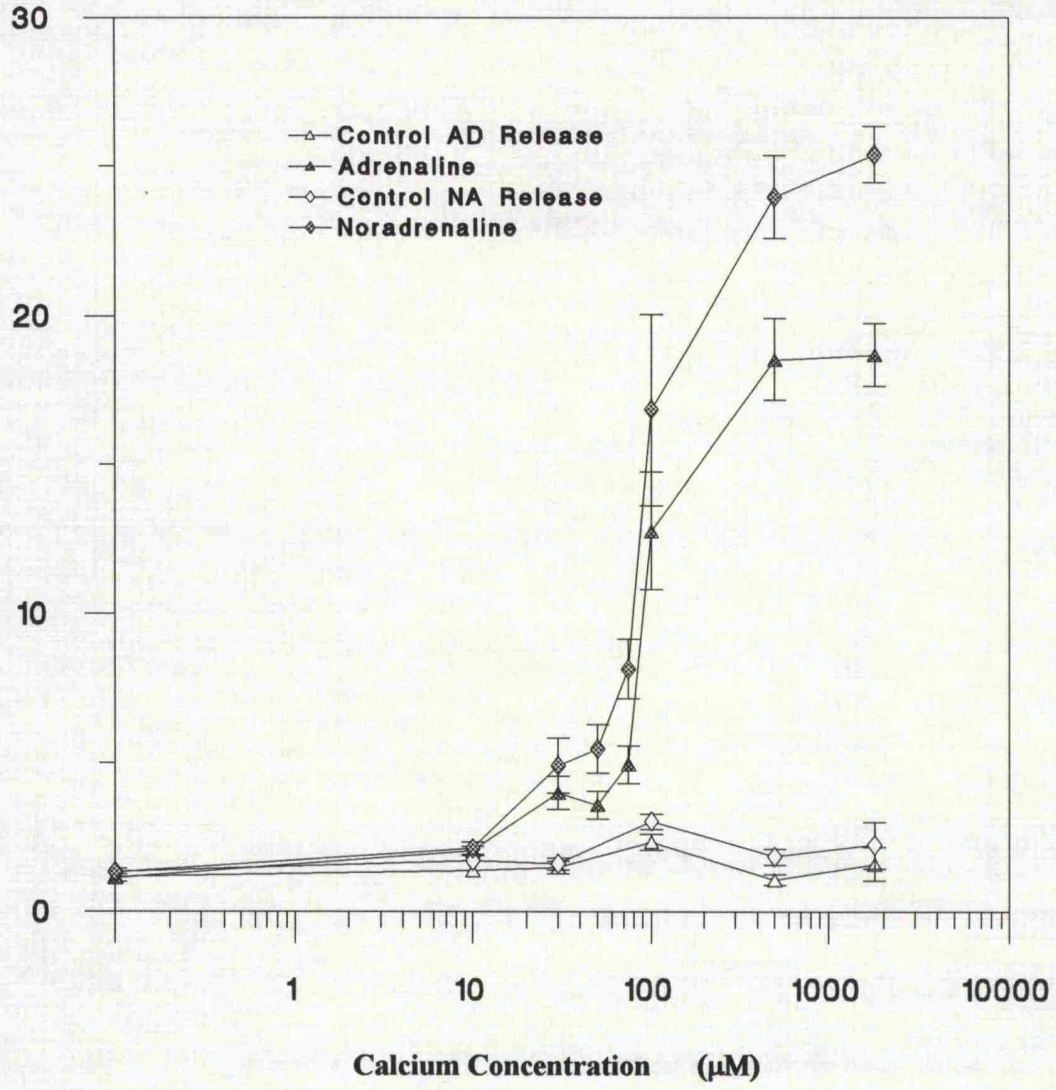


Fig. 3.2. Calcium dependency of nicotine stimulated catecholamine release. Chromaffin cells were washed twice in BSS buffered in increasing concentrations of calcium before being stimulated with nicotine (30 μM) in the same calcium concentration for 3 minutes. Release is expressed as percentage of total cell content (AD: Adrenaline, NA: Noradrenaline) and results are mean ± S.E.M. (each value represents a mean of at least three experiments, $n \geq 3$). Nicotine release at 30 μM was significantly different from control ($P < 0.05$).

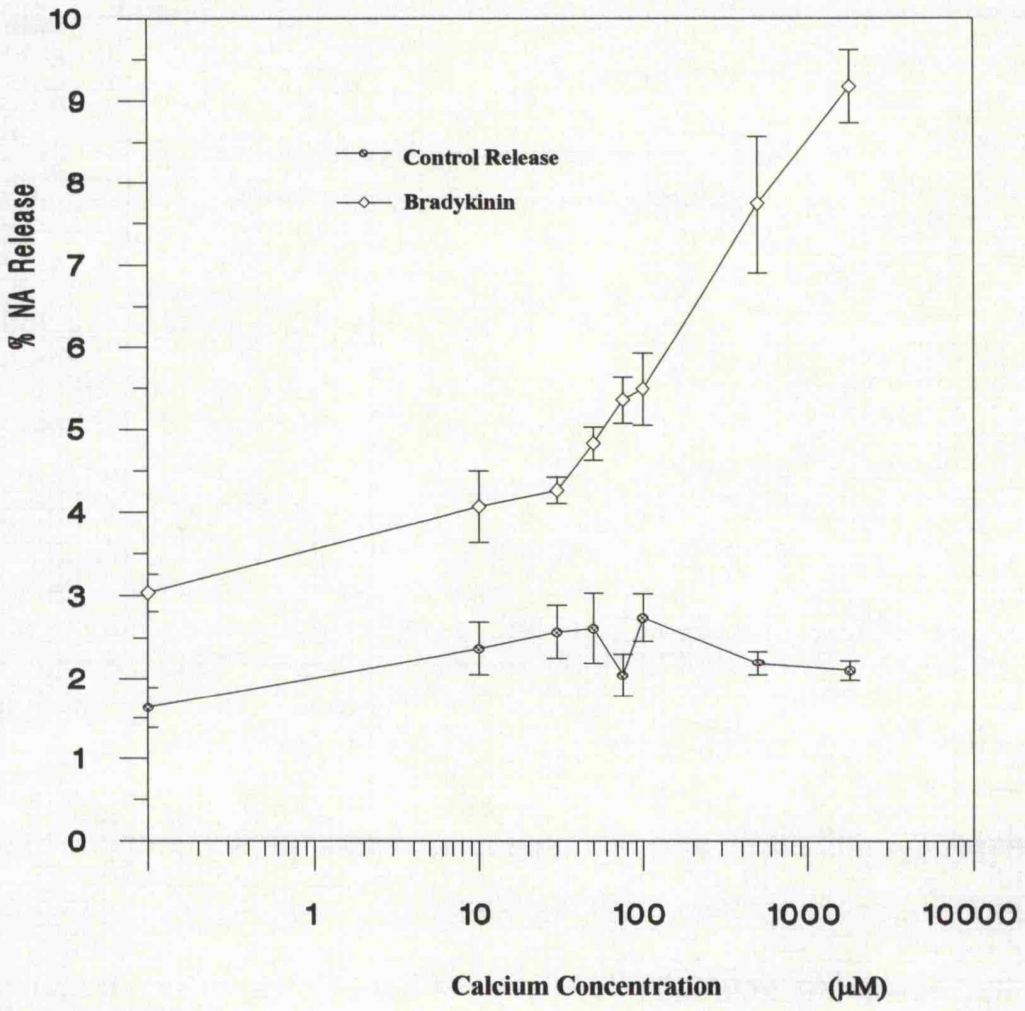


Fig. 3.3. Calcium dependency of bradykinin stimulated noradrenaline release. Chromaffin cells were washed twice in BSS buffered in increasing concentrations of calcium before being stimulated with maximal dose of bradykinin (100 nM) in the same calcium concentration for 3 minutes. Release is expressed as a percentage of total cell content and results are mean ± S.E.M. (each value represents a mean of at least three experiments determined in triplicate, $n \geq 3$, $P<0.05$). The bradykinin stimulated release seen in the presence of 1 mM EGTA is significant ($P<0.001$). Control release did not significantly change over the calcium concentration used here.

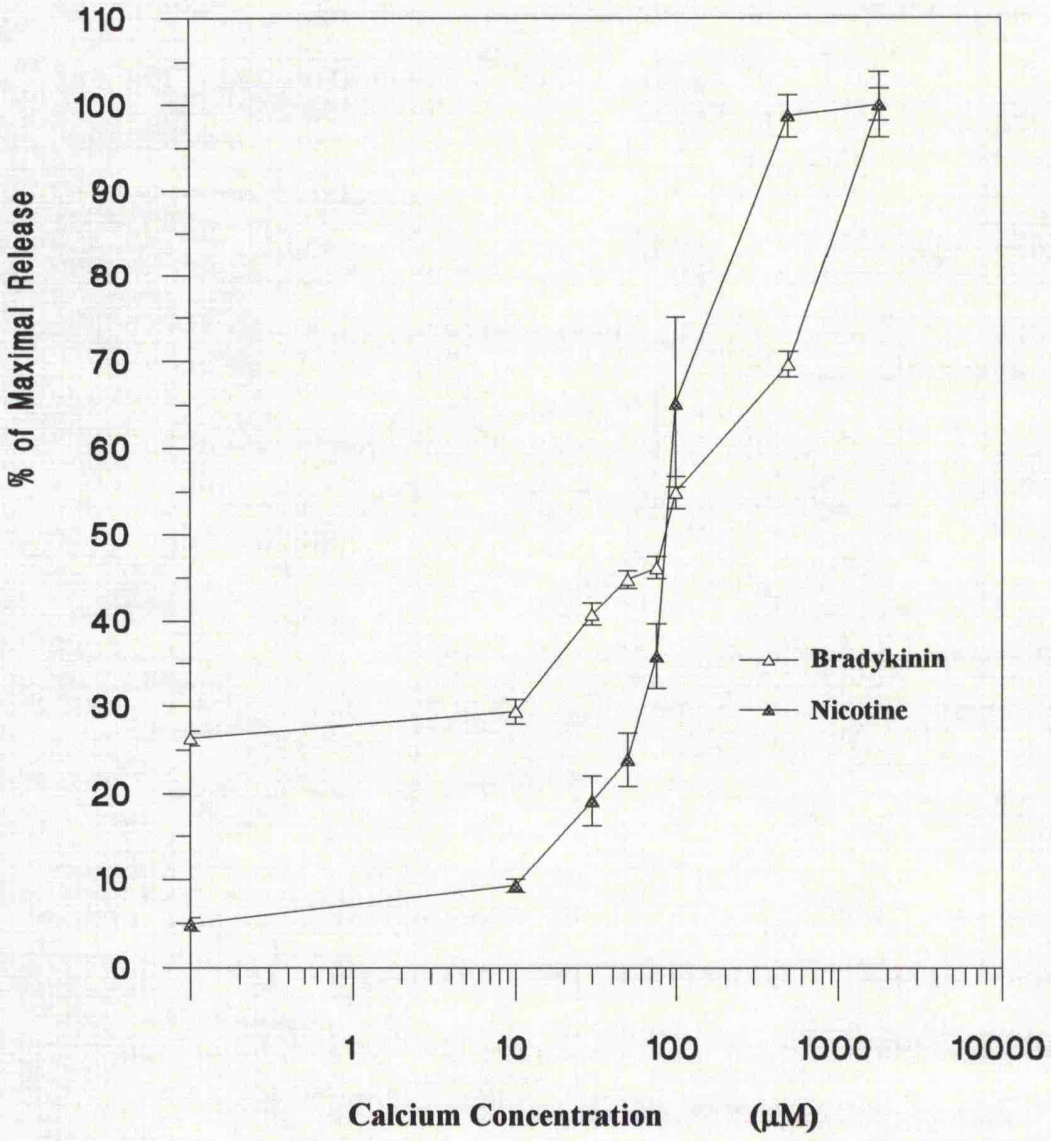


Fig. 3.4. Comparison of calcium dependency of nicotine (30 μM) and bradykinin (100 nM) stimulated noradrenaline release. Chromaffin cells were stimulated with maximal dose of nicotine and bradykinin for three minutes. Results are expressed as percentage release and are mean ± S.E.M. (n=3), each determined in triplicate.

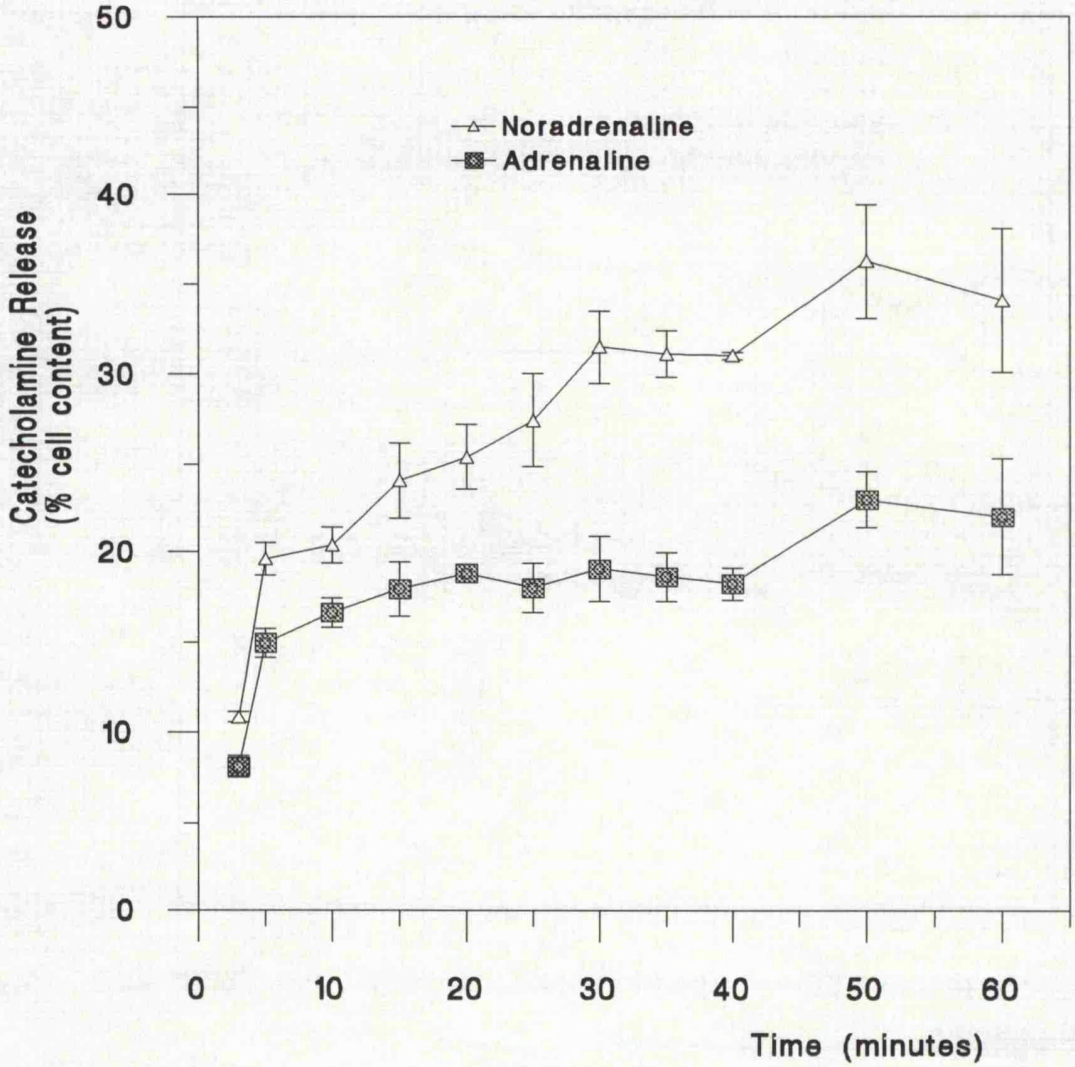


Fig. 3.5. Time course of Pneumolysin permeabilisation on catecholamine release from chromaffin cells. Cells in multiwells were washed twice in BSS and permeabilised with Pneumolysin (500 ng/ml) for 20 minutes. Release is expressed as a percentage of total cell content and results are mean ± S.E.M. of one experiment determined in triplicate. Calcium was present at 1.8 mM.

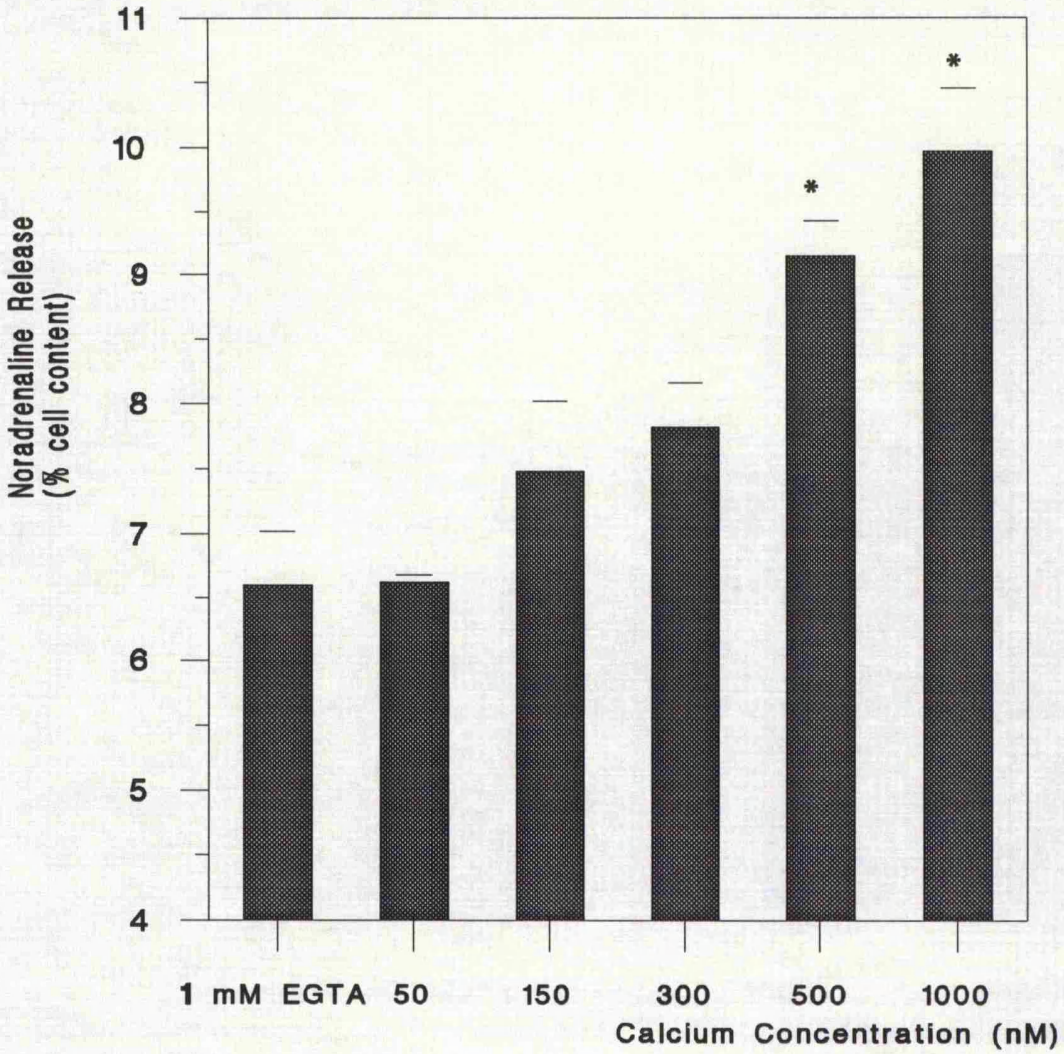


Fig. 3.6. Calcium stimulation of noradrenaline release in chromaffin cells. Cells in multiwells were washed twice in BSS buffered in increasing calcium concentrations and permeabilised with Pneumolysin (500 ng/ml) for 20 minutes. Release is expressed as a percentage of total cell content and results are mean ± S.E.M. of three experiments each determined in triplicate, except release at 50 nM which is from one experiment. Significant differences from average control release (1 mM EGTA) are shown (*P<0.001).

3.3 DISCUSSION

Secretion in response to depolarising stimuli such as nicotine and K^+ has been widely studied in chromaffin cells (see Burgoyne, 1984; and 1991). A very rapid influx of calcium across the plasma membrane, leading to an extremely large calcium gradient in the cell is believed to be the most important source for triggering the exocytotic process.

It has been also shown that nicotine stimulates Ca^{2+} entry across the chromaffin cell plasma membrane (Burgoyne, 1991). In this study nicotine, in the presence of 1.8 mM Ca^{2+}, evoked a large increase in catecholamine release. Results presented here clearly demonstrate that nicotinic response was abolished when extracellular calcium was buffered below 100 nM, indicating that Ca^{2+} entry is the signal which triggers secretion in response to nicotine.

In addition to depolarising stimuli like K^+ and nicotine other non-cholinergic agonists, like the kinin nonapeptide bradykinin, have been shown to stimulate catecholamine release in chromaffin cells. Bradykinin receptors have been reported to be linked to phosphoinositide metabolism. Previous reports from our laboratory have shown that bradykinin produced a large accumulation of inositol phospholipids (linear for at least 60 minutes) with an EC_{50} of 0.5 nM, quite consistent with the EC_{50} of 1.86 nM that stimulates secretion (Plevin and Boarder, 1988; Owen et al., 1989b). Bradykinin stimulation also increased $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ production which were dependent on the presence of extracellular calcium (Challiss et al., 1991).

It is also generally accepted that Ca^{2+} enters the cell in response to bradykinin (O'Sullivan and Burgoyne, 1989; O'Sullivan et al., 1989). Although bradykinin has been shown to stimulate Ca^{2+} entry through voltage sensitive calcium channels in other cells (NG108-15, Brown and Higashida, 1988), use of DHP calcium channel blockers, such as nitrendipine, indicate that bradykinin evoked secretion from chromaffin cells was not inhibited, indicating that Ca^{2+} entry was not through L-type channels in chromaffin cells (Owen et al., 1989b). Bradykinin stimulated Ca^{2+} entry was also

found not to be via N-type voltage-sensitive calcium channels indicating that an alternative route is present in chromaffin cells.

Results from this study showed that bradykinin stimulated release was calcium dependent but in a different manner from that of nicotine (see Fig. 3.4). Noradrenaline release in response to bradykinin was reduced at low $[Ca^{2+}]_i$ levels but clearly not abolished. A 26.29% $\pm$ 0.85 (or 17.82% $\pm$ 1.73 when the control release was subtracted) of the bradykinin release was independent of the presence of extracellular calcium.

Results presented here agree with those reported by O'Sullivan and Burgoyne, 1989, where they showed that bradykinin response is decreased in the presence of 1mM EGTA to 30% of that in the presence of calcium. The bradykinin evoked secretion in the absence of extracellular calcium was 8 times smaller than that evoked by nicotine in agreement with data published recently (Augustine and Neher, 1992) comparing bradykinin response with depolarisation stimulation in chromaffin cells. However, other reports showed that the bradykinin response is completely abolished in the absence of extracellular calcium (Kim and Westhead, 1989). In my study the bradykinin response in the absence of extracellular calcium was 3.04%$\pm$ 0.22 of total cell content (1.47%$\pm$ 0.17 when control release was subtracted), which probably suggests that previous reports may not have been able to detect the relatively small amounts of catecholamine secretion.

Data presented by O'Sullivan and Burgoyne, 1989; and O'Sullivan et al., 1989, showed that challenge of chromaffin cells with bradykinin mobilised calcium from intracellular stores, and a part of the evoked secretion was independent of extracellular calcium. This evidence may suggest that bradykinin stimulation of chromaffin cells divides into two phases of Ca^{2+} rise, an initial phase which is due to calcium mobilisation from intracellular stores and a second phase due to calcium entry. Their data also suggested that nicotine and high K^+ stimulation resulted in Ca^{2+} entry through the VSCCs followed by an intracellular $[Ca^{2+}]_i$ rise in the region where the internal Ca^{2+} stores are located, indicating that Ca^{2+} influx via the VSCCs resulted in

Ca^{2+} mobilisation from internal stores. Similar results have been obtained in cerebellar granule cells, where a major component of the NMDA and high K^+ evoked sustained phase was related to Ca^{2+} release from intracellular stores, since it was reduced after prior addition of thapsigargin and ryanodine (Simpson et al., 1993; but also see Irving et al., 1992).

A large number of studies have demonstrated that depolarisation can stimulate Ca^{2+} mobilisation from intracellular caffeine-sensitive Ca^{2+} stores in neuronal cells (Kuba, 1980; Lipscombe et al., 1988; Thayer et al., 1988a; Friel and Tsien, 1992a and b). Caffeine has also been shown to stimulate catecholamine secretion in bovine adrenal chromaffin cells in the presence of extracellular calcium (Cheek et al., 1990), which was blocked by prior addition of ryanodine (Teraoka et al., 1991). Use of ryanodine and caffeine to deplete the caffeine-sensitive Ca^{2+} store did not affect the response to nicotine and K^+, but the filling state of the store had an effect on the shape of the Ca^{2+} transient in response to depolarising stimuli and agonists linked to Ins(1,4,5)P_3 production (Cheek et al., 1990; Stauderman and Murawsky, 1991; Stauderman et al., 1991). Experiments in PC12 cells (Barry and Cheek, 1994) indicated that inhibition of the CICR resulted in reduced ATP responses without affecting the responses to depolarising stimuli (high K^+). Depletion of the caffeine-sensitive stores enhanced the recovery of the K^+ -induced rise in Ca^{2+}, indicating that the role of the caffeine-sensitive Ca^{2+} store is to remove calcium from the cytosol acting as a calcium sink in response to depolarising stimuli.

Studies on sympathetic neurones (Friel and Tsien, 1992a; Hua et al., 1993) demonstrated that depletion of the caffeine stores decreased K^+ depolarisation responses. Their data also suggested that Ca^{2+} entry in response to depolarising stimuli rapidly recovered in the presence of caffeine due to Ca^{2+} uptake in the caffeine-sensitive Ca^{2+} stores, indicating that in this cell type caffeine can act as sink and a source of calcium. The role of caffeine-sensitive stores in these cells was dependent on their filling state, acting as a source of Ca^{2+} when they were relatively full or a sink when they were depleted. The role of CICR mechanism and the caffeine-sensitive Ca^{2+}

store in response to depolarisation differs between various cell types and experimental approaches. The magnitude of depolarisation is also important since there is a large Ca^{2+} entry through a limited number of channels in response to small depolarising stimuli. Since Ca^{2+} entry is localised it may be difficult for the cytoplasmic buffers to buffer the calcium (Neher and Augustine, 1992). Large depolarising stimuli would result in the opening of a greater number of Ca^{2+} channels with each channel allowing a smaller more diffuse influx (Chad and Eckert, 1984; Simon and Llinas, 1985; see also Batty and Cheek, 1994). Under these circumstances it would easier for the cells to buffer the calcium.

The difference between the bradykinin and nicotinic stimulation could be explained using the theory proposed by Llinas et al., 1992. This theory maintains that the clustering of voltage sensitive Ca^{2+} channels results in 'microdomains' of $[Ca^{2+}]_i$ reaching high levels of 200-300 μM in the giant squid synapse. At the same time in chromaffin cells (Augustine and Neher, 1992; Neher and Augustine, 1992) it was reported that Ca^{2+} concentration at the secretory sites could be in excess of 10 μM, in a matter of microseconds, during depolarisation of the cell. Recent data shows that Ca^{2+}- activated proteins involved in vesicle fusion have a relatively low affinity for calcium (Brose at el., 1992). Therefore, these localised high levels of calcium, which cannot be detected with standard Ca^{2+} concentration techniques, are in excess of those determined as an average cytosolic signal.

The effect of calcium in permeabilised chromaffin cells is studied in this Chapter. Calcium at 1.8 mM evoked release of 25% of total cell catecholamine content. Very low calcium concentrations of between 500 nM and 1 μM appeared to stimulate secretion, which was reduced by approximately 80%. The amount of catecholamine release was small but significantly above basal. A logical explanation would be that Pneumolysin in these experiments permeabilised either: chromaffin granules, or intracellular stores. However, Pneumolysin is believed to permeabilise the plasma membrane without affecting the intracellular stores or granules. Secretion with 500 nM and 1 μM $[Ca^{2+}]_i$ is consistent with catecholamine release in response to

bradykinin in the absence of extracellular calcium indicating that these $[Ca^{2+}]_i$ levels were sufficient to stimulate part of the total secretion obtained in the presence of 1.8 mM $[Ca^{2+}]_i$. Co-localisation of intracellular stores with the exocytotic machinery may be responsible for the secretion in the absence of extracellular Ca^{2+}.

Experiments in single adrenal chromaffin cells demonstrated that histamine stimulation resulted in an initial rise in $[Ca^{2+}]_i$ due to Ca^{2+} mobilisation from intracellular stores followed by Ca^{2+} influx over the entire surface of the cell where angiotensin II stimulated Ca^{2+} release from internal stores was followed by localised Ca^{2+} entry restricted at the same region of the cell (Cheek et al., 1993a). The Ca^{2+} influx in response to these two agonists was not through VSCCs. For angiotensin II there is evidence suggesting that calcium entry is due to receptor activation since Ca^{2+} entry was not abolished after prior depletion of the intracellular Ca^{2+} stores with ionomycin (Stauderman and Pruss, 1989). Findings from this data indicate that different spatial localisation of agonist induced Ca^{2+} entry probably reflects differences in their potency to stimulate secretion.

It is still not known how bradykinin, histamine, and angiotensin II stimulate Ca^{2+} entry. In this study nicotine evoked secretion appeared to be different to that of bradykinin (Fig. 3.4). Different $[Ca^{2+}]_i$ levels were required to stimulate secretion in response to these two agonists. Nicotine stimulation results in Ca^{2+} entry through the VSCCs (Holz et al., 1982; Kilpatrick et al., 1982) and through the nicotinic receptor (Boarder et al., 1987), whereas the bradykinin response was not affected by L- and N-type channels blockers (Owen et al., 1989a and b). Although voltage-sensitive-calcium- channels have been widely studied and well characterised, little is known about calcium channels linked to intracellular Ca^{2+} stores. Recently an ion channel (Icrac: calcium release- activated current) has been proposed to activate Ca^{2+} entry after depletion of intracellular stores in rat mast cells (Hoth and Penner, 1992). Their findings showed that the Icrac current is not inhibited by heparin, is voltage independent and highly selective for Ca^{2+}. Differences in the $[Ca^{2+}]_i$ levels required to stimulate secretion in response to nicotine and bradykinin as illustrated in Fig. 3.4

probably reflect differences in the characteristics of the calcium channels responsible for calcium entry mediated by those two agonists (for more discussion on Ca^{2+} entry see Chapter 6).

Data presented in this Chapter suggested that bradykinin release of noradrenaline was partially independent on the presence of extracellular calcium. Similar evidence has been shown in SH-SY5Y cells (Vaughan et al., 1993; Atchenson et al., 1994; Purkiss et al., 1995). There is also evidence that pretreatment with thapsigargin abolishes the bradykinin evoked secretion, indicating that Ca^{2+} mobilisation is responsible for the bradykinin extracellular Ca^{2+} independent secretion (Purkiss et al., 1995).

Although the major part of the exocytotic process is due to Ca^{2+} entry across the chromaffin cell plasma membrane, intracellular Ca^{2+} stores also appear to generate a Ca^{2+} signal leading to exocytosis. Further work is required to investigate the localisation of the intracellular calcium stores in relation to the secretion vesicles and to establish their possible role in secretion. The nature of the intracellular Ca^{2+} stores in bovine adrenal chromaffin cells will be investigated in subsequent Chapters.

CHAPTER 4:

CHARACTERISATION OF INOSITOL-1,4,5-TRISPHOSPHATE-SENSITIVE AND CAFFEINE-SENSITIVE Ca^{2+} STORES IN ELECTRICALLY PERMEABILISED BOVINE ADRENAL CHROMAFFIN CELLS.

4.1 INTRODUCTION

Agonists linked to Ins(1,4,5)P_3 production can stimulate secretion in chromaffin cells in the absence of extracellular calcium, suggesting that intracellular Ca^{2+} stores may involved in exocytosis in chromaffin cells (O'Sullivan and Burgoyne, 1989; Augustine and Neher, 1992). It is now well documented that many cells possess Ins(1,4,5)P_3-sensitive Ca^{2+} stores (Thayer et al., 1988b; Henzi and MacDermott, 1992; Berridge, 1993a). Studies on digitonin-permeabilized bovine adrenal chromaffin cells (Stoehr et al., 1986), using the fluorescent probe quin-2 provided first evidence for Ins(1,4,5)P_3-sensitive stores. This was later verified by Kao in 1988 and others (Cheek et al., 1991; Robinson and Burgoyne, 1991a).

Many cells possess Ins(1,4,5)P_3-sensitive and caffeine-sensitive Ca^{2+} stores, as a significant number of studies have now revealed (Thayer et al., 1988a and b; Malgaroli et al., 1990; Zacchetti et al., 1991). The presence of caffeine-sensitive Ca^{2+} stores indicates the presence of a ryanodine receptor and the mechanism of Ca^{2+}-induced- Ca^{2+} release (CICR) in sarcoplasmic reticulum of skeletal and cardiac muscle (Fabiato, 1983; Endo, 1977). Recent studies demonstrated the existence of ryanodine receptors in neurons (Walton et al., 1991; McPherson et al., 1991), indicating that a CICR mechanism may be present in neuronal cells (Lipscombe et al., 1988; Berridge, 1993a and b).

Calcium released from the Ins(1,4,5)P_3-sensitive Ca^{2+} store can activate Ca^{2+} mobilisation from the ryanodine-sensitive Ca^{2+} stores (CICR), or from the Ins(1,4,5)P_3-sensitive Ca^{2+} store by the co-agonist effect of Ca^{2+} on the Ins(1,4,5)P_3 receptor (Bezprozvanny et al., 1991). Various theories proposed to explain Ca^{2+} oscillations and Ca^{2+} waves in cells assume that the Ins(1,4,5)P_3 and the caffeine-sensitive Ca^{2+} stores are distinct. However, the evidence for this assumption is inconclusive. Differences in Ca^{2+} store distribution in different cell types could contribute to the confusion.

The existence of caffeine-sensitive stores is also well documented in bovine adrenal chromaffin cells (Burgoyne et al., 1989b; Cheek et al., 1990) as discussed in Chapter 1. The caffeine-sensitive Ca^{2+} stores are diffusely distributed throughout the chromaffin cell (Burgoyne et al., 1989b; Cheek et al, 1990), whereas the Ins(1,4,5)P_3-sensitive store has been shown to be a spatially localised store in close proximity to the nucleus, as studies using video digital imaging of single fura-2 loaded chromaffin cells have shown (O'Sullivan et al., 1989).

The relationship between the Ins(1,4,5)P_3- and the caffeine-sensitive stores is not yet clear. Data from studies using fura-2 to monitor $[Ca^{2+}]_i$ changes in digitonin-permeabilised adrenal chromaffin cells suggests that chromaffin cells possess two distinct non overlapping Ca^{2+} stores sensitive either to Ins(1,4,5)P_3 or caffeine (Robinson and Burgoyne, 1991a). They also suggest that the Ins(1,4,5)P_3- and caffeine-sensitive stores possess different Ca^{2+}-ATPases as video imaging studies of fura-2 loaded cells have shown (Burgoyne et al., 1989b). Other studies using fura-2 AM to monitor intracellular cytosolic Ca^{2+} changes in intact bovine adrenal chromaffin cells show that the Ins(1,4,5)P_3- and the caffeine-sensitive stores overlap and that the Ins(1,4,5)P_3-sensitive store is smaller than the caffeine-sensitive store (Liu et al., 1991). This report also suggests a major role for the caffeine-sensitive stores in buffering cytosolic Ca^{2+}. Studies in single bovine adrenal chromaffin cells loaded with fura-2 and α-toxin-permeabilised cells indicated that three stores were present, one Ins(1,4,5)P_3-sensitive, the second caffeine-sensitive and a third sensitive to both (Stauderman et al., 1991).

There is therefore enough evidence to suggest that adrenal chromaffin cells possess two non-mitochondrial Ca^{2+} stores, one sensitive to Ins(1,4,5)P_3 and the other sensitive to caffeine. In order to characterise these two pools and to examine the relationship between the Ins(1,4,5)P_3-sensitive and caffeine-sensitive intracellular Ca^{2+} stores a Ca^{2+}- selective electrode was used to monitor cytosolic Ca^{2+} changes (Chapter 2, section 6.1).

The main problem when trying to characterise the intracellular Ca^{2+} stores is that there are only a few pharmacological compounds that can be used to differentiate between different Ca^{2+} stores. Other problems like membrane permeability can be overcome by use of permeabilised cells. Several chemical compounds have been used in an attempt to either stimulate or inhibit Ca^{2+} release from different Ca^{2+} stores. Two compounds that proved to be of use are Ins(1,4,5)PS_3 and Ins(4,5)SP_2. The Ins(1,4,5)P_3 analogue Ins(1,4,5)PS_3 has been shown to be a very useful pharmacological tool since it is a full agonist, as far as Ca^{2+} release is concerned, and appears to be resistant to metabolism by 5- phosphatase and 3- kinase (Safrany et al., 1991b). The introduction of phosphorothioate groups at the 4- and 5- position has been reported to markedly diminish recognition by Ins(1,4,5)P_3 5-phosphatase and also to reduce recognition by 3-kinase (S. Safrany, 1993-PhD Thesis, University of Leicester; Safrany et al., 1994), indicating that Ins(4,5)SP_2 is also a poorly metabolised synthetic analogue of Ins(1,4,5)P_3.

A series of control experiments were carried out using SH-SY5Y human neuroblastoma cells in order to test the method, since the Ins(1,4,5)P_3-induced Ca^{2+} release in these cells had already been partially characterised with the use of a Ca^{2+}-selective electrode (Wojcikiewicz et al., 1990).

4.2 RESULTS

In the experiments described in this Chapter electrically permeabilized bovine adrenal chromaffin cells were used (as described in Chapter 2 section 6.1). A Ca^{2+}-selective electrode monitored changes in intracellular $[Ca^{2+}]_i$. In the later experiments the changes in Ca^{2+} levels were recorded by the Ca^{2+}- selective electrode (Clapper and Lee, 1985) were converted to actual amounts of $[Ca^{2+}]_i$ by using quin-2 fluorescence (Wojcikiewicz et al., 1990).

The experiments performed in this Chapter with the use of Ca^{2+}-selective electrode were carried out at a temperature of 20^0 C to slow the formation and metabolism of Ins(1,4,5)P_3. At this temperature Ins(1,4,5)P_3 gave a reproducible increase in Ca^{2+} as seen previously (Stoehr et al., 1986; Kao, 1988).

The electrically permeabilized chromaffin cells accumulate Ca^{2+} in intracellular compartments in the presence of 2.0 mM ATP. After the addition of ATP the levels of $[Ca^{2+}]_i$ were decreased from 552 nM $\pm$ 7.76 to 97.34 nM $\pm$ 3.56 nM (mean $\pm$ S.E.M., n=12, see also Fig. 4.1) indicating Ca^{2+} uptake in the intracellular stores. In this study Ins(1,4,5)P_3 was used at 3 μM, following the work of Kao, 1988 in digitonin permeabilised chromaffin cells, which indicated that 3 μM of Ins(1,4,5)P_3 resulted in maximal response. Ins(1,4,5)P_3 (3 μM) also evoked maximal release in saponin permeabilised chromaffin cells (see Ins(1,4,5)P_3 dose- response in Chapter 5). This dose also resulted in maximal calcium release in permeabilised SH-SH5Y cells as shown by Safrany et al., 1990.

Challenge with Ins(1,4,5)P_3 stimulated a rapid rise to a peak of $[Ca^{2+}]_i$ (87.71 nM $\pm$ 5.83, mean $\pm$ S.E.M, n=7) above basal. The $[Ca^{2+}]_i$ rise in response to Ins(1,4,5)P_3 was transient and $[Ca^{2+}]_i$ levels returned to the baseline within two or three minutes (Fig. 4.1, see also Table 4.1). It appears that Ins(1,4,5)P_3 was rapidly metabolised by 5-phosphatase and 3-kinase (Nahorski, 1988; Shears, 1992) so Ca^{2+} was resequestered into intracellular compartments in the absence of Ins(1,4,5)P_3. The Ins(1,4,5)P_3 evoked Ca^{2+} release was not diminished after sequential additions of

Ins(1,4,5)P_3 suggesting that the Ca^{2+} released was taken up in the Ins(1,4,5)P_3-sensitive Ca^{2+} stores (Fig. 4.2).

The tri-methylxanthine caffeine was used at different concentrations. It has been previously reported (Liu et al., 1991) that 40 mM of caffeine resulted in maximum response in chromaffin cells. Other studies in chromaffin cells used 5 mM (Robinson and Burgoyne, 1991a) or 10 mM (Stauderman et al., 1991). High doses of caffeine have been reported to have other non specific effects such as inhibition of the Ins(1,4,5)P_3 production and possibly function (Missiaen et al., 1992; Toescu et al., 1992), blockage of the Ins(1,4,5)P_3 Ca^{2+} release channel (Parker and Ivorra, 1991; Brown et al., 1992), and inhibition of voltage-sensitive-Ca^{2+} channels (Hughes et al., 1990). In order to avoid the non specific effects of high doses of caffeine on the Ins(1,4,5)P_3-induced Ca^{2+} release caffeine (15 mM) was used in most of the experiments described in this Chapter.

Studies using a Ca^{2+} selective electrode have the disadvantage of not allowing the removal of the compounds used. Caffeine stimulation resulted in a peak Ca^{2+} elevation followed by a lower sustained phase. Caffeine, after prior addition of Ins(1,4,5)P_3 also resulted in a peak followed by a lower sustained phase (see Fig. 4.1 and Fig. 4.3). Since Ins(1,4,5)P_3 in this study was rapidly metabolised and the Ca^{2+} was resequestered in the Ins(1,4,5)P_3-sensitive Ca^{2+} stores without affecting sequential additions of Ins(1,4,5)P_3 most of our experiments were designed to challenge chromaffin cells firstly with Ins(1,4,5)P_3.

Caffeine (15 mM), after prior addition of Ins(1,4,5)P_3 elicited a peak increase of intracellular Ca^{2+} levels of 81 $\pm$ 5.25 nM (mean $\pm$ S.E.M., n=6) followed by a lower sustained phase, indicating that a small part of the Ca^{2+} released was resequestered into caffeine insensitive Ca^{2+} stores; the rest remained giving sustained Ca^{2+} levels above the baseline resulting in a plateau phase. Addition of caffeine (15 mM-20 mM) did not deplete the caffeine-sensitive Ca^{2+} store since as illustrated in Fig. 4.4 subsequent addition of caffeine (20 mM) induced a further Ca^{2+} release.

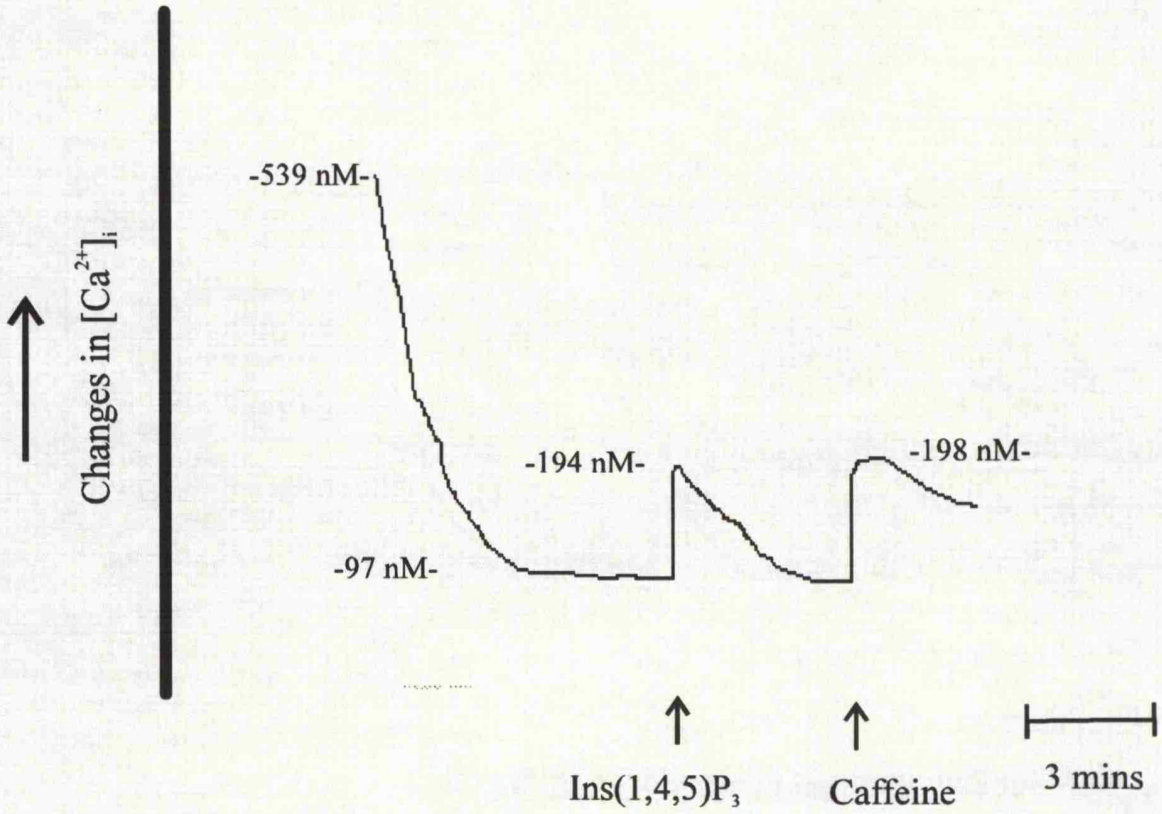


Fig. 4.1. Effect of Ins(1,4,5)P_3 and caffeine on Ca^{2+} release from electrically permeabilised chromaffin cells. Addition of 2 mM ATP resulted in a drop of $[Ca^{2+}]_i$ levels in this experiment from 539 nM to 97 nM indicating that Ca^{2+} was sequestered in intracellular Ca^{2+} stores. Typical responses to Ins(1,4,5)P_3 (3 μM) and caffeine (15 mM) are shown.

Table 4.1. Effect of Ins(1,4,5)P_3 and caffeine on Ca^{2+} release from electrically permeabilised chromaffin cells.

Stimulus	**Changes in intracellular Ca^{2+} levels. $\Delta[Ca^{2+}]_{free}$ in nM.**	**n**
Ins(1,4,5)P_3(3 μM)	87.71 ± 5.83	7
Ins(1,4,5)P_3 (3 μM) after caffeine	41.00 ± 7.81	5
Caffeine(15 mM) after Ins(1,4,5)P_3	81 ± 8.16	6
Caffeine after Ins(4,5)SP_2	Still able to produce Ca^{2+} release	3

The changes in Ca^{2+} in electrically permeabilised chromaffin cells were monitored with a Ca^{2+}-selective electrode. Changes recorded by the Ca^{2+} -selective electrode were converted to changes in the levels of free intracellular calcium. The resting levels of $[Ca^{2+}]_i$ were between 80-100 nM. The Ins(1,4,5)P_3 concentration was the maximal 3 μM and caffeine was used at 15 mM which is not the maximal dose. Results expressed are mean ± S.E.M, n = number of experiments.

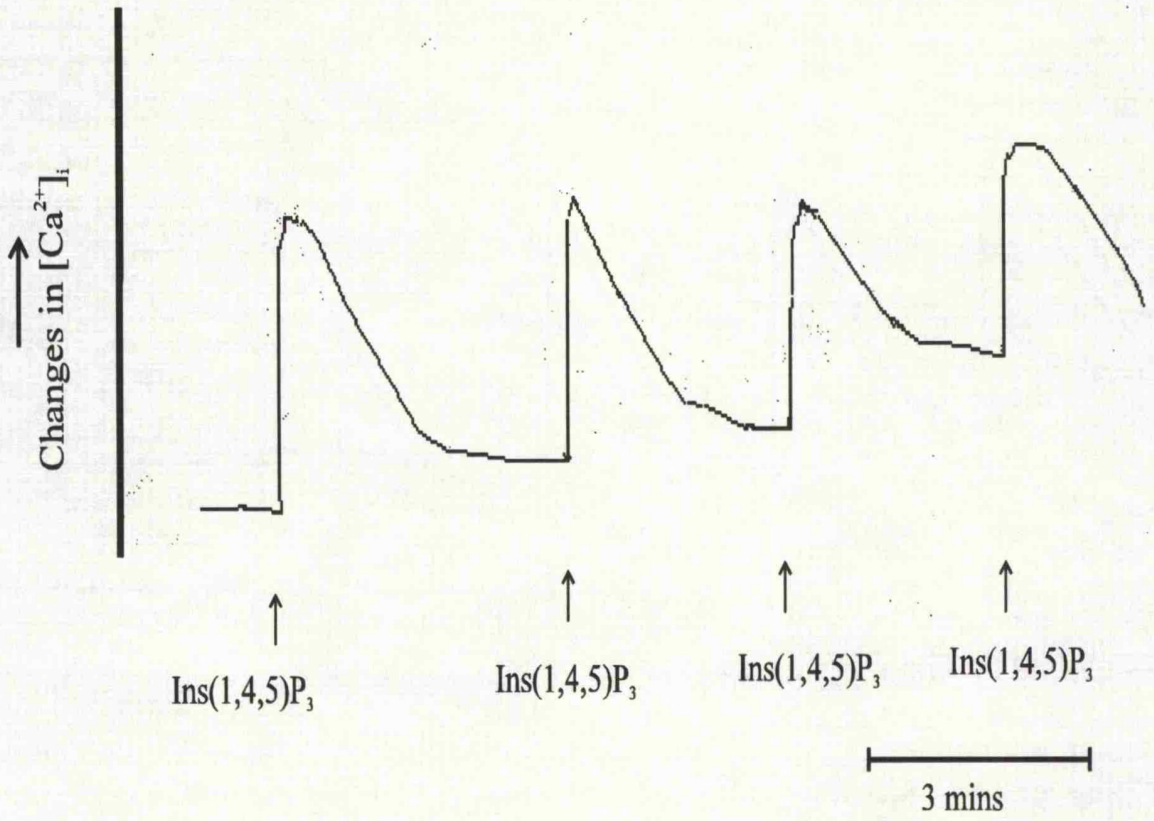


Fig. 4.2. Effect of repetitive additions of Ins(1,4,5)P_3 on Ca^{2+} release from electrically permeabilised chromaffin cells.

$[Ca^{2+}]_i$ levels were around 100 nM after prior addition of 2 mM ATP. Subsequent additions of Ins(1,4,5)P_3 (3 μM) resulted in Ca^{2+} mobilisation from intracellular Ca^{2+} stores. The rise in $[Ca^{2+}]_i$ levels was transient and returned to the baseline within 3 minutes. The trace is representative of 6 experiments. The size of the Ins(1,4,5)P_3 evoked responses were not significantly different over these experiments.

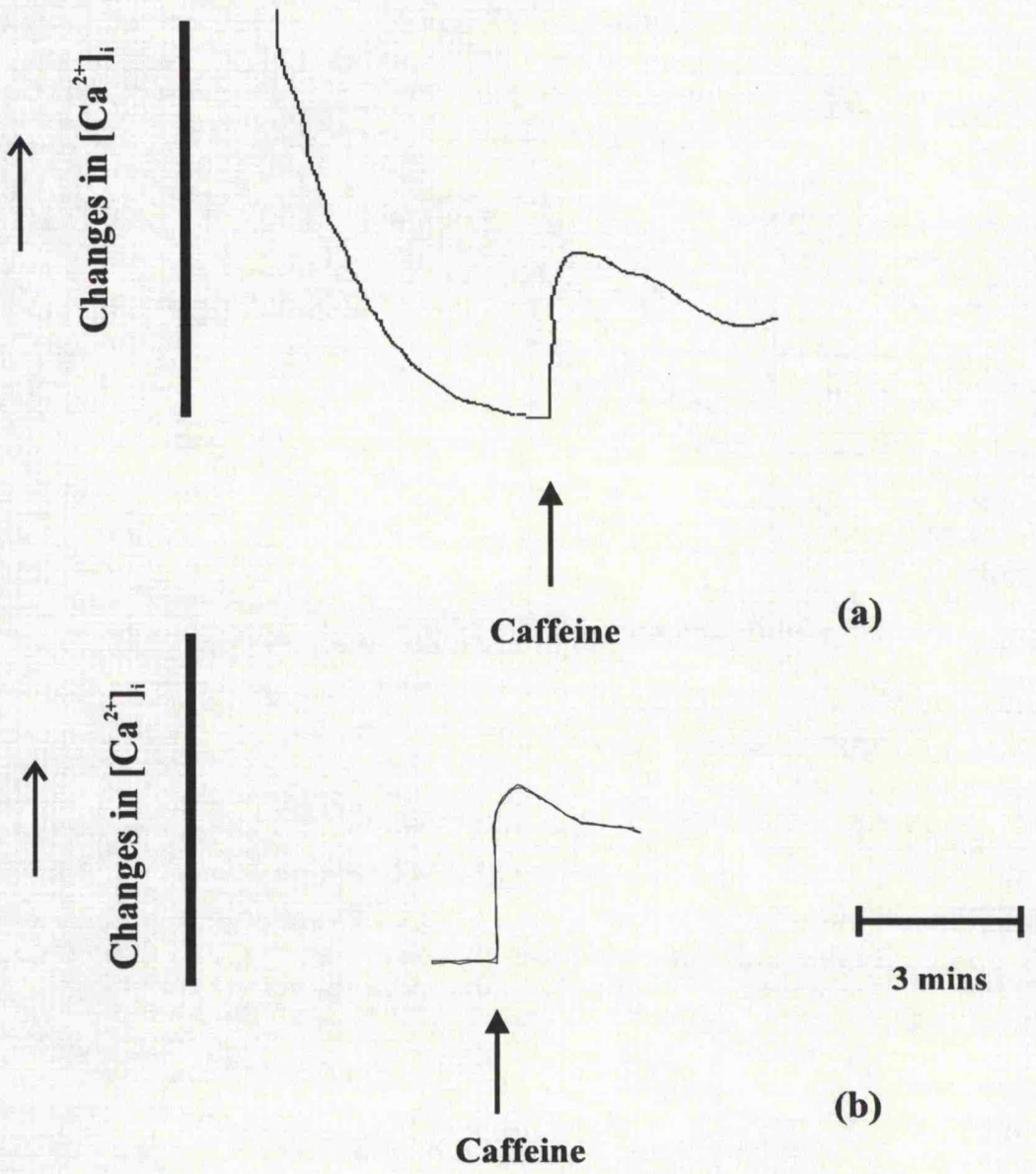


Fig. 4.3. Effect of caffeine on intracellular Ca^{2+} release from electrically permeabilised chromaffin cells. A Ca^{2+}- selective electrode was used to monitor changes in $[Ca^{2+}]_i$. The resting level of $[Ca^{2+}]_i$ was at about 100 nM after the addition of 2.0 mM ATP. Typical responses of permeabilized adrenal chromaffin cells to caffeine (a) and caffeine after prior addition of Ins(1,4,5)P_3 are shown (b). The Ins(1,4,5)P_3 concentration was 3 μM and caffeine was used at 15 mM.

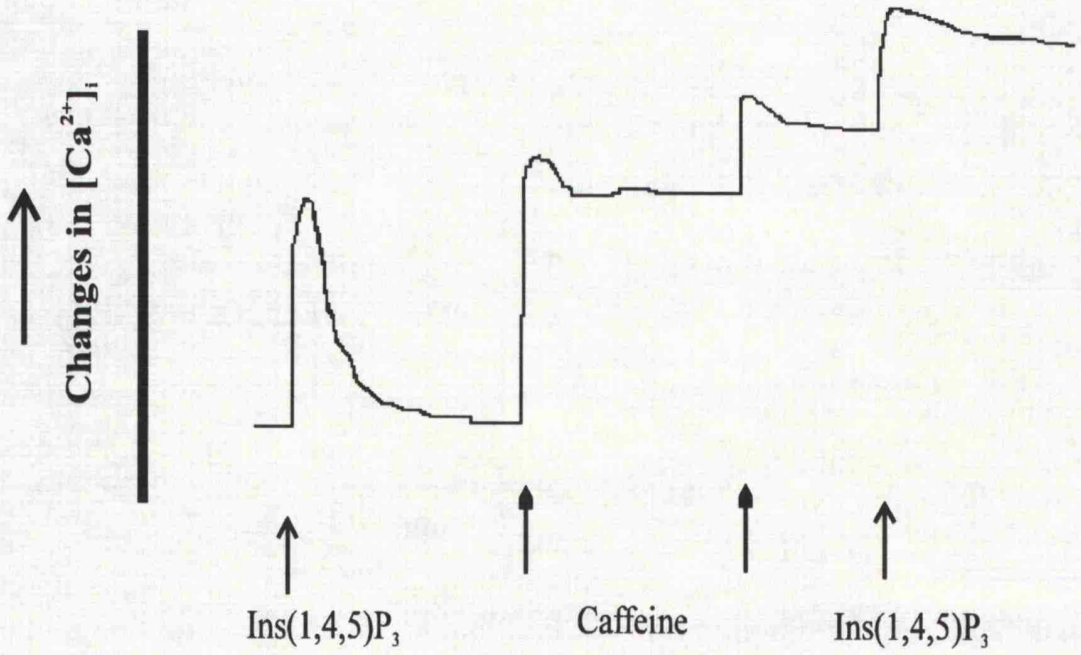


Fig. 4.4. Effect of Ins(1,4,5)P_3 after prior addition of caffeine on intracellular Ca^{2+} release from electrically permeabilised chromaffin cells.

From a resting level of $[Ca^{2+}]_i$ of about 100 nM Ins(1,4,5)P_3 induced a transient Ca^{2+} release. Chromaffin cells were then challenged (twice) with caffeine (20 mM each) which elicited sustained Ca^{2+} release. Subsequent addition of Ins(1,4,5)P_3 evoked Ca^{2+} mobilisation from intracellular Ca^{2+} stores. Ins(1,4,5)P_3 concentration was 3 μM and caffeine concentration was 20 mM. This trace is representative of three similar experiments.

A number of Ca^{2+}- selective electrode experiments were carried out using SH-SY5Y human neuroblastoma cells at the same time in order to compare the results with those obtained from chromaffin cells. These cells were used at a density of 1-2 mg of protein/ml (similar to that of chromaffin cells). Evidence that SH-SY5Y neuroblastoma cells possess, like chromaffin cells, an Ins(1,4,5)P_3-sensitive store were found. Surprisingly, caffeine was also able to release Ca^{2+} from intracellular stores in these cells (see Fig. 4.5a and 4.6). The Ins(1,4,5)P_3- induced Ca^{2+} release was transient falling to the basal levels within 2-3 min. The Ca^{2+} increase, in response to Ins(1,4,5)P_3, was found to be larger (136.2 $\pm$ 9.5, mean $\pm$ S.E.M., n=5) than that in chromaffin cells.

Ins(1,4,5)P_3 stimulated Ca^{2+} release even after prior addition of a high dose of caffeine (40 mM) in chromaffin cells (Fig. 4.4) and SH-SY5Y cells (Fig. 4.5a). In chromaffin cells the Ins(1,4,5)P_3 induced calcium release was significantly reduced ($P<0.001$) after prior addition of caffeine (15 mM). The Ins(1,4,5)P_3 response was reduced by 55% in the presence of caffeine (15 mM) compared to that in the absence (41 nM $\pm$ 7.81 (n=5) and 87.71 nM $\pm$ 5.83 (n=7), respectively, mean $\pm$ S.E.M., see also Table 4.1).

Previous studies on chromaffin cells suggested that elevated Ca^{2+} levels may be responsible for the reduced Ins(1,4,5)P_3 response after prior addition of caffeine (Robinson and Burgoyne, 1991a). Experiments on both SH-SY5Y cells (Fig. 4.5b) and chromaffin cells (4.7) where $CaCl_2$ was added to elevate $[Ca^{2+}]_i$ levels achieved with caffeine (40 mM) were carried out. The Ins(1,4,5)P_3-induced Ca^{2+} release was not reduced with prior addition of $CaCl_2$ (see Fig. 4.5b and 4.7). These findings indicate that Ca^{2+} mobilisation from the caffeine-sensitive store was responsible for the reduced, subsequent responses to Ins(1,4,5)P_3. The effect of Ca^{2+} on Ins(1,4,5)P_3-induced Ca^{2+} release from permeabilised chromaffin cells is also studied in Chapter 5.

In order to further examine the relationship between the Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores the efficacious but non or poorly metabolised analogues Ins(1,4,5)PS_3 and Ins(4,5)PS_2 were used. These analogues are very useful

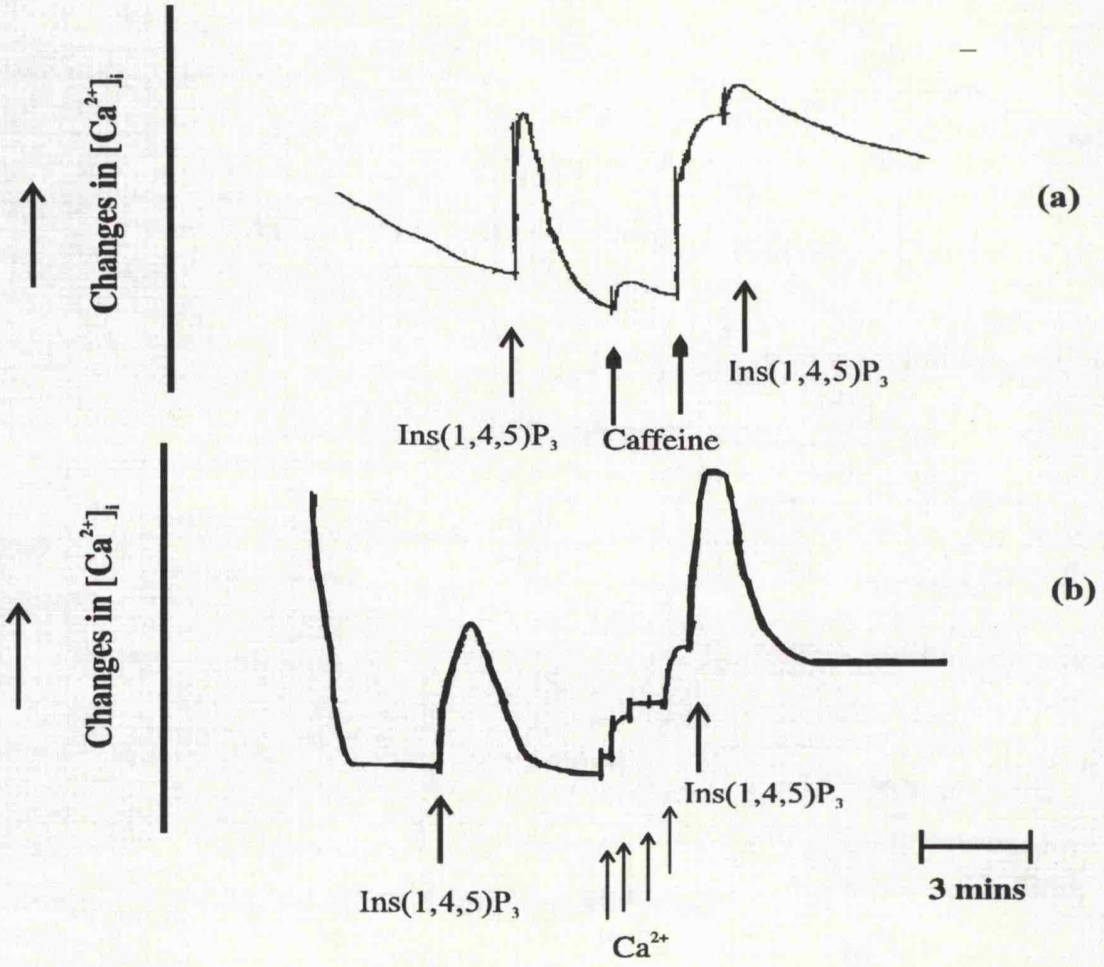


Fig. 4.5. (a) Effect of Ins(1,4,5)P_3 and caffeine on Ca^{2+} release from electrically permeabilised SH-SY5Y cells. Challenge with Ins(1,4,5)P_3 (3 mM) evoked Ca^{2+} release from SH-SY5Y cells. Subsequent additions of caffeine (20 mM + 40 mM) resulted in Ca^{2+} mobilisation from intracellular Ca^{2+} stores. Stimulation with Ins(1,4,5)P_3 after prior additions of caffeine resulted in further Ca^{2+} release.

(b) Effect of $[Ca^{2+}]_i$ levels on Ins(1,4,5)P_3 induced Ca^{2+} release from permeabilised SH-SY5Y cells. Repetitive additions of Ca^{2+} were used to increase the $[Ca^{2+}]_i$ levels. Ins(1,4,5)P_3 concentration was 3 μM. Ca^{2+} was added at 1 μM at a time since it gave the desired free $[Ca^{2+}]_i$ levels.

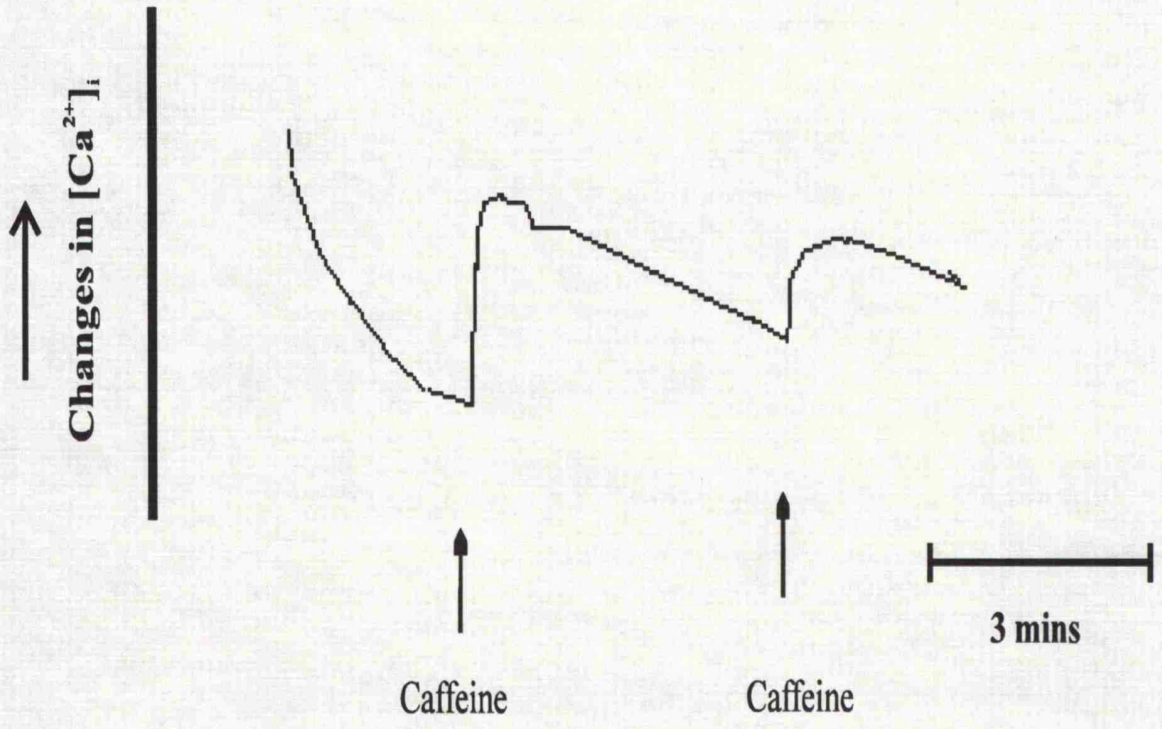


Fig. 4.6. Effect of caffeine on Ca^{2+} release from electrically permeabilised SH-SY5Y cells. Ca^{2+} sequestered in the intracellular Ca^{2+} stores of SH-SY5Y.
Challenge of the permeabilised cells with caffeine (15 mM) evoked Ca^{2+} mobilisation. Subsequent addition of 10 mM caffeine resulted in a further Ca^{2+} release. Caffeine induced Ca^{2+} release was present in all the experiments performed on SH-SY5Y cells (n>5).

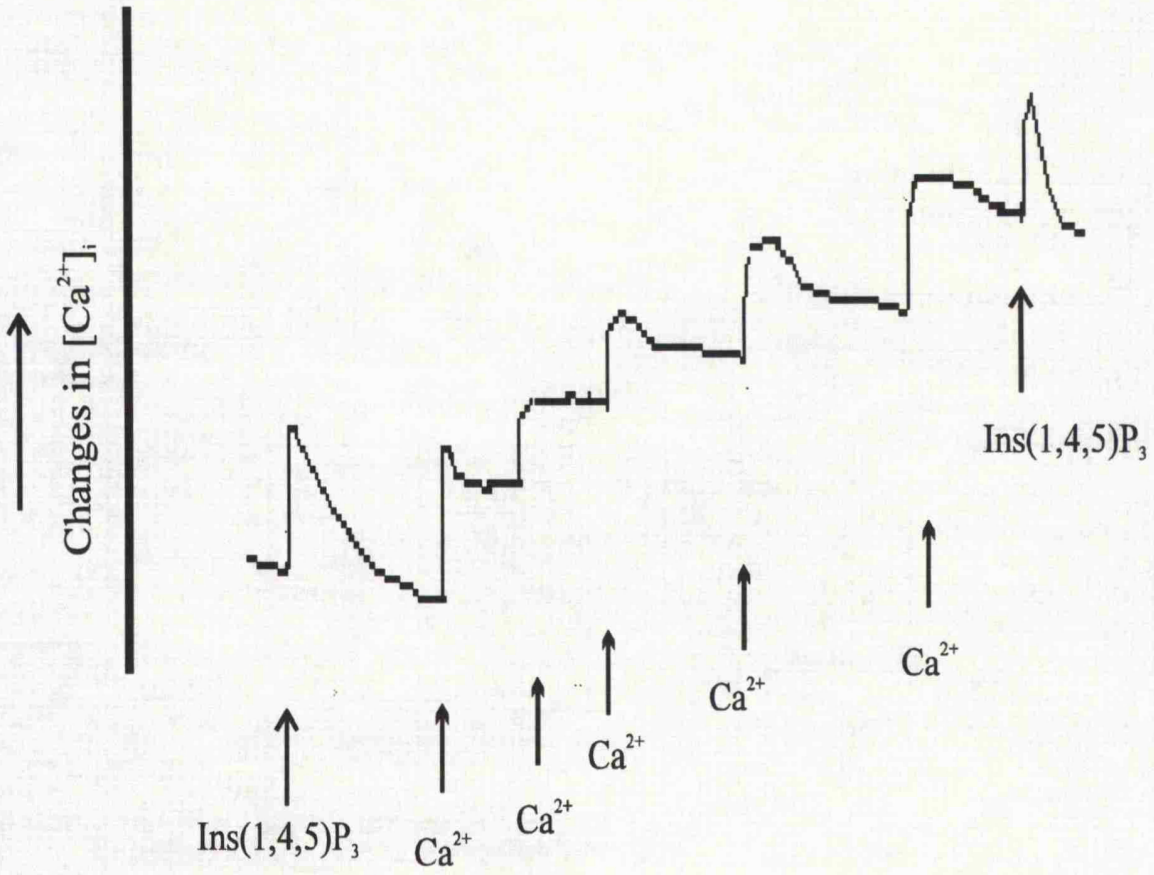


Fig. 4.7. Effect of elevated $[Ca^{2+}]_i$ levels on Ins(1,4,5)P_3 induced Ca^{2+} release from electrically permeabilised chromaffin cells.

Repetitive additions of Ca^{2+} were used to increase the $[Ca^{2+}]_i$ levels. Ins(1,4,5)P_3 concentration was 3 μM. Ca^{2+} was added at 1 μM at a time since it gave the desired free $[Ca^{2+}]_i$ levels. This trace is representative of three similar experiments.

pharmacological tools when attempting to characterise intracellular Ca^{2+} stores. The Ins(1,4,5)PS_3 compound has been shown to be resistant to metabolism by 5-phosphatase and 3-kinase enzymes whilst still being a full agonist with 2.5 μM resulting in half maximal release (Safrany et al., 1991b). In the Ca^{2+}-selective electrode experiments presented in this Chapter, the other compound Ins(4,5)PS_2 appeared to have similar characteristics (Fig. 4.8) to the Ins(1,4,5)PS_3, such as the resistance to metabolism, in agreement with previous studies (S. Safrany, 1993-PhD. Thesis; University of Leicester; Safrany et al., 1994).

The Ins(4,5)PS_2 analogue has been previously shown in SH-SY5Y cells to stimulate Ca^{2+} mobilisation with an EC_{50}=1.3 μM lower than that of Ins(1,4,5)PS_3 (EC_{50}= 2.5 μM) where all phosphate groups have been replaced with phosphorothioate groups (S. Safrany, 1993-PhD Thesis, University of Leicester). The Ins(4,5)PS_2 analogue was found to be a potent inhibitor of the Ins(1,4,5)P_3 5-phosphatase with similar Ki (dissociation constant for an inhibitor-enzyme complex) affinity to Ins(1,4,5)PS_3 analogue, 1.4 μM and 1.7 μM respectively. In the same study (S. Safrany, 1993-PhD Thesis, University of Leicester) Ins(4,5)PS_2 was also found to be a poor substrate for Ins(1,4,5)P_3 3-kinase, although there is no introduction of phosphorothioate group into the 3-position, with a Ki = 46 μM, indicating that the introduction of phosphorothioate group into the 4-position diminished Ins(1,4,5)P_3 3-kinase recognition. The rate of phosphorylation was about 10% of the rate of Ins(1,4,5)P_3.

The poorly metabolised Ins(1,4,5)P_3 analogues Ins(1,4,5)PS_3 and Ins(4,5)PS_2 elicited a sustained Ca^{2+} release as shown in Fig. 4.8 consistent with the resistance of these compounds to metabolic enzymes (Safrany et al., 1991b; S. Safrany, 1993-PhD Thesis, University of Leicester; Safrany et al., 1994). These results also indicate that Ca^{2+} mobilised from the Ins(1,4,5)P_3-sensitive Ca^{2+} store was not re-sequestered into caffeine-sensitive Ca^{2+} stores. Maximal response of Ins(4,5)SP_2 was obtained with 15 μM which depleted the Ca^{2+} content of the Ins(1,4,5)P_3-sensitive Ca^{2+} store, since a further addition of Ins(4,5)PS_2 (10 μM) or Ins(1,4,5)P_3 (3 μM) did not induce any

further Ca^{2+} release in seven similar experiments (Fig. 4.9a illustrates representative experiments). Caffeine (not maximal dose), subsequently added was still able to elicit Ca^{2+} release (n=8), although the Ins(1,4,5)P_3-sensitive store was already depleted (Fig. 4.9b). These data therefore provide strong evidence that chromaffin cells possess a caffeine-sensitive Ca^{2+} store which is not depleted by and hence is not sensitive to Ins(1,4,5)P_3.

The results presented here demonstrate that permeabilised chromaffin cells possess Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores. Partial depletion of the caffeine-sensitive store affected the Ins(1,4,5)P_3 response (see Table 1), indicating that a communication between these two stores may exist. It has been reported that guanosine 5' triphosphate (GTP) may have a role in altering the sensitivity of the Ins(1,4,5)P_3 receptor in streptolysin-O permeabilised bovine adrenal chromaffin cells (Föhr et al., 1991). Their data suggests that the Ins(1,4,5)P_3- induced Ca^{2+} release was diminished after sequential additions of Ins(1,4,5)P_3 and GTP was able to fully recover the sensitivity to Ins(1,4,5)P_3 (Föhr et al., 1991). This possibility was also examined in electrically permeabilised chromaffin cells by using a Ca^{2+} selective electrode. Different concentrations of GTP were used but they did not stimulate Ca^{2+} release or affect the Ins(1,4,5)P_3- induced Ca^{2+} release (n=6) (see Fig. 4.10).

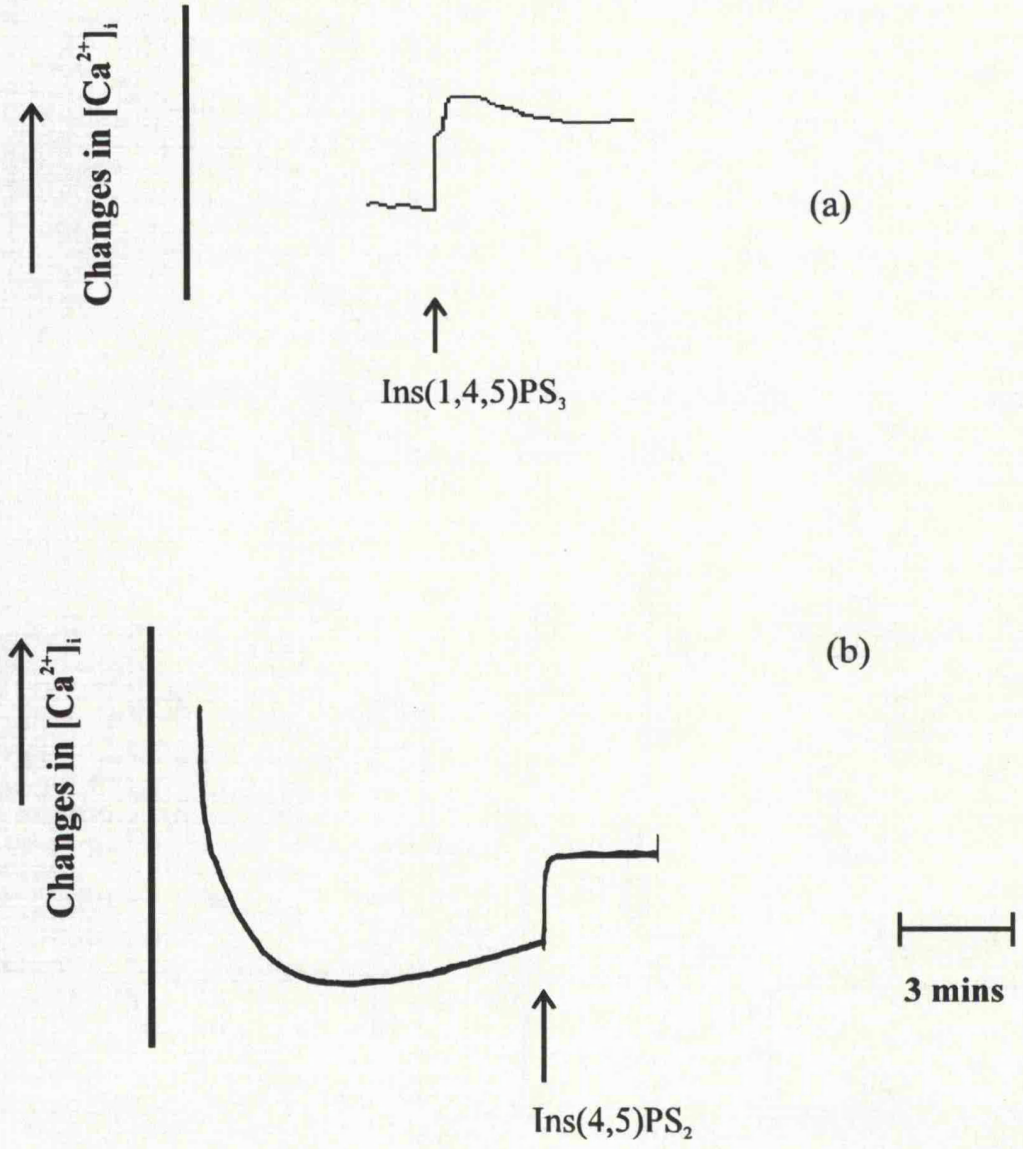


Fig. 4.8. Effect of Ins(1,4,5)PS_3 and Ins(4,5)PS_2 on Ca^{2+} release from electrically permeabilised chromaffin cells. Typical responses to Ins(1,4,5)PS_3 (7 μM) (a) and Ins(4,5)PS_2 (30 μM) (b) are shown. Both of these poorly metabolised analogues resulted in a rapid sustained Ca^{2+} release. The traces presented here are representative of three (a) and six (b) similar experiments.

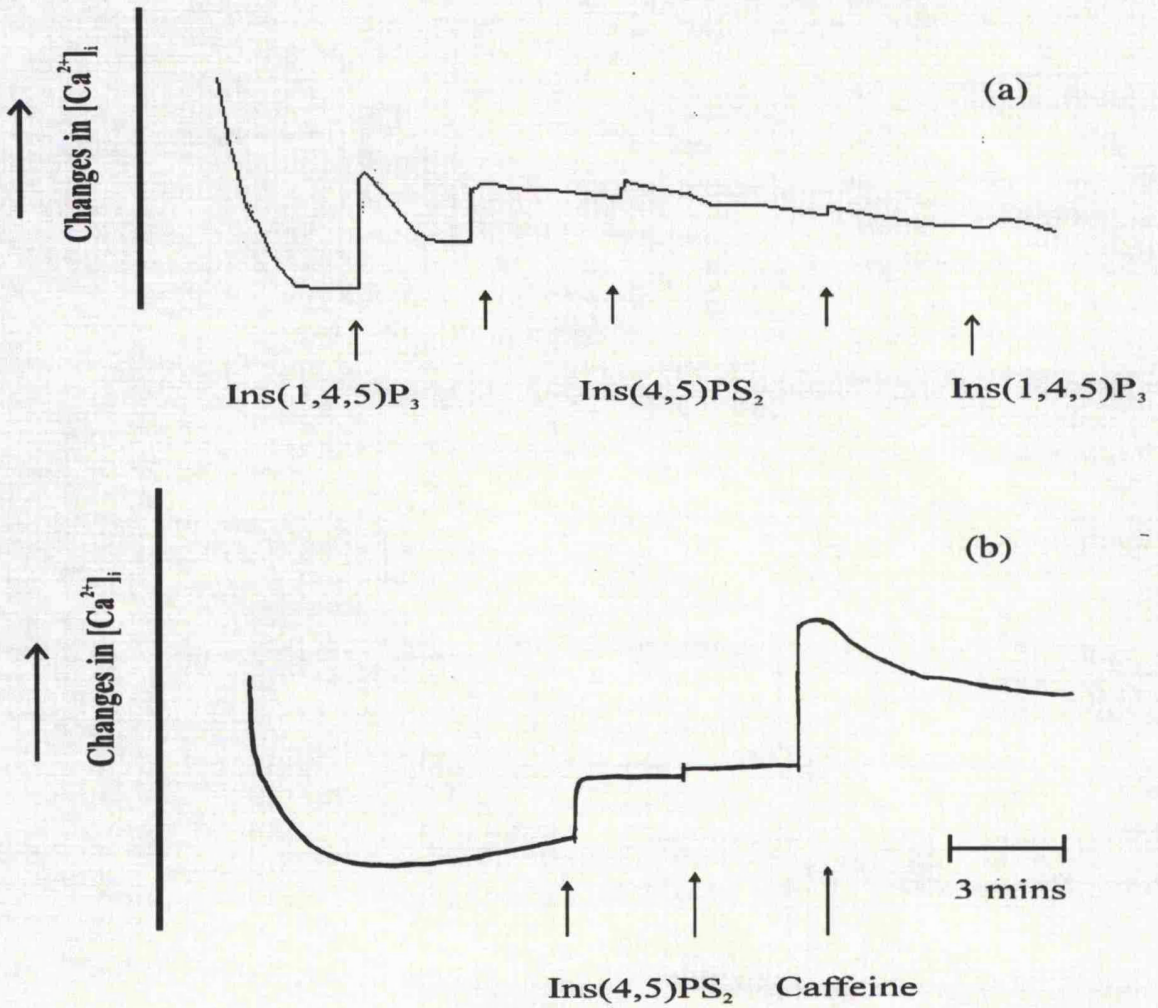


Fig. 4.9. Effect of Ins(1,4,5)P_3 and caffeine-induced Ca^{2+} release from permeabilised chromaffin cells. (a) Three repetitive additions of Ins(4,5)PS_2 (5 μM each) depleted the Ins(1,4,5)P_3-sensitive Ca^{2+} stores since subsequent addition of Ins(1,4,5)P_3 had no effect.

(b) Addition of supramaximal Ins(4,5)PS_2 (30 μM) resulted in a sustained Ca^{2+} release. Subsequent addition of Ins(4,5)PS_2 (10 μM) had no effect. Caffeine (25 mM) was able to stimulate Ca^{2+} release after depletion of the Ins(1,4,5)P_3-sensitive stores. Traces presented here are representative of n $\geq$3 experiments.

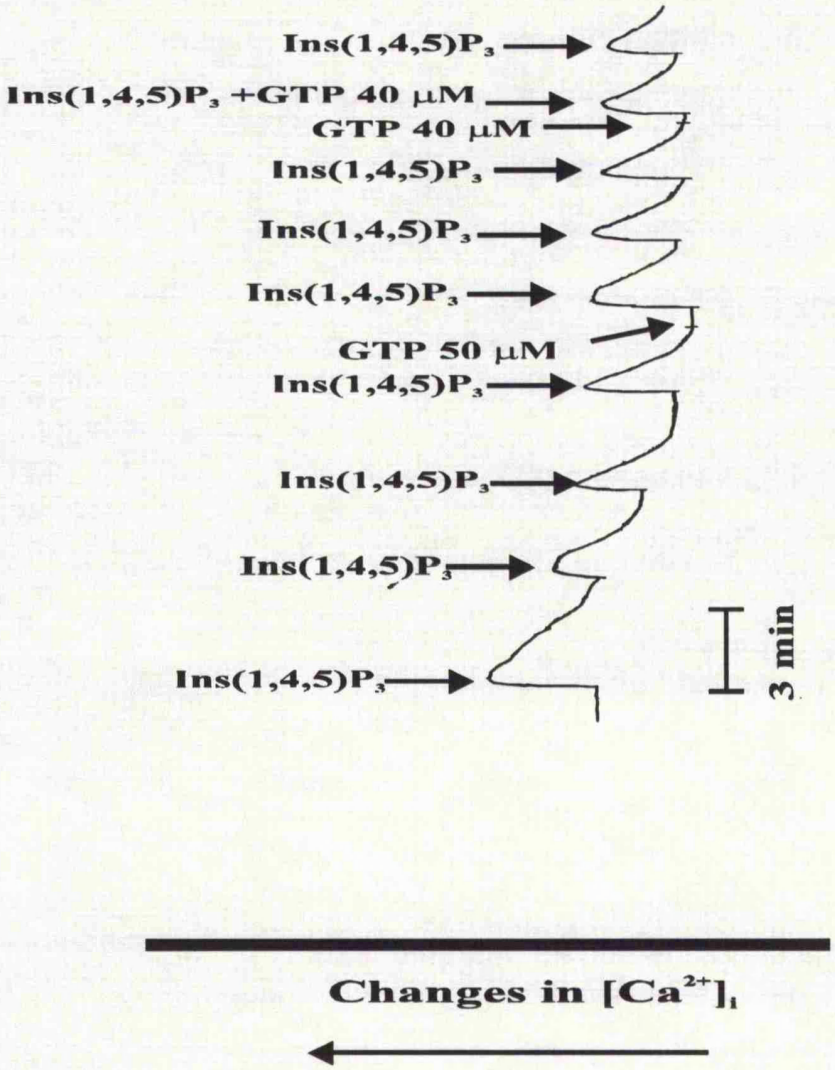


Fig. 4.10. Effect of guanosine 5' triphosphate (GTP) on Ins(1,4,5)P_3- induced Ca^{2+} release from electrically permeabilised chromaffin cells. Sequential additions of 3 μM Ins(1,4,5)P_3 did not have any dramatic effect on the magnitude of the Ins(1,4,5)P_3-induced Ca^{2+} responses. Total concentration of GTP added was 130 μM. ATP was present at 2.0 mM. Ins(1,4,5)P_3 concentration was 3 μM. The trace is representative of six experiments.

4.3 DISCUSSION

Data presented in this Chapter demonstrates that bovine adrenal chromaffin cells possess an Ins(1,4,5)P_3-sensitive and an Ins(1,4,5)P_3-insensitive, but caffeine-sensitive Ca^{2+} store. The Ins(1,4,5)P_3-induced Ca^{2+} release (87.71 nM $\pm$ 5.83, Ca^{2+} release) was transient and Ca^{2+} was re-sequestered in intracellular stores of electrically permeabilised chromaffin cells. Findings from this study suggest that Ca^{2+} mobilised from the Ins(1,4,5)P_3-sensitive Ca^{2+} stores was re-accumulated into the same stores, since challenge of the chromaffin cells with poorly metabolised analogues resulted in a sustained release which was not taken up into intracellular Ca^{2+} stores. Caffeine resulted in a sustained Ca^{2+} response (81 nM $\pm$ 5.25, Ca^{2+} release) and the $[Ca^{2+}]_i$ levels remained above the baseline. The responses to Ins(1,4,5)P_3 and caffeine (15 mM) were not found to be significantly different. Caffeine was able to release Ca^{2+} after prior application of Ins(1,4,5)P_3 and Ins(1,4,5)P_3 was still able to induce Ca^{2+} release after prior addition of caffeine.

The major two routes of Ins(1,4,5)P_3 metabolism involve dephosphorylation by a 5-phosphatase and phosphorylation by a 3-kinase (Nahorski, 1988; Nahorski and Potter, 1989). A large number of synthetic analogues resistant to metabolism have been synthesised (see Nahorski and Potter, 1992). Ins(1,4,5)PS_3 is a full agonist and resistant to metabolic routes.

In this study Ins(4,5)PS_2 was used which produce a sustained Ca^{2+} transient like the Ins(1,4,5)PS_3, indicating that it was also resistant to metabolism. Ins(4,5)PS_2 evoked Ca^{2+} mobilisation from the Ins(1,4,5)P_3-sensitive Ca^{2+} store since a further application of Ins(1,4,5)P_3 did not result in Ca^{2+} release. Ins(1,4,5)P_3 response was abolished after prior addition of the poorly metabolised Ins(1,4,5)P_3 analogues, Ins(1,4,5)SP_3 and Ins(4,5)SP_2, suggesting that the Ins(1,4,5)P_3-sensitive store had been depleted. Results presented in this study have shown that even when the Ins(1,4,5)P_3-sensitive store has been emptied caffeine was still able to release Ca^{2+} indicating an Ins(1,4,5)P_3- insensitive but exclusively caffeine-sensitive Ca^{2+} store.

Addition of Ins(1,4,5)P_3 even after prior additions of high doses of caffeine resulted in Ca^{2+} release. Ins(1,4,5)P_3-induced Ca^{2+} release was significantly reduced by 55% after prior addition of caffeine (15 mM) suggesting that partial depletion of the caffeine-releasable stores affected the Ins(1,4,5)P_3-sensitive store. The remaining response was due to the Ca^{2+} mobilising effect of Ins(1,4,5)P_3 demonstrating that chromaffin cells also possess an exclusively Ins(1,4,5)P_3-sensitive calcium store. The part of the Ins(1,4,5)P_3 response that was abolished in the presence of caffeine (15 mM) indicates the existence of an intracellular store that is sensitive to both Ins(1,4,5)P_3 and caffeine. Data presented here is in agreement with evidence reported by Stauderman et al., 1991 suggesting a large degree of overlap between the two stores. The mechanism by which depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores affects the caffeine induced Ca^{2+} mobilisation will be examined in more detail in subsequent Chapters.

Data reported by Föhr et al., (1991) demonstrated that repeated additions of low doses of Ins(1,4,5)P_3 (0.15 μM) resulted in decreased responses to Ins(1,4,5)P_3. A second addition of Ins(1,4,5)P_3 elicited only ~50% of the signal achieved by the first one. Their data also demonstrated that 20 μM of GTP fully restored the sensitivity to Ins(1,4,5)P_3 and therefore Ins(1,4,5)P_3 was able to induce the initial release of Ca^{2+}. Further additions of Ins(1,4,5)P_3 did not alter the amount of Ca^{2+} released. This data led Föhr et al., (1991) to report a possible role for GTP in permeabilised chromaffin cells. They suggested that the presence of GTP could reverse the desensitisation of the Ins(1,4,5)P_3 receptor or, alternatively, that GTP reactivates communications between the Ins(1,4,5)P_3-sensitive and -insensitive Ca^{2+} stores. GTP has also been reported to enlarge the Ins(1,4,5)P_3 releasable store in an manner independent of signal transducing G proteins (Gill et al., 1988). Data presented in Fig. 4.3 showed that repetitive additions of Ins(1,4,5)P_3 resulted in similar Ca^{2+} release. Addition of GTP did not appear to affect this response. Since Ins(1,4,5)P_3 response was not decreased by time thus GTP seems to play no part in Ca^{2+} release due to Ins(1,4,5)P_3 in chromaffin cells. GTP (10 μM) did not affect the Ins(1,4,5)P_3 response in a study with

digitonin permeabilised chromaffin cells (Robinson and Burgoyne, 1991a). Further, a similar lack of effect of GTP has been reported in SH-SY5Y cells (Wojcikiewicz et al., 1990).

In a previous study using digitonin-permeabilised chromaffin cells it was concluded that the Ins(1,4,5)P_3 and caffeine stores were distinct and non-overlapping (Robinson and Burgoyne, 1991a). Their findings indicated that Ins(1,4,5)P_3-induced Ca^{2+} release was inhibited after prior addition of caffeine but they proposed that this was not due to depletion of the caffeine-sensitive store but to the inhibitory effect of the increased Ca^{2+} levels, although these levels were not measured in that study. Elevated $[Ca^{2+}]_i$ levels have been shown to inhibit the binding of Ins(1,4,5)P_3 to its receptor (Danoff et al., 1988; Supattapone et al., 1988b) and to inhibit Ins(1,4,5)P_3-induced Ca^{2+} release in *Xenopus* oocytes (Parker and Ivorra, 1990) and hepatocytes (Ogden et al., 1990). Caffeine (15 mM and 40 mM) induced calcium release in this study was never above 200 nM and 300 nM above basal, respectively, indicating that the rise in $[Ca^{2+}]_i$ levels was not responsible for the inhibitory effect of caffeine on the Ins(1,4,5)P_3 induced calcium release in the experiments described in this Chapter. However, higher $[Ca^{2+}]_i$ levels did have an effect on the Ins(1,4,5)P_3 response as described in Chapter 5.

The inhibitory effects of caffeine on Ins(1,4,5)P_3 generation (Missiaen et al., 1992) and the Ins(1,4,5)P_3 Ca^{2+} release channel (Parker and Ivorra, 1991; Brown et al., 1992) could be responsible for the reduced Ins(1,4,5)P_3 response as described in this Chapter. However, in experiments described in Chapter 5 pretreatment of chromaffin cells with ryanodine had a similar effect on the Ins(1,4,5)P_3 induced Ca^{2+} release from permeabilised chromaffin cells, indicating that Ins(1,4,5)P_3 response was diminished due to the depletion of the caffeine-sensitive Ca^{2+} store. Results presented in this Chapter provide strong evidence for the presence of an exclusive Ins(1,4,5)P_3-sensitive Ca^{2+} store and an exclusive caffeine-sensitive Ca^{2+} store. The existence of a third store sensitive to both Ins(1,4,5)P_3 and caffeine is also apparent.

Findings on the SH-SY5Y cells suggested that an Ins(1,4,5)P_3-sensitive Ca^{2+} store is present and the size of the Ins(1,4,5)P_3-releasable pool is larger than that for chromaffin cells. Previous studies showed that challenge of SH-SY5Y cells with Ins(1,4,5)P_3 resulted in release of almost 80% of the total Ca^{2+} releasable from the intracellular stores with an EC_{50} for Ins(1,4,5)P_3 of between 50 nM and 320 nM (Safrany et al., 1990 and 1991a and b; Wojcikiewicz and Nahorski, 1991; Wilcox et al., 1993a). These cells also possess a putative Ins(1,3,4,5)P_4 receptor (Gawler et al., 1990 and 1991). Ins(1,3,4,5)P_4 is proposed to have a Ca^{2+} mobilising role since it has been demonstrated that it can directly stimulate Ca^{2+} release with an EC_{50} of 2.1 μM from intracellular stores via interaction with the Ins(1,4,5)P_3 receptor (Wilcox et al., 1993a, b and c).

Findings in this study also indicated that caffeine stimulation resulted in Ca^{2+} mobilisation from these cells, indicating that a ryanodine receptor may be present in these cells. This ryanodine receptor should be expected to resemble the type 2 cardiac form since type 1 ryanodine receptors have been reported only in the cerebellum and type 3 are believed to be insensitive to caffeine. Further experiments will be required to investigate the role of the caffeine-sensitive Ca^{2+} stores in SH-SY5Y cells and to examine if they are part of the Ins(1,4,5)P_3-sensitive Ca^{2+} store as in PC12 cells (Zacchetti et al., 1991) or whether they are separate with a degree of overlap as in chromaffin cells. Experiments on permeabilised SH-SY5Y cells (previously described in Chapter 2) demonstrated that Ins(1,4,5)P_3 was able to mobilise almost 80% of the total Ca^{2+} released by ionomycin. This data indicates that 20% of the Ca^{2+} sequestered in intracellular Ca^{2+} stores was not mobilised by Ins(1,4,5)P_3 and may represent the caffeine-sensitive Ca^{2+} stores in SH-SY5Y cells.

The major question arising from the existence of Ins(1,4,5)P_3- and caffeine-sensitive stores is what the relationship is between these two stores. In PC12 cells caffeine- and Ins(1,4,5)P_3-sensitive Ca^{2+} stores were believed to co-exist (Zachetti et al., 1991), since bradykinin inhibited the effects of subsequent addition of caffeine and caffeine inhibited the effects of subsequent addition of bradykinin. Recent studies

demonstrated that agonist stimulation inhibited subsequent caffeine response but caffeine was not able to inhibit the agonist induced Ca^{2+} release, suggesting that two stores are present in PC12 cells, one uniquely sensitive to Ins(1,4,5)P_3 and the other sensitive to both Ins(1,4,5)P_3 and caffeine (Reber et al., 1993; Barry and Cheek, 1994).

In chromaffin cells previous studies (Robinson and Burgoyne, 1991a) proposed that two distinct non-overlapping stores are present while others (Cheek et al., 1991; Stauderman et al., 1991; Liu et al., 1991) proposed different degrees of overlapping between these stores. Findings from this Chapter agree with the two pool model indicating that a degree of overlapping also exists between these stores.

The following figure 4.8 summarises Ca^{2+} homeostasis in electrically permeabilised chromaffin cells. It demonstrates the presence of the Ins(1,4,5)P_3-sensitive and caffeine-sensitive Ca^{2+} stores. It can be seen that Ins(1,4,5)P_3 stimulates Ca^{2+} release from the Ins(1,4,5)P_3-sensitive Ca^{2+} store. Caffeine is shown to release Ca^{2+} from the caffeine-sensitive Ca^{2+} store. The two Ca^{2+} sensitive stores have been plotted separately although they actually overlap. Communication exist between the Ins(1,4,5)P_3-sensitive and the caffeine-sensitive Ca^{2+} stores. CICR may be responsible for this communication. The possible role of GTP in this communication, is also displayed although no evidence was found in this study to support this role.

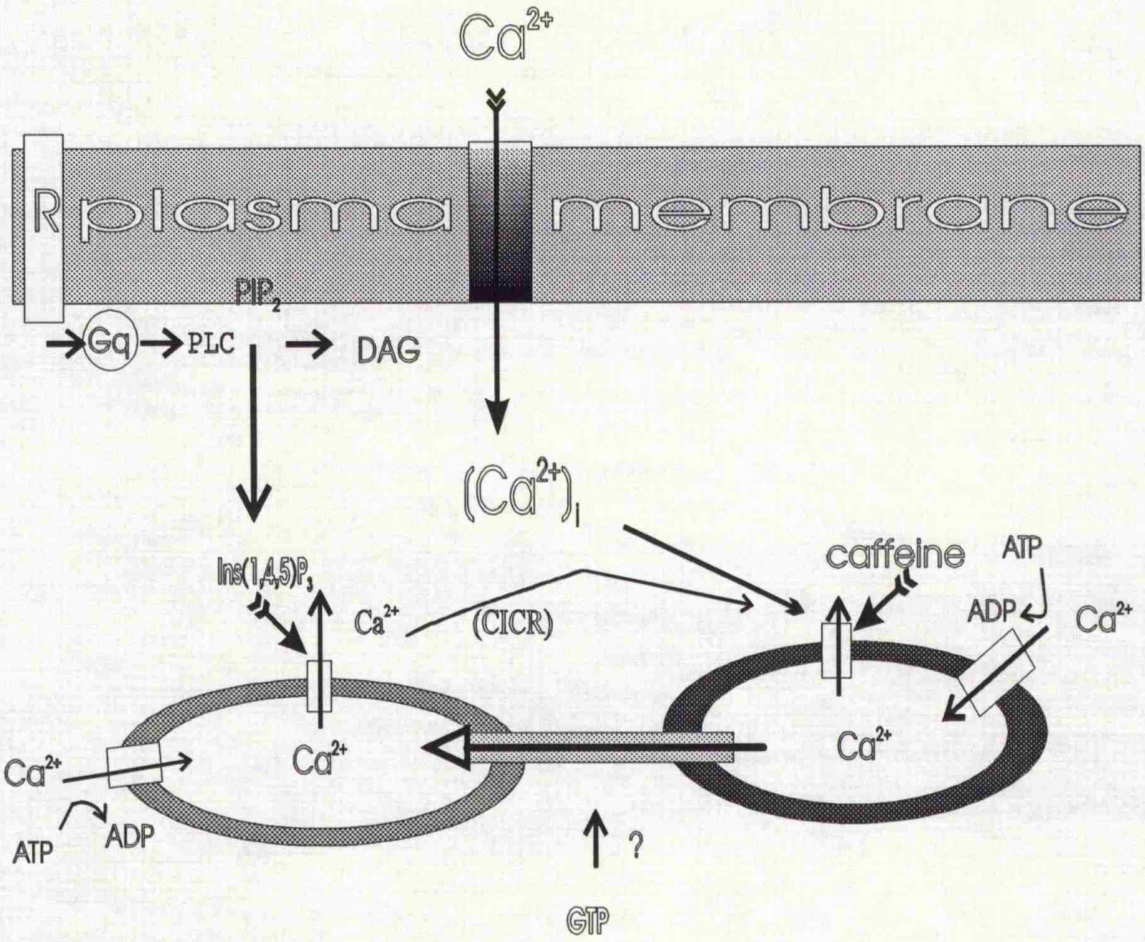


Fig. 4.8. This diagram is a schematic representation of Ca^{2+} homeostasis in bovine adrenal chromaffin cells. It also summarises the characteristics of the Ca^{2+} stores within the chromaffin cells. It can be seen that chromaffin cells possess at least two Ca^{2+} stores one sensitive to $Ins(1,4,5)P_3$ and the other to caffeine. This diagram also illustrates the proposed hypothesis of the mechanism by which CICR and GTP may be involved in Ca^{2+} homeostasis by engaging in communication between the two Ca^{2+} stores.

CHAPTER 5:

FURTHER CHARACTERISATION OF THE INOSITOL-1,4,5-TRISPHOSPHATE-SENSITIVE AND CAFFEINE SENSITIVE Ca^{2+} STORES IN SAPONIN-PERMEABILISED CHROMAFFIN CELLS.

5.1 INTRODUCTION.

Evidence for the existence of two distinct Ca^{2+} stores in chromaffin cells has been presented in the previous Chapter (Chapter 4). This data was obtained by using a Ca^{2+}-selective electrode to monitor changes in intracellular Ca^{2+} levels. One of these pools appeared to be sensitive to Ins(1,4,5)P_3 and the other sensitive to the tri-methyl xanthine caffeine. The possible communications between these two stores was also examined. The data presented in this Chapter was obtained from saponin permeabilised chromaffin cells. Chromaffin cells were loaded with $^{45}CaCl_2$ (as described in Chapter 2 section 8) and the effects of Ins(1,4,5)P_3 and caffeine and agonists such as bradykinin on $^{45}CaCl_2$ release studied.

As described previously, agonist activation of its receptor stimulates phospholipase C activation via a G protein link. The phospholipase C activation stimulates hydrolysis of phosphatidylinositol-4,5 bisphosphate (PtdIns-4,5P_2) to diacylglycerol (DAG), a protein kinase activator, and Ins(1,4,5)P_3. All the following steps between receptor activation and Ins(1,4,5)P_3 formation are well documented and the role of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores is well studied.

Previous data from chromaffin cells indicated that Ins(1,4,5)P_3 generating agonists result in spatially restricted rise in $[Ca^{2+}]_i$ initiated in a region between the nucleus and the plasma membrane close to one pole of the cells (Cheek et al., 1989b and c; O'Sullivan et al., 1989). Immunological studies have revealed that chromaffin cells possess two distinct Ca^{2+}-ATPase-like proteins, the first is 100 kDa which is diffusely distributed, the second being 140 kDa which is restricted around the nucleus of the cell (Burgoyne et al., 1989b). Their data also suggests that the 100 kDa protein is not connected to the Ins(1,4,5)P_3-sensitive store and proposes that the calciosome which has been shown to possess a 100 kDa Ca^{2+}-ATPase protein (Hashimoto et al., 1988) is the caffeine-sensitive store in chromaffin cells. The existence of the caffeine-sensitive stores in chromaffin cells is of wide interest because it may represent the nonmuscle cell equivalent of the Ca^{2+} -induced Ca^{2+} release (CICR) stores. If that is

the case their existence may be involved in Ca^{2+} homeostasis within the chromaffin cell and have a role in Ca^{2+} signalling.

Caffeine is believed to stimulate calcium mobilisation from the ryanodine-gated Ca^{2+} channels by lowering the threshold for Ca^{2+} induced Ca^{2+} (CICR) release. The ryanodine receptor protein has been shown to be distinct from the Ins(1,4,5)P_3 receptor protein. [^{3}H]ryanodine binding sites have been reported in chromaffin cell membrane preparations (Stauderman and Murawsky, 1991) with a similar affinity (K_d= 3.3) to those in striated muscle, but a 10,000 fold lower binding site density. The plant alkaloid ryanodine has been a very useful pharmacological tool since it blocks the Ca^{2+} release channels of the sarcoplasmic reticulum of skeletal and cardiac muscle cells in an open state (Rousseau et al., 1987). Ryanodine, it has been demonstrated, is able to inhibit the caffeine-induced Ca^{2+} release from the muscle CICR store by blocking the Ca^{2+} channels in a low conductance state, and by inhibiting the agonist effect of caffeine (Fill and Coronado, 1988; Lai et al., 1988;) without affecting the Ins(1,4,5)P_3 induced Ca^{2+} release in smooth muscle cells (Iino et al., 1988). Furthermore, recent reports demonstrate that ryanodine did not inhibit the Ins(1,4,5)P_3 receptor (Ehrlich et al., 1994).

Very recent data shows that another endogenous compound can stimulate Ca^{2+} release (see Berridge, 1993b; Galione, 1993 and 1994). This putative second messenger is the cyclic-adenosine 5'-diphosphate ribose (cyclic ADP-ribose) which was first discovered by Dargie et al., in 1990 when studying Ca^{2+} release by microsomes derived from the endoplasmic reticulum of sea urchin eggs (Dargie et al., 1990; Galione et al., 1991). Cyclic ADP-ribose is a low molecular weight metabolite of the pyridine nucleotide, nicotinamide adenine dinucleotide (NAD^+). This NAD metabolite has been reported to stimulate Ca^{2+} mobilisation from the ryanodine-sensitive Ca^{2+} stores in sea urchin eggs. The possibility of cyclic ADP-ribose inducing Ca^{2+} release from the caffeine-sensitive Ca^{2+} stores in saponin permeabilised chromaffin cells was examined.

Ins(1,4,5)P_3 receptor is a prominent substrate for protein kinases (Ferris et al., 1991; Ferris and Snyder, 1992). Evidence has been shown for PKC, calmodulin-dependent (CaM) kinase and cAMP-dependent kinase (PKA) (Ferris et al., 1991; see also Chapter 1). The effect of phosphorylation of the Ins(1,4,5)P_3 receptor is controversial since phosphorylation of the Ins(1,4,5)P_3 receptors expressed in brain cells reduced their sensitivity, whereas in peripheral tissues it had the opposite effect (Berridge, 1993a; Pozzan et al., 1994). The effect of phosphorylation of the Ins(1,4,5)P_3 receptor on the Ins(1,4,5)P_3-induced Ca^{2+} release from saponin permeabilised chromaffin cells was also studied. The phorbol ester TPA (12-0-tetradecanoylphorbol 13- acetate), an exogenous activator of protein kinase C, and forskolin, which activates adenylate cyclase and raises cAMP concentration, were employed for this purpose.

Ins(1,4,5)P_3 receptor is also believed to contain bindings sites for ATP which have been proposed to be located near the phosphorylation sites (Maeda et al., 1991; Mikoshiba et al., 1993). The cerebellar Ins(1,4,5)P_3 receptors have been proposed to contain two amino acid sequences for ATP binding (Ferris and Snyder, 1992). The regulatory effect of ATP on the Ins(1,4,5)P_3 receptor is independent of phosphorylation. Early studies on the Ins(1,4,5)P_3 receptor channel from aortic sarcoplasmic reticulum demonstrated that ATP (100 μM) enhanced the open probability of the channel in the presence of Ins(1,4,5)P_3 (Ehrlich and Watras, 1988). However, the regulatory role of ATP on the Ins(1,4,5)P_3 receptor is not clear since enhancement of the Ins(1,4,5)P_3 induced Ca^{2+} release occurs below normal cytosolic ATP concentrations (Ferris and Snyder, 1992; Pozzan et al., 1994). The ryanodine receptor channel is activated by millimolar ATP (Smith et al., 1985; Meissner, 1994). The effect of ATP on the Ins(1,4,5)P_3 and caffeine induced Ca^{2+} release was examined.

Ca^{2+} is believed to be the most important modulator of the Ins(1,4,5)P_3 receptor. Studies on the cerebellum microsomal Ins(1,4,5)P_3- gated Ca^{2+} channel first indicated that the Ins(1,4,5)P_3- induced calcium release displayed a bell-shaped

calcium response curve reaching a peak at 250-300 nM $[Ca^{2+}]_i$ (Bezprozvanny et al., 1991) and this was later confirmed by others (Iino and Endo, 1992). Intracellular levels of Ca^{2+} have been reported to influence the Ins(1,4,5)P_3- and the caffeine- induced Ca^{2+} release. The effect of ATP and Ca^{2+} on the Ins(1,4,5)P_3- and caffeine- induced Ca^{2+} mobilisation from intracellular stores of chromaffin cells was also examined.

5.2 RESULTS.

In the experiments described in this Chapter saponin-permeabilised plated chromaffin cells were used (see Chapter 2 section 8). Saponin has been previously shown to have a high affinity for cholesterol and causes permeability by interacting with the cholesterol rich plasma membranes (Glauert et al., 1952). It has much less effect on the endoplasmic reticulum and the mitochondria (Korn, 1969). Treatment with saponin disrupts the continuity of the plasma membrane producing chromaffin cells that are permeable to small molecules but not to large proteins (Brooks and Treml, 1983 and 1984; Livett, 1984), without affecting the total cellular catecholamine content and the morphology of chromaffin cells.

Chromaffin cells plated in 24-well multiwell plates have been widely used for secretion experiments but not for $^{45}CaCl_2$ release studies. The permeability of the chromaffin cells was measured with Azur- A. This compound enabled us to examine the permeability of chromaffin cells by visual test under the microscope since it penetrates permeabilised cells and colours the interior blue. Data presented in Table 5.1 demonstrates that 0.5 mg/ ml or 0.1 mg/ ml of saponin permeabilised about 95% of chromaffin cells when present for 8 minutes without losing any cells.

Experiments presented at Chapter 3 examining the role of extracellular Ca^{2+} in catecholamine secretion were performed at 37^0C. Experiments investigating the role of intracellular Ca^{2+} stores in intact cells are also performed at 37^0C. All the experiments presented in this Chapter were carried out at a temperature of 37^0C and were performed as described in Chapter 2, section 8. At this temperature (37^0C) $Ins(1,4,5)P_3$ consistently induced Ca^{2+} mobilisation from intracellular Ca^{2+} stores. The $Ins(1,4,5)P_3$-induced Ca^{2+} release from saponin-permeabilised chromaffin cells was dose-dependent as expected (see Fig. 5.1). The first $Ins(1,4,5)P_3$ response was obtained at 0.3 μM. Maximal $Ins(1,4,5)P_3$ response was obtained with 3 μM and

upwards, with an EC_{50} of 619 nM $\pm$ 94 (n= 6). Maximally effective concentrations of Ins(1,4,5)P_3 were able to release 44.17% $\pm$ 2.45 of the total $^{45}Ca^{2+}$ sequestered into the intracellular Ca^{2+} stores (n= 42). The Ins(1,4,5)P_3-induced Ca^{2+} release was also time dependent (Fig. 5.2). The shortest time that Ins(1,4,5)P_3-induced Ca^{2+} release was measured was at 30 seconds. The results in Fig. 5.2 indicate that release was rapid, beginning before this 30 seconds time point. Ins(1,4,5)P_3 stimulation for 3 minutes resulted in maximal response.

Caffeine was also able to mobilise Ca^{2+} from intracellular Ca^{2+} stores from saponin permeabilised chromaffin cells in a concentration-dependent manner with an EC_{50} of 16.59 mM $\pm$ 1.33 (n= 7, see Fig. 5.3). The maximal concentration used was 50 mM. Caffeine (50 mM) resulted in release of 26.48% $\pm$ 3.25 of the total $^{45}Ca^{2+}$ sequestered into the intracellular Ca^{2+} stores (n= 42). In the experiments described in this Chapter 50 mM of caffeine was used (despite the fact that high doses should be considered with caution, due to the multiple indirect effects of caffeine as discussed in Chapter 4), since this is the dose required to deplete the ryanodine-sensitive Ca^{2+} stores.

Thapsigargin has been shown to inhibit the Ca^{2+}-ATPase responsible for refilling the Ins(1,4,5)P_3-sensitive Ca^{2+} stores (Thastrup et al., 1989 and 1990). Thapsigargin has been previously reported to inhibit agonist evoked Ca^{2+} release in chromaffin cells (Cheek et al., 1989a). In order to examine the effect of thapsigargin, chromaffin cells were pretreated with thapsigargin (1 μM) for four minutes. Subsequent addition of Ins(1,4,5)P_3 did not result in Ca^{2+} release, indicating that the Ins(1,4,5)P_3-sensitive Ca^{2+} store had been depleted. Application of caffeine (50 mM) to the chromaffin cells after their Ins(1,4,5)P_3-releasable pools had been emptied evoked Ca^{2+} response showing that an exclusive caffeine-sensitive Ca^{2+} store is present in chromaffin cells (Fig. 5.4). With this method it is difficult to statistically compare the size of the responses due to the different conditions of $^{45}Ca^{2+}$ loading. Less $^{45}Ca^{2+}$ was sequestered into the intracellular Ca^{2+} stores when chromaffin cells

Concentration of saponin in mg/ml	Time (mins)	% of permeabilised chromaffin cells
1.0 mg/ ml	1	> 95 %
1.0 mg/ ml	3	Increasing loss of cells ⇓
1.0 mg/ ml	5	Increasing loss of cells ⇓
1.0 mg/ ml	8	Increasing loss of cells ⇓
1.0 mg/ ml	10	Increasing loss of cells ⇓
1.0 mg/ ml	12	All cells lost
0.5 mg / ml	1	> 60 %
0.5 mg / ml	3	> 70 %
0.5 mg / ml	5	> 90 %
0.5 mg / ml	8	> 95 %
0.5 mg / ml	10	Increasing loss of cells ⇓
0.5 mg / ml	12	All cells lost
0.1 mg / ml	1	> 50 %
0.1 mg / ml	3	> 60 %
0.1 mg / ml	5	> 75 %
0.1 mg / ml	8	~ 80- 90 %
0.1 mg / ml	10	> 95 %
0.1 mg / ml	12	> 95 %

Table 5.1. Effect of different saponin concentrations, applied for different durations, on permeabilisation of chromaffin cells. It appears that 0.5 mg/ ml or 0.1 mg/ ml of saponin could be sufficient to permeabilise chromaffin cells without any loss of cells occurring.

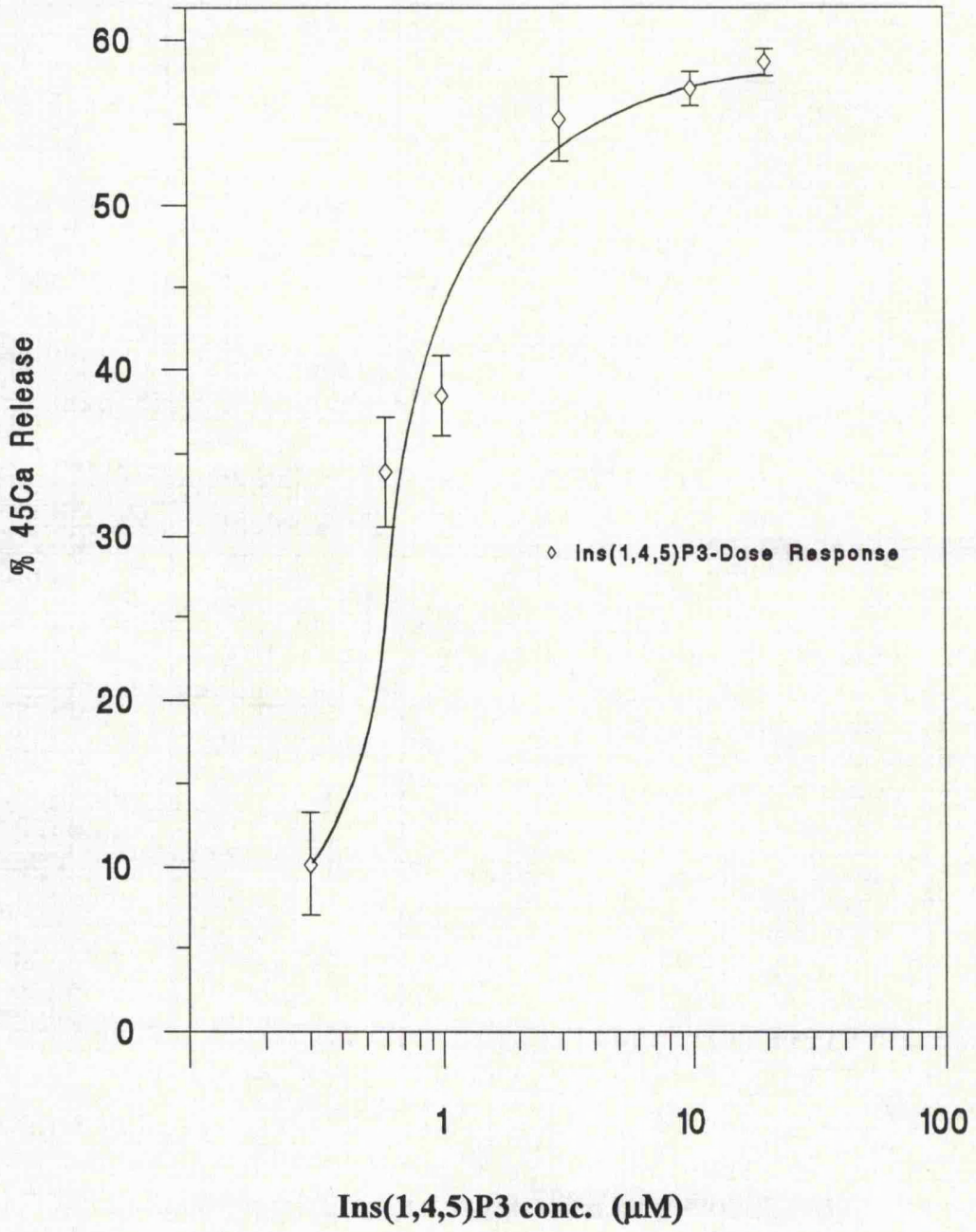


Fig. 5.1. Ins(1,4,5)P_3 dose-response in chromaffin cells. This figure shows that Ins(1,4,5)P_3 response was dose- dependent in saponin permeabilised chromaffin cells. The $[Ca^{2+}]_i$ levels were buffered to around 120 nM. ATP was present at 5 mM. The cells were stimulated with Ins(1,4,5)P_3 for 3 mins. Results are mean values $\pm$ S.E.M. of six experiments each performed in triplicate.

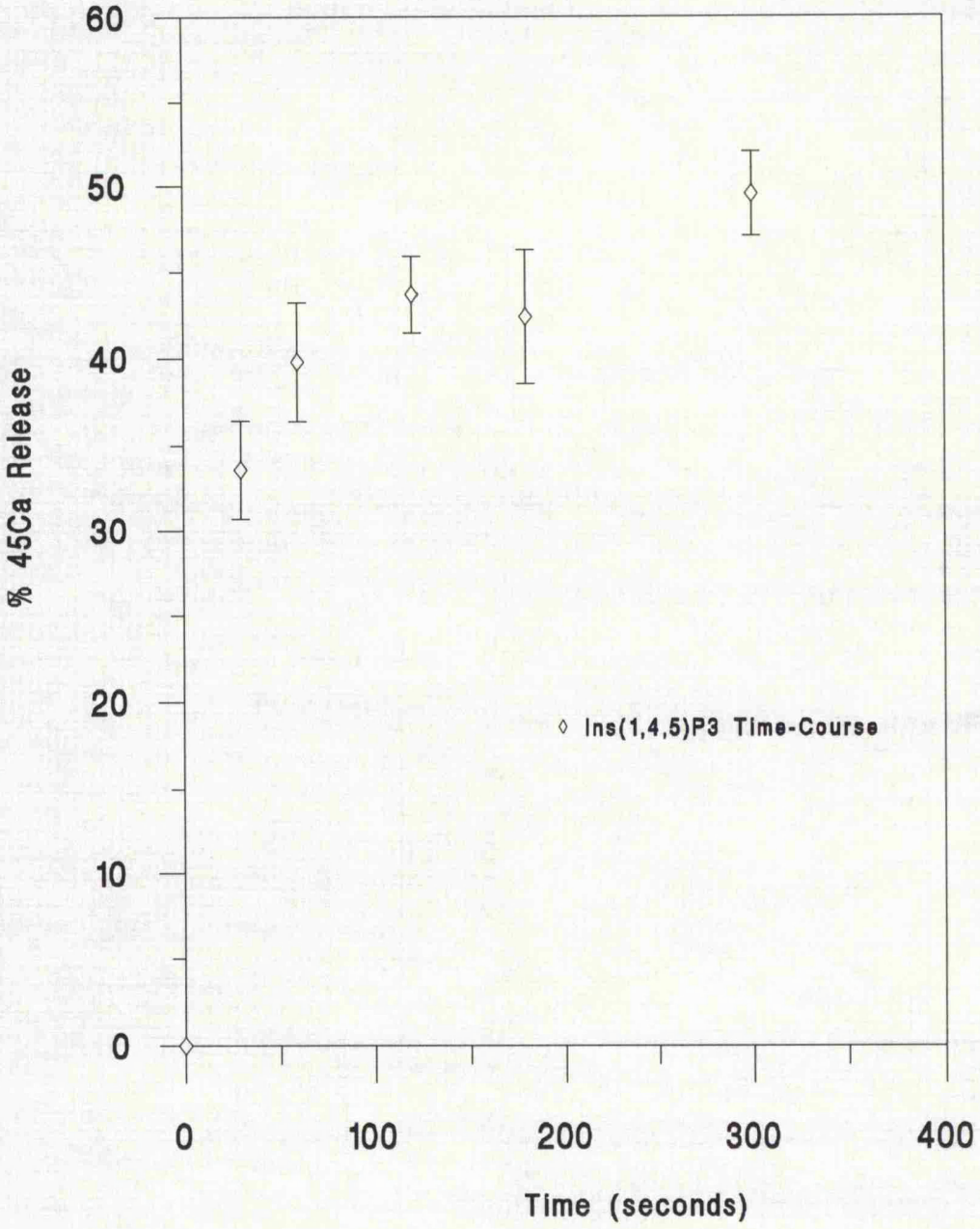


Fig. 5.2. Ins(1,4,5)P_3 time course on saponin permeabilised chromaffin cells. This graph shows that Ins(1,4,5)P_3 response was time dependent. ATP was present at 5 mM. Ins(1,4,5)P_3 concentration was 3 μM. $[Ca^{2+}]_i$ levels were buffered to around 120 nM. Results are mean values ± S.E.M. of four experiments each performed in triplicate.

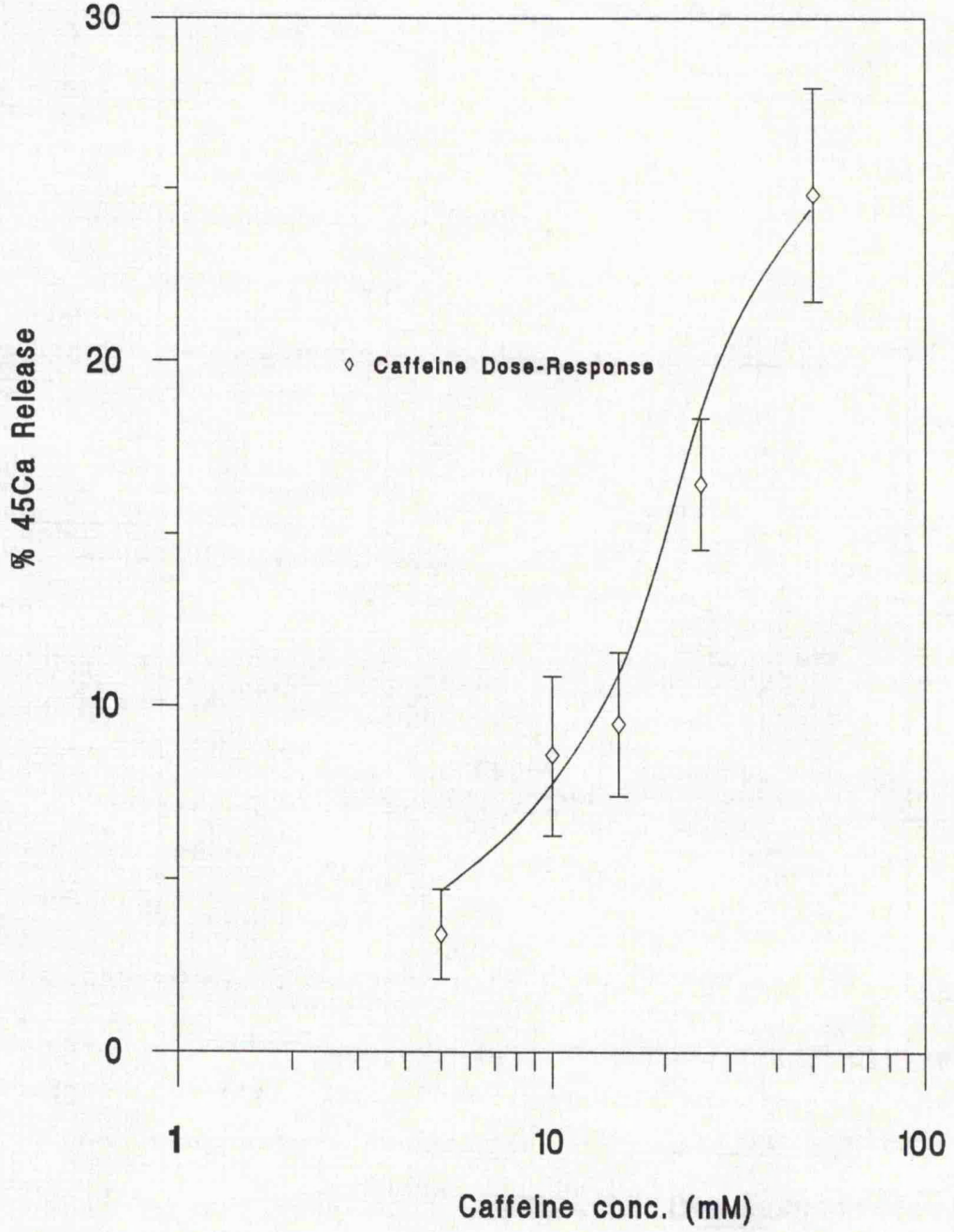


Fig. 5.3. Caffeine dose response in permeabilised chromaffin cells. The $[Ca^{2+}]_i$ levels were buffered (around 100 nM) and ATP was present at 5 mM. The cells were stimulated with caffeine for 3 mins. Results are mean values ± S.E.M. of seven experiments each performed in triplicate.

were loaded with $^{45}Ca^{2+}$ in the presence of thapsigargin, indicative of depletion of some stores. The importance of these findings is that they demonstrate that although Ins(1,4,5)P_3 was not able to mobilise Ca^{2+} from intracellular stores, caffeine was, indicating the existence of two distinct intracellular stores.

When chromaffin cells were pretreated (during the $^{45}Ca^{2+}$ loading period) with caffeine (50 mM), in order to inhibit $^{45}Ca^{2+}$ loading in to the caffeine-releasable Ca^{2+} stores, subsequent addition of Ins(1,4,5)P_3 evoked Ca^{2+} release from internal stores suggesting that an exclusive Ins(1,4,5)P_3-sensitive Ca^{2+} store is also present in saponin permeabilised chromaffin cells (Fig. 5.4). Further evidence that chromaffin cells possess Ins(1,4,5)P_3- and caffeine- sensitive Ca^{2+} stores is illustrated in Fig. 5.5 where it is shown that addition of both Ins(1,4,5)P_3 and caffeine resulted in a larger response than Ins(1,4,5)P_3 or caffeine alone. Differential proportions of the $^{45}Ca^{2+}$ were mobilised by Ins(1,4,5)P_3, caffeine and Ins(1,4,5)P_3/Caffeine in individual experiments. However, in each individual experiment (n=7) the responses to Ins(1,4,5)P_3/Caffeine were greater than each of these agonists when added alone but less than the product of these agonists responses. These findings also suggest that saponin permeabilised chromaffin cells possess both Ins(1,4,5)P_3- and caffeine- sensitive stores and indicate that these stores may overlap.

Ryanodine is believed to bind to the ryanodine receptor and block it in an open conductance state (Rousseau et al., 1987). When chromaffin cells were treated with ryanodine (10 μM) when they were loaded with $^{45}Ca^{2+}$ it was found that the amount of $^{45}Ca^{2+}$ taken up by the permeabilised chromaffin cells was significantly reduced (see Fig. 5.6). This is a clear indication that ryanodine-sensitive stores exist in chromaffin cells and that ryanodine prevents them sequestering Ca^{2+}. No caffeine-induced Ca^{2+} release after prior treatment with ryanodine was detected (Fig. 5.6) indicating that caffeine is acting on the ryanodine receptor. Although the ryanodine releasable stores had been depleted, Ins(1,4,5)P_3 was still able to release Ca^{2+} from the Ins(1,4,5)P_3-sensitive Ca^{2+} stores, demonstrating that exclusive Ins(1,4,5)P_3-sensitive Ca^{2+} stores exist in saponin permeabilised chromaffin cells. If the Ins(1,4,5)P_3- and ryanodine-

sensitive Ca^{2+} stores were distinct in chromaffin cells depletion of the ryanodine-sensitive pools should not affect the Ins(1,4,5)P_3-induced Ca^{2+} mobilisation. Pretreatment with ryanodine during $^{45}Ca^{2+}$ loading reduced the amount of $^{45}Ca^{2+}$ that was sequestered into the intracellular stores. The amount of $^{45}Ca^{2+}$ accumulated into the Ins(1,4,5)P_3-sensitive Ca^{2+} stores should be expected to be the same since ryanodine has no effect on the Ins(1,4,5)P_3 receptor. Under these conditions challenge of chromaffin cells with Ins(1,4,5)P_3 should stimulate mobilisation of the same amount of $^{45}Ca^{2+}$ from the Ins(1,4,5)P_3-sensitive Ca^{2+} stores, although the total $^{45}Ca^{2+}$ taken up into the intracellular stores is reduced. The percentage of the $^{45}Ca^{2+}$ released from the Ins(1,4,5)P_3-sensitive Ca^{2+} stores to the total $^{45}Ca^{2+}$ sequestered into the stores should be increased if the Ins(1,4,5)P_3 and caffeine-sensitive Ca^{2+} stores were distinct without any functional overlap.

Findings from these experiments demonstrate that pretreatment with ryanodine resulted in a reduced Ins(1,4,5)P_3-induced Ca^{2+} mobilisation, indicating that ryanodine- and Ins(1,4,5)P_3- sensitive Ca^{2+} stores do partially overlap in saponin permeabilised chromaffin cells.

5.2.1 Role of Cyclic ADP-Ribose.

It has now been reported that cyclic ADP-ribose stimulates Ca^{2+} mobilisation from the ryanodine Ca^{2+} sensitive stores in smooth muscle cells (Berridge, 1993b; Galione, 1993). The interesting possibility that cyclic ADP-ribose could have a regulatory role on in the ryanodine-sensitive Ca^{2+} channels in permeabilised chromaffin cells was examined.

Over a substantial series of experiments it was found that there was no reliable effect of cyclic ADP-ribose on Ca^{2+} release or on the caffeine-induced Ca^{2+} release in saponin-permeabilised chromaffin cells. This conclusion was reached despite the fact that a limited number (n=4) of early experiments, did appear to show that cyclic ADP-ribose has an effect on caffeine-induced Ca^{2+} release.

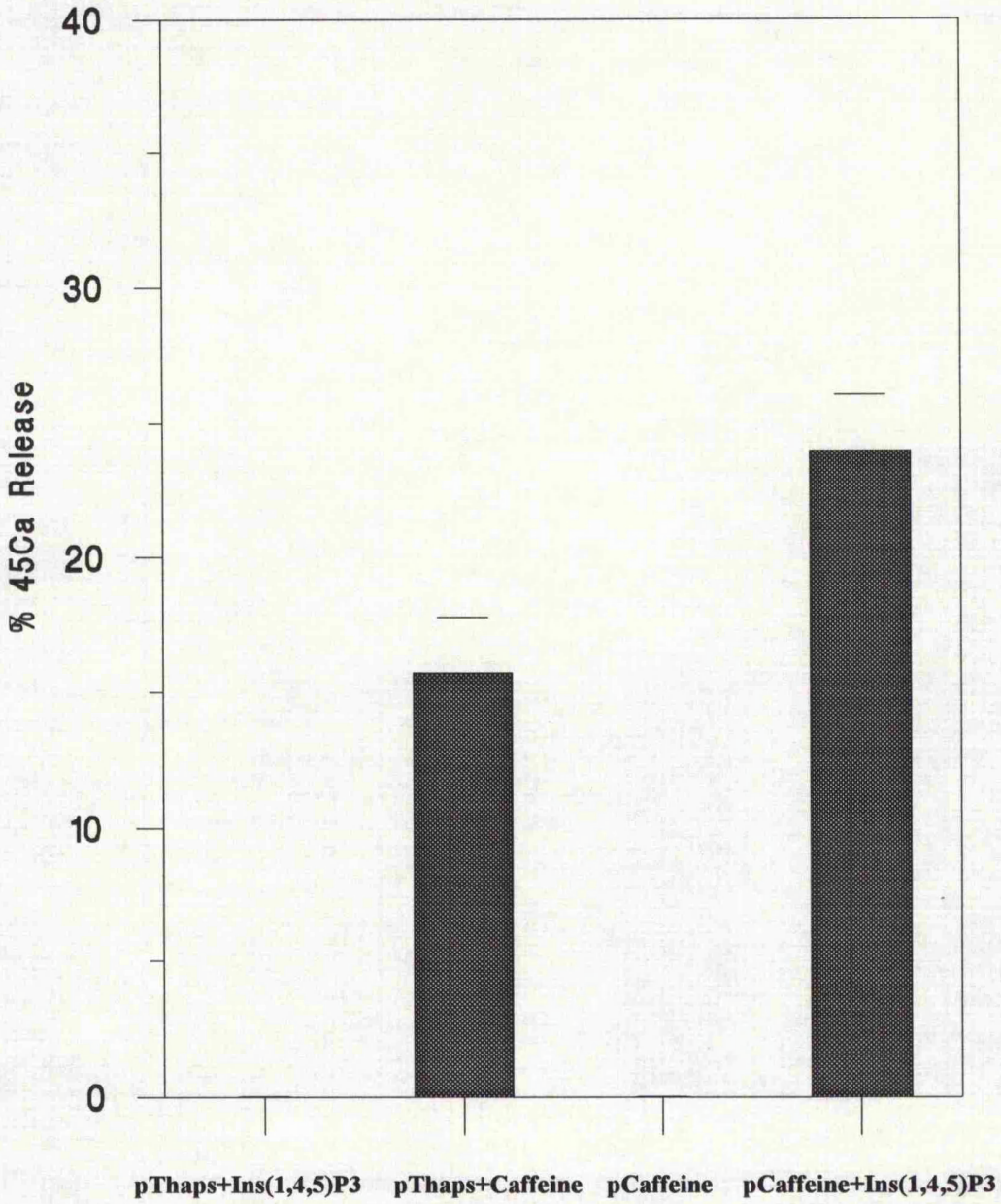


Fig. 5.4. Effect of thapsigargin and caffeine pretreatment on Ins(1,4,5)P_3 and caffeine induced Ca^{2+} release from permeabilised chromaffin cells. Cells were pretreated with thapsigargin (1μM) for four minutes and caffeine (50 mM) for eight minutes. The $[Ca^{2+}]_i$ levels were buffered (around 100 nM) and ATP was present at 5 mM. Results are expressed as percentage release of Ca^{2+} and are mean values ± S.E.M. of $n \geq 5$ experiments each performed in triplicate. p: pretreatment with thapsigargin or caffeine during the loading of chromaffin cells with $^{45}Ca^{2+}$.

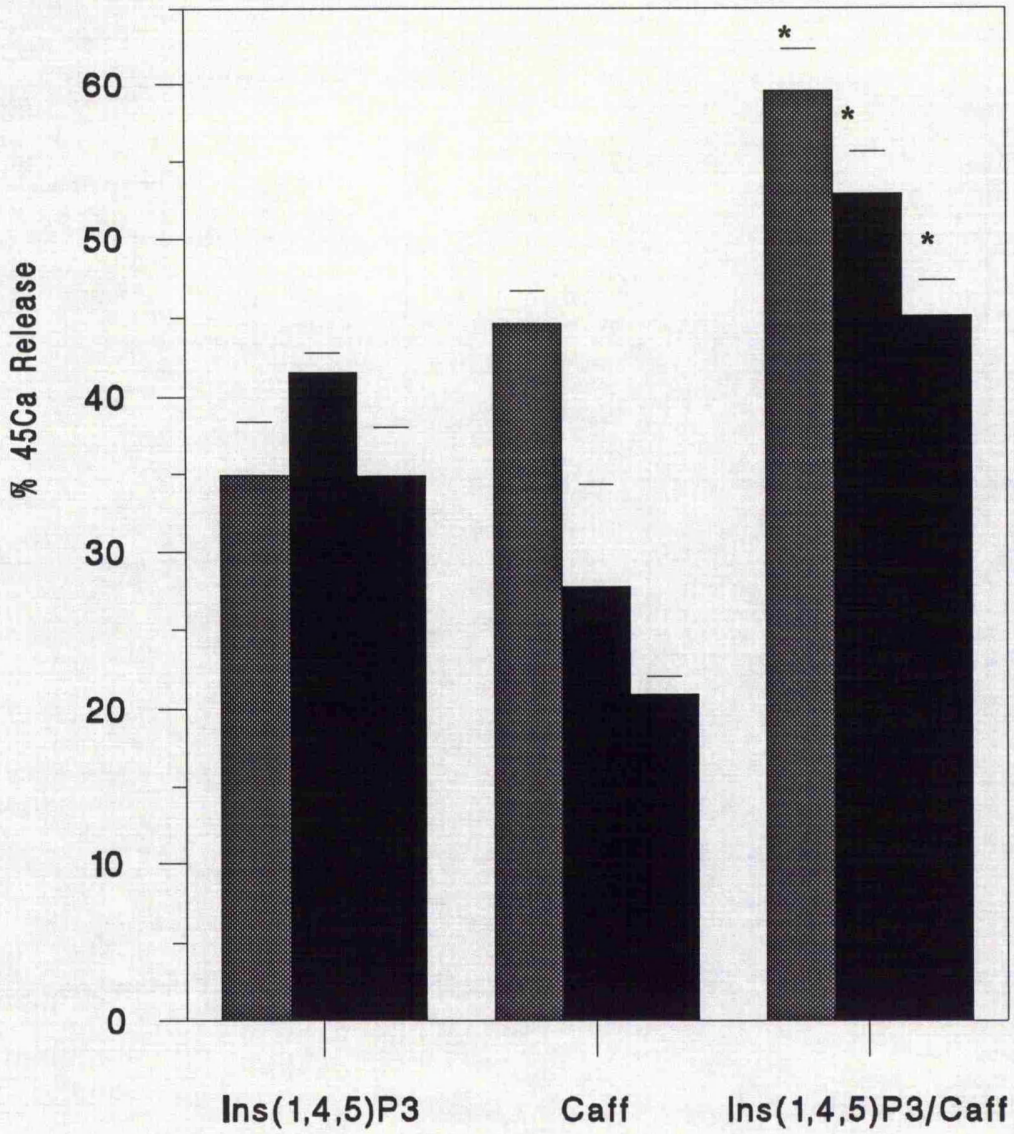


Fig. 5.5. Effect of Ins(1,4,5)P_3 and caffeine on Ca^{2+} release from permeabilised chromaffin cells. Cells were stimulated with Ins(1,4,5)P_3 (3 μM) and caffeine (50 mM) for three minutes. The $[Ca^{2+}]_i$ levels were buffered (around 100 nM) and ATP was present at 5 mM. Results are expressed as percentage of $^{45}Ca^{2+}$ released compared with the total $^{45}Ca^{2+}$ sequestered in chromaffin cells Three separate experiments performed in triplicate are shown (mean values ± S.E.M.) and are representative of seven similar experiments. *: significantly larger than stimuli alone ($P<0.05$)

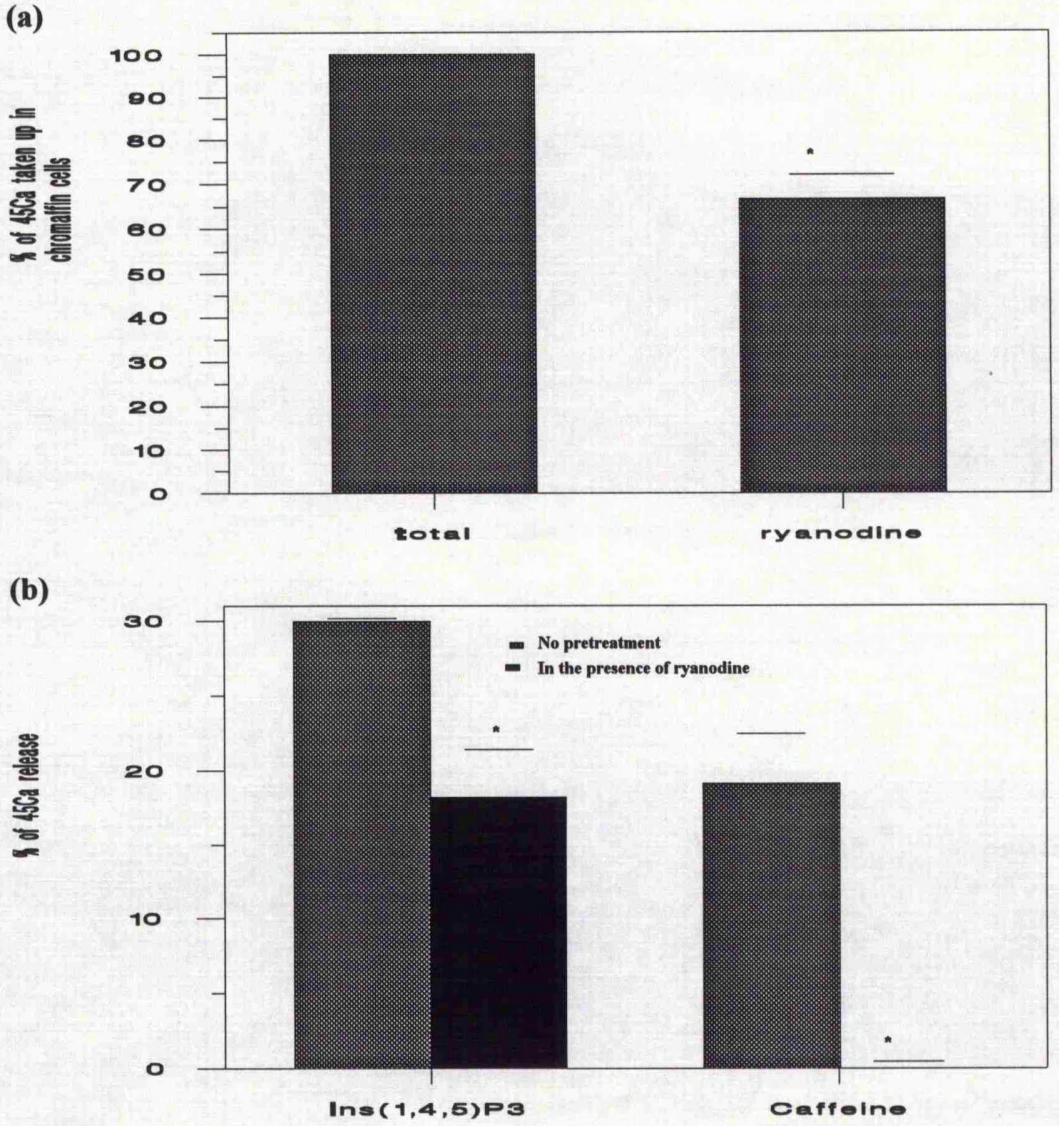


Fig. 5.6. Effect of ryanodine on Ins(1,4,5)P_3- and caffeine induced Ca^{2+} release from saponin-permeabilised chromaffin cells. Chromaffin cells were pretreated with ryanodine for 8 mins and loaded with $^{45}CaCl_2$ in the presence of ryanodine when it was applied. Ryanodine was also present when cells were stimulated with the test drug. (a) Shows the amount of $^{45}CaCl_2$ taken up in chromaffin cells in the presence and absence of ryanodine. (b) Ins(1,4,5)P_3 and caffeine response in the presence of ryanodine. Ins(1,4,5)P_3 concentration was 3 μM; caffeine concentration was 50 mM. Ryanodine concentration was 10 μM. $[Ca^{2+}]_i$ levels were buffered at around 500 nM. Results are mean values ± S.E.M. ($P<0.05$) of three experiments each performed in triplicate.

One of these experiments is shown on Fig. 5.7. However, in over 10 experiments that followed various concentrations of cyclic ADP-ribose up to 1 μM were used and their effect on varying concentrations of caffeine was examined. Different combinations of intracellular $[Ca^{2+}]_i$ levels and presence, or absence, of ATP were tested with no effect. The possibility of cyclic ADP-ribose enhancing the caffeine response was also examined by testing its effect on low doses of caffeine which did not produce any Ca^{2+} release either alone or with cyclic ADP-ribose. Fig. 5.7 provides an example where there was an apparent effect of caffeine (5 mM) only in the presence of cyclic ADP-ribose. Subsequent experiments failed to confirm this.

5.2.2 Phosphorylation of the Ins(1,4,5)P_3 Receptor.

Various mechanisms have been proposed to regulate the Ins(1,4,5)P_3 receptor channel. There is evidence that phosphorylation of the Ins(1,4,5)P_3 receptor by cAMP-dependent protein kinase (PKA), a calmodulin-dependent kinase (CaMKII) and PKC modulates its function. The possibility of TPA (protein kinase C activator) and forskolin (a cAMP activator) affecting the Ins(1,4,5)P_3-induced Ca^{2+} release from saponin-permeabilised chromaffin cells was examined. In this study both were used at maximal concentrations and found not to have any effect on the $^{45}CaCl_2$ released by different doses of Ins(1,4,5)P_3 in bovine adrenal permeabilised chromaffin cells (see Fig. 5.8 and 5.9). These results suggest that phosphorylation of the Ins(1,4,5)P_3-receptor did not affect the Ins(1,4,5)P_3-induced Ca^{2+} mobilisation. It is also possible that the amount of PKC and cAMP was reduced due to leakage since permeabilised cells were used in this study.

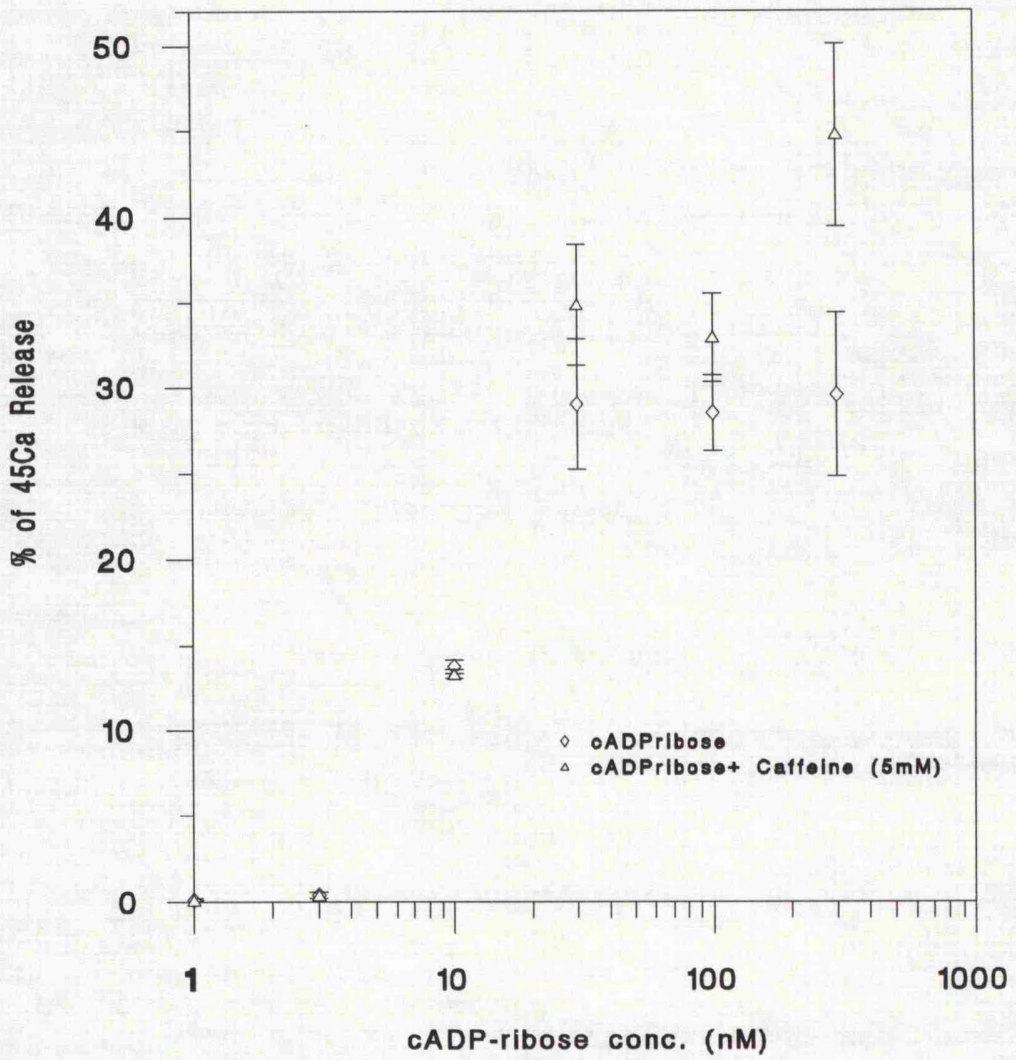


Fig. 5.7. Effect of cyclic-ADP-ribose on caffeine-induced Ca^{2+} release from saponin permeabilised chromaffin cells. Cyclic-ADP-ribose over a large number of experiments did not appear to affect the caffeine response (n>10). This figure shows one of several experiments (n=4) where cyclic-ADP-ribose seemed to have an effect on the Ca^{2+} released by caffeine. Caffeine concentration was 5 mM. Cyclic-ADP-ribose was used at different concentrations of 1, 3, 10, 30, 100 and 300 nM. No ATP was added in this experiment. $[Ca^{2+}]_i$ levels were buffered to 500 nM. Caffeine and cyclic-ADP-ribose stimulation was carried out for 3 mins.

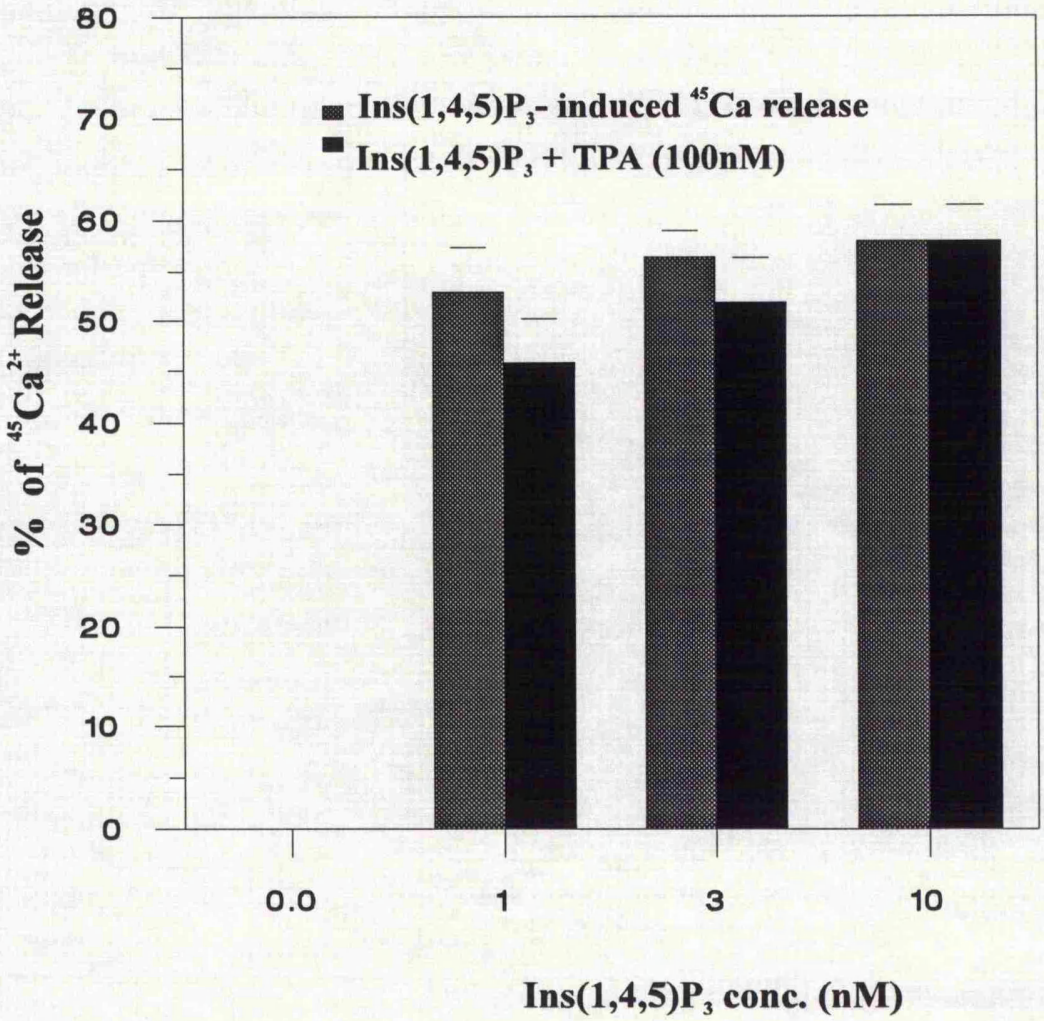


Fig. 5.8. Effect of TPA on Ins(1,4,5)P_3- induced Ca^{2+} release from saponin permeabilised chromaffin cells. Cells were pre-treated with TPA for 8 minutes and loaded with $^{45}CaCl_2$ in the presence of TPA when it was applied. TPA did not appear to affect the Ins(1,4,5)P_3 response. TPA had no significant effect on the Ca^{2+} released by 1, 3, or 10 μM of Ins(1,4,5)P_3 ($P>0.05$). TPA concentration was 100 nM. ATP was present at 5 mM. $[Ca^{2+}]_i$ levels were buffered to 120 nM. Ins(1,4,5)P_3 was left to stimulate the cells for 1 min. Results are mean values ± S.E.M. of three experiments each performed in triplicate.

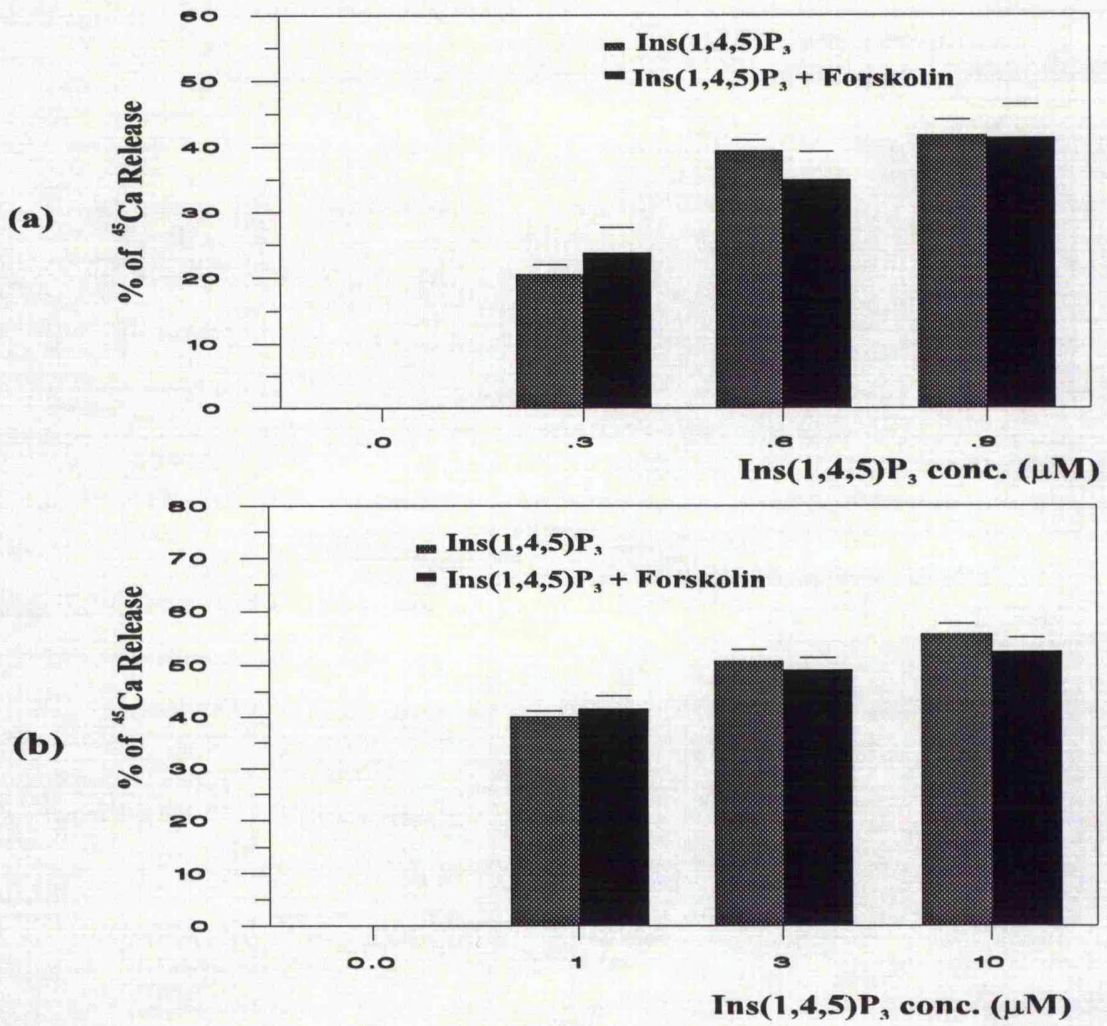


Fig. 5.9. Effect of forskolin on Ins(1,4,5)P_3-induced Ca^{2+} release from saponin-permeabilised chromaffin cells. (a) Forskolin had no significant effect on Ca^{2+} released by low doses of Ins(1,4,5)P_3 (0.3, 0.6, and 0.9 μM, P>0.05). Figure (b) demonstrates that this was also the case for higher doses of Ins(1,4,5)P_3 (1, 3, 10 μM). Forskolin concentration was 10 μM. ATP was present at 5 mM. $[Ca^{2+}]_i$ levels were buffered at around 120 nM. Ins(1,4,5)P_3 stimulation was present for 1 min. Results are mean values ± S.E.M. of three experiments each performed in triplicate.

5.2.3 Agonist Effects.

Attempts were made to study the effect of agonist induced Ca^{2+} release from saponin-permeabilised chromaffin cells. Measurement of total inositol phosphates was performed as described in Chapter 2 section 10. Fig. 5.10 shows that bradykinin is able to stimulate inositol phosphate production. Although bradykinin did stimulate inositol phosphate production, the findings were not clear as to where bradykinin could induce a Ca^{2+} release from permeabilised chromaffin cells. A limited number of experiments indicated Ca^{2+} release from internal stores but most of them exhibited no effect. Previous reports from our laboratory have shown that bradykinin induced a rapid increase and decrease in Ins(1,4,5)P_3 concentration (Challiss et al., 1991) which were not due to receptor desensitisation since bradykinin produced a linear (for at least 60 minutes) accumulation of inositol phospholipids (Plevin and Boarder, 1988). The most logical explanation for bradykinin having no effect on Ins(1,4,5)P_3-induced Ca^{2+} release is that permeabilisation of the cells disturbed associations which exist in intact cells.

5.2.4 Role of ATP.

ATP has been reported to have a biphasic effect on the Ins(1,4,5)P_3 receptor. At concentrations between 1 and 100 μM (Ferris and Snyder, 1992; Ehrlich and Watras, 1988) ATP increases the ability of Ins(1,4,5)P_3 to mobilise Ca^{2+} from intracellular stores, while, at concentrations higher than 100 μM it has no effect on the Ins(1,4,5)P_3-induced Ca^{2+} release. However, Ca^{2+} release from the ryanodine-sensitive Ca^{2+} store is enlarged with millimolar ATP concentrations (Smith et al., 1985; Meissner, 1994).

The effect of different concentrations of ATP and intracellular $[Ca^{2+}]_i$ levels on the Ins(1,4,5)P_3- and caffeine- induced Ca^{2+} release was examined. Data indicates that different concentrations of ATP affect the Ins(1,4,5)P_3- and the caffeine- induced Ca^{2+}

release (see Fig. 5.11). The Ins(1,4,5)P_3 response appeared to be enlarged when physiological concentrations of 5 mM ATP were used, but the caffeine response was diminished. In conditions where no added ATP was present Ca^{2+} release in response to caffeine was increased, but the response to Ins(1,4,5)P_3 was diminished (see Fig. 5.11). Findings in this Chapter do not agree with the proposed effect of ATP binding on the Ins(1,4,5)P_3 and the ryanodine receptor indicating that ATP had a different effect on the Ins(1,4,5)P_3 and caffeine on Ca^{2+} release in saponin-permeabilised chromaffin cells.

5.2.5 Role of Ca^{2+}.

Calcium has been proposed to have a regulatory role on both the Ins(1,4,5)P_3 and the ryanodine receptor. The effects of various $[Ca^{2+}]_i$ levels on the Ins(1,4,5)P_3- and caffeine- induced Ca^{2+} release were also studied. Raising the free Ca^{2+} levels in the loaded and permeabilised cells from 100 nM to 800 nM had opposite effects on the release elicited by the two stimuli. Release in response to Ins(1,4,5)P_3 was reduced, while that in response to caffeine was increased (see Fig. 5.12). There was no effect on the amount of $^{45}Ca^{2+}$ released by ionomycin. These findings indicate that Ca^{2+} levels differently regulate Ins(1,4,5)P_3- and caffeine-induced Ca^{2+} mobilisation from saponin-permeabilised chromaffin cells.

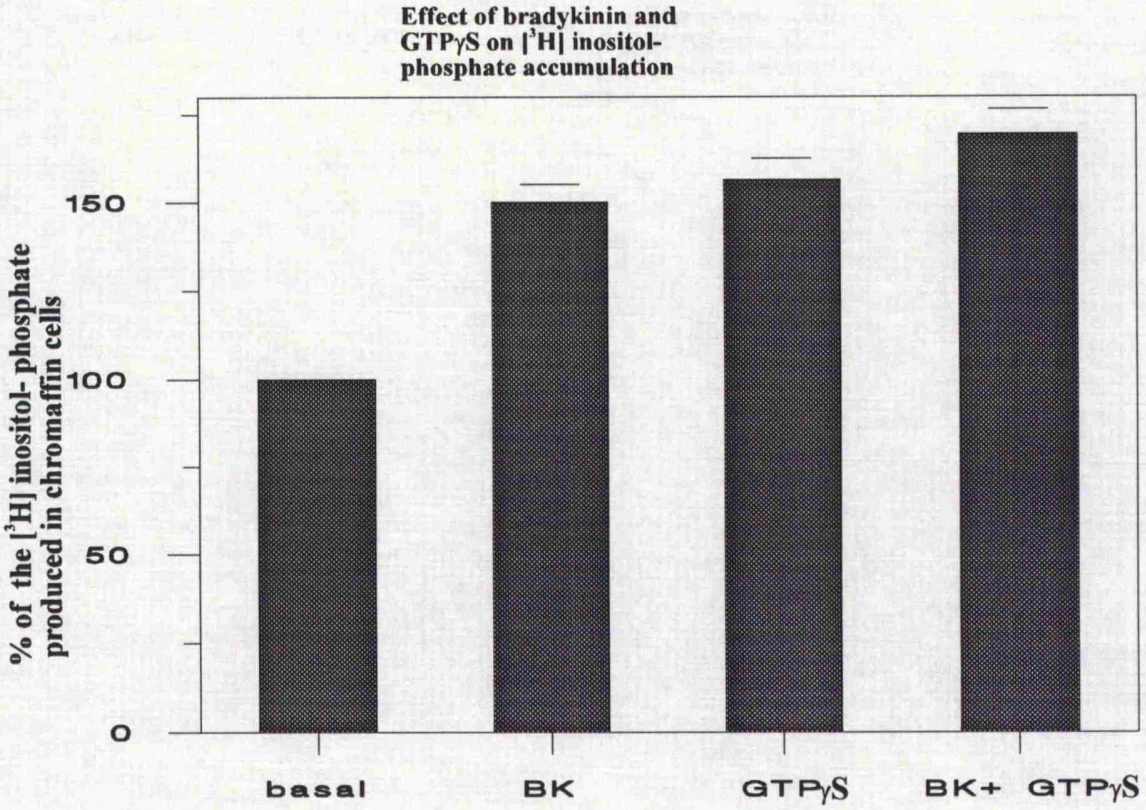


Fig. 5.10. Effect of bradykinin and GTPγS on [^{3}H] inositol phosphate production from saponin permeabilised chromaffin cells. Chromaffin cells were preloaded with 4 μCi/ well of [^{3}H] inositol phosphates. This figure shows that bradykinin and GTPγS stimulate production of total inositol phosphates. Bradykinin concentration was 100 nM. GTPγS concentration was 100 μM. Both bradykinin and GTPγS stimulation was for 5 mins. Results are mean values ± S.E.M. of one experiment representative of three similar experiments each performed in triplicate. Results were significantly different from control ($P<0.05$).

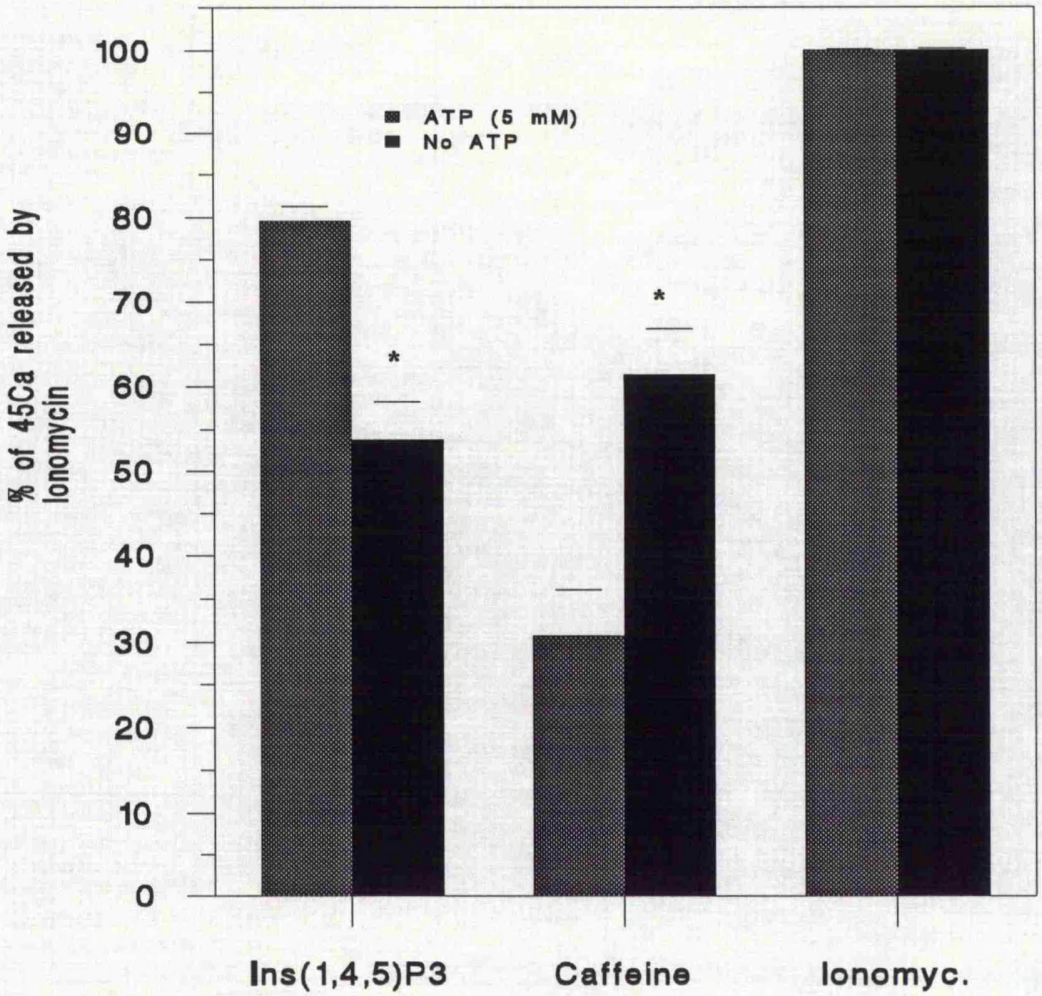


Fig. 5.11. Effect of ATP on Ins(1,4,5)P_3- and caffeine-induced Ca^{2+} release from saponin-permeabilised chromaffin cells. Ins(1,4,5)P_3 response was decreased in the absence of added ATP. Caffeine response was less in the presence of 5 mM ATP. $^{45}CaCl_2$ release is expressed as a percentage of the $^{45}CaCl_2$ released by Ionomycin. In these experiments Ionomycin resulted in release of 68.59% $\pm$ 4.7 and 61.69% $\pm$ 2.9 of the $^{45}Ca^{2+}$ sequestered into the intracellular Ca^{2+} stores of chromaffin cells in the presence or absence of ATP, respectively (mean $\pm$ S.E.M., n=5). $[Ca^{2+}]_i$ was buffered to less than 100 nM. Ins(1,4,5)P_3 concentration was 3 μM and caffeine was 50 mM. Ionomycin concentration was 1 μM. The cells were stimulated for 4 mins with Ins(1,4,5)P_3 and caffeine. Results are mean $\pm$ S.E.M. of five experiments each determined in triplicate ($P<0.05$).

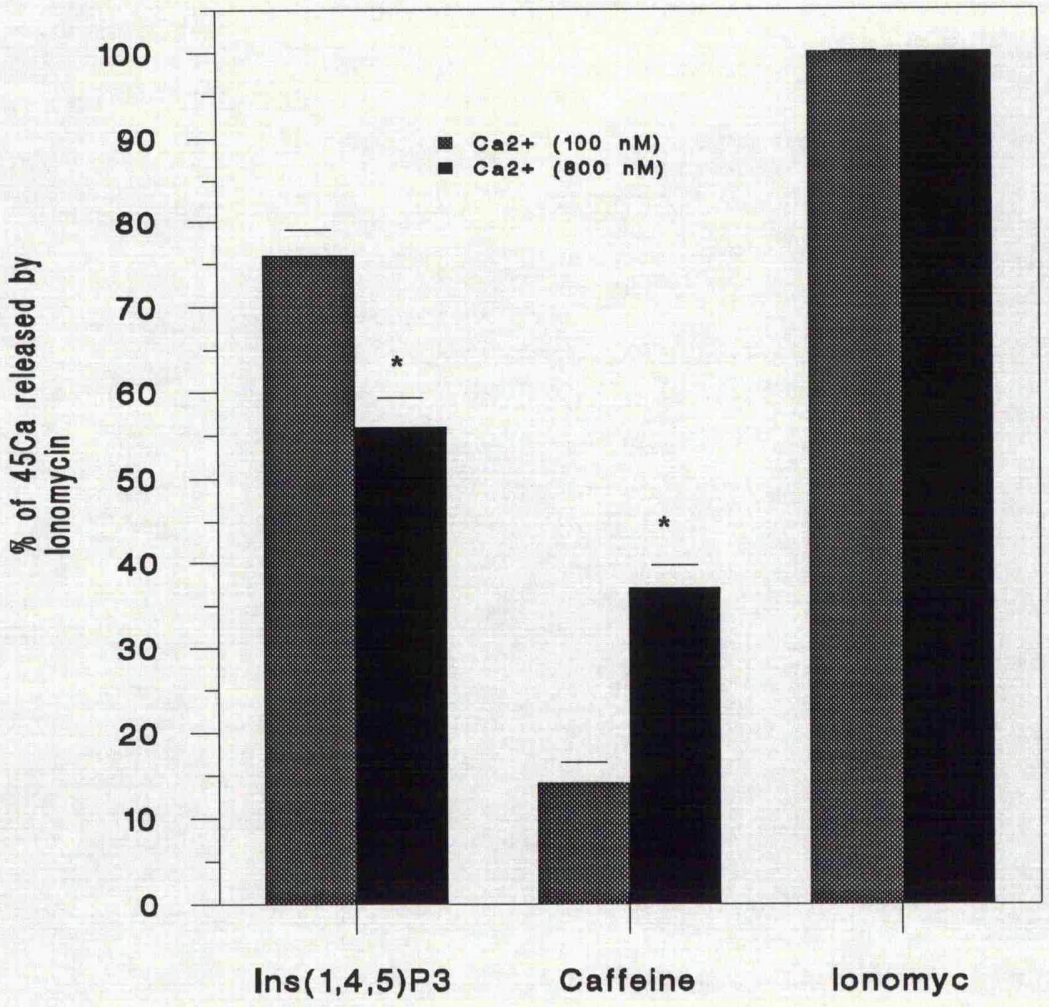


Fig. 5.12. Effect of $[Ca^{2+}]_i$ on Ins(1,4,5)P_3- and caffeine- induced Ca^{2+} release from saponin permeabilised chromaffin cells. The Ca^{2+} release is expressed as a percentage of the $^{45}CaCl_2$ released by ionomycin. In these experiments Ionomycin resulted in release of 66.16% $\pm$ 5.7 and 63.11% $\pm$ 3.4 of the $^{45}Ca^{2+}$ sequestered into the intracellular Ca^{2+} stores of chromaffin cells in the presence of low (100 nM) or high (800 nM) levels of $[Ca^{2+}]_i$, respectively (mean $\pm$ S.E.M., n=6). Ins(1,4,5)P_3 concentration was 3 μM and caffeine concentration was 50 mM. Ionomycin concentration was 1 μM. Ins(1,4,5)P_3 and caffeine stimulation was for 4 mins. Results are mean $\pm$ S.E.M. of six experiments each determined in triplicate ($P<0.05$).

5.3 DISCUSSION

The results presented in the previous Chapter confirm that bovine adrenal chromaffin cells possess at least two distinct Ca^{2+} stores, one sensitive to Ins(1,4,5)P_3 and the other sensitive to caffeine. It has been previously reported that 3-4 μM of Ins(1,4,5)P_3 gave maximal Ca^{2+} responses in chromaffin cells (Stoehr et al., 1986; Kao, 1988) but the Ins(1,4,5)P_3 response has not been otherwise characterised in these cells. The Ins(1,4,5)P_3-induced Ca^{2+} release was dose- and time-dependent manner, as data in Fig. 5.1 and 5.2 demonstrated. When saponin-permeabilised chromaffin cells were challenged with Ins(1,4,5)P_3 for 30 seconds, Ca^{2+} mobilisation from intracellular stores was detected, consistent with the rapid-time course for Ins(1,4,5)P_3 binding to its receptor (see Berridge, 1993a). In the previous Chapter it was shown that electrically-permeabilised chromaffin cells exhibit both 3-kinase and 5-phosphatase activities in agreement with findings from SH-SY5Y cells (Safrany et al., 1991a and b). Results presented here demonstrated that binding of Ins(1,4,5)P_3 to the Ins(1,4,5)P_3 receptor channel stimulates Ca^{2+} release, and that release was maintained for a time period of 5 minutes. These findings contrast with Ca^{2+}-selective electrode studies (Chapter 4) and indicate that the effect of the 3-kinase and 5-phosphatase was minimal because the number of cells used in these experiments was much lower (30 fold less) than the number used in the Ca^{2+}-selective electrode studies. High concentrations of cellular metabolic enzymes also resulted in rapid transient Ca^{2+} elevation in response to Ins(1,4,5)P_3 in studies on electrically permeabilised SH-SY5Y cells (Safrany et al., 1991b)

Maximal response was obtained with 3 μM Ins(1,4,5)P_3 in agreement with previous data (Kao, 1988). Previous studies reported that maximal release occurred at 4 μM, with half maximal release occuring at 1 μM (Stoehr et al., 1986). In this study the EC_{50} for Ins(1,4,5)P_3 was 619 $\pm$ 94 nM which is similar to 500 nM reported in digitonin permeabilised chromaffin cells (Kao, 1988). The efficacy of Ins(1,4,5)P_3 in mobilising Ca^{2+} from permeabilised chromaffin cells agrees with findings from other

cell types (Berridge, 1987), although Ins(1,4,5)P_3 stimulates Ca^{2+} release with a lower EC_{50} in SH-SY5Y cells (Safrany et al., 1990 and 1991a and b; Wojcikiewicz and Nahorski, 1991; Wilcox et al., 1993a).

In this study caffeine evoked Ca^{2+} release from permeabilised chromaffin cells and maximal release was obtained with 50 mM caffeine. The EC_{50} for caffeine was 16.59 $\pm$ 1.33 mM, indicating that caffeine has a low affinity for stimulating Ca^{2+} release in chromaffin cells. Since the completion of this study evidence supporting these findings has been reported. In a study using populations of fura-2 loaded chromaffin cells (Cheek et al., 1993b) maximal caffeine response occurred at around 40 mM with an EC_{50} of 11 mM and 13 mM in the presence and absence of extracellular Ca^{2+} respectively. Findings from this study and their data (Cheek et al., 1993b) demonstrate that the doses of caffeine (5-10 mM) used in previous studies (Cheek et al., 1991; Stauderman et al., 1991; Robinson and Burgoyne, 1991a) were not maximal. Although caffeine has a number of indirect effects, as discussed in Chapter 4, it was necessary to use maximal dose in order to deplete the caffeine-sensitive Ca^{2+} stores and to examine the relationship between the two stores.

Addition of 1 μM thapsigargin during the loading of chromaffin cells with $^{45}CaCl_2$ resulted in a much smaller uptake, as was expected due to its inhibiting action on the Ca^{2+}-ATPase of the Ins(1,4,5)P_3-sensitive stores. Addition of Ins(1,4,5)P_3 after prior treatment with thapsigargin did not induce a Ca^{2+} release, indicating that thapsigargin, by blocking the Ca^{2+}-ATPase, did not allow the Ins(1,4,5)P_3-sensitive stores to sequester $^{45}CaCl_2$. A maximal dose of caffeine resulted in a further Ca^{2+} mobilisation indicating the presence of an exclusive caffeine-sensitive Ca^{2+} store. These findings indicate that the Ins(1,4,5)P_3-sensitive Ca^{2+} stores had been depleted during the four minutes of thapsigargin treatment, whereas the caffeine-sensitive Ca^{2+} stores had not, in agreement with the two pool model. These results do not necessarily suggest that Ins(1,4,5)P_3- and caffeine- sensitive Ca^{2+} stores possess different Ca^{2+}-ATPases. Another explanation could be that these results reflect differences in the nature of these stores. It is possible that Ins(1,4,5)P_3-sensitive Ca^{2+} stores leak Ca^{2+}

more rapidly than the caffeine-sensitive stores. Whatever the case is the results described in this study point to the existence of two different Ca^{2+} stores.

The sarco(endo)plasmic reticulum Ca^{2+}-ATPase proteins (SERCA) are responsible for Ca^{2+} accumulation in the intracellular Ca^{2+} stores. Three isoforms have been identified, indicating that functional differences between these pumps may exists. The Ins(1,4,5)P_3-sensitive store contains a Ca^{2+}-ATPase which is believed to be a member of the SERCA family, as recent data has shown all the Ca^{2+}-ATPases belonging to that family are sensitive to thapsigargin (Lytton et al., 1991).

Findings from this data may agree with the possibility that chromaffin cells possess two distinct Ca^{2+}-ATPases for each of the Ins(1,4,5)P_3- and caffeine-sensitive stores (Burgoyne et al., 1989b). The question of which Ca^{2+}-ATPase is expressed in the caffeine-sensitive Ca^{2+} stores still remains unclear. Other explanations suggesting that these stores may use different Ca^{2+}-uptake mechanisms can also be taken into consideration since in pancreatic acinar cells a $2H^+/Ca^{2+}$ exchange mechanism has been proposed to be responsible for Ca^{2+} sequestration in the Ins(1,4,5)P_3-sensitive Ca^{2+} stores (Thevenod et al., 1989). There is no evidence to support such a mechanism in chromaffin cells.

When the caffeine-sensitive Ca^{2+} store had been depleted, challenge of chromaffin cells with Ins(1,4,5)P_3 evoked Ca^{2+} release, indicating that permeabilised chromaffin cells also possess an exclusive Ins(1,4,5)P_3-sensitive Ca^{2+} store. Stimulation of chromaffin cells with both Ins(1,4,5)P_3 (3 μM) and caffeine (50 mM) simultaneously (Ins(1,4,5)P_3/caffeine) resulted in Ca^{2+} release which was larger than that obtained with Ins(1,4,5)P_3 or caffeine alone. These results indicate that two Ca^{2+} stores exist in chromaffin cells. If these two pools were distinct and non-overlapping the response to Ins(1,4,5)P_3/caffeine should not be significantly different from the product of Ins(1,4,5)P_3 and caffeine when added alone. However, findings from this study indicated that Ins(1,4,5)P_3/caffeine response was significantly different from the product (Ins(1,4,5)P_3 + caffeine). This data suggests that Ins(1,4,5)P_3- and caffeine-

sensitive Ca^{2+} stores are not separate and distinct but a degree of functional overlap between these two stores also exists in saponin-permeabilised chromaffin cells.

Although caffeine has been widely used in the study of ryanodine receptors other effects of caffeine have been recently demonstrated. These effects involve inhibition of Ins(1,4,5)P_3 generation and increase of cAMP concentration (Missiaen et al., 1992; Zacchetti et al., 1991), suggesting that the use of caffeine should always be undertaken with caution. In this study ryanodine was also used in order to investigate where the caffeine effect was due to mobilising Ca^{2+} release from the ryanodine-sensitive Ca^{2+} stores and not to indirect effects of caffeine. Ryanodine, as discussed previously, has been reported to selectively inhibit the caffeine-induced Ca^{2+} release. This effect of ryanodine on the caffeine-evoked Ca^{2+} release has been shown in chromaffin cells (Teraoka et al., 1991; Robinson and Burgoyne 1991a). Their data showed that ryanodine alone had no effect on Ca^{2+} release but inhibited the caffeine response, indicating that ryanodine-sensitive Ca^{2+} stores exist in chromaffin cells. High affinity binding sites (K_d= 3.3 nM) for ryanodine with low binding site density (25 fmol/mg of protein) were detected in membranes from chromaffin cells (Stauderman and Murawsky, 1991) demonstrating the existence of ryanodine-sensitive Ca^{2+} release mechanism in chromaffin cells.

Experiments in Fig. 5.6 illustrated that caffeine response was completely abolished after prior treatment with ryanodine (10 μM), indicating the existence of a ryanodine receptor, and that caffeine stimulated calcium release from the ryanodine-sensitive Ca^{2+} store in permeabilised chromaffin cells. Ins(1,4,5)P_3 was still able to stimulate Ca^{2+} mobilisation after prior depletion of the caffeine-sensitive Ca^{2+} stores by ryanodine indicating that permeabilised chromaffin cells possess an exclusive Ins(1,4,5)P_3-sensitive Ca^{2+} store.

If the Ins(1,4,5)P_3- and caffeine- sensitive Ca^{2+} stores were distinct depletion of the ryanodine-sensitive Ca^{2+} store should not affect the Ins(1,4,5)P_3-induced Ca^{2+} release. As illustrated in Fig. 5.6 in the presence of ryanodine less Ca^{2+} was

sequestered into the intracellular Ca^{2+} stores due to the fact that ryanodine prevented filling of the caffeine-sensitive Ca^{2+} stores. There is also no evidence to demonstrate that ryanodine affects the filling of the Ins(1,4,5)P_3-sensitive Ca^{2+} store. Since less $^{45}Ca^{2+}$ was sequestered into the intracellular stores in the presence of ryanodine, and the amount of $^{45}Ca^{2+}$ mobilised by Ins(1,4,5)P_3 should not be affected by the presence of ryanodine (if these two stores were distinct and non-overlapping) the percentage of the Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ mobilisation should be expected to be increased. Findings presented in Fig. 5.6 demonstrate that the Ins(1,4,5)P_3 response was significantly reduced (almost 40%) after pretreatment with ryanodine, indicating that Ins(1,4,5)P_3- and ryanodine- sensitive Ca^{2+} stores may partially overlap in saponin-permeabilised chromaffin cells. Findings from this Chapter support findings from Chapter 4 and clearly demonstrate that chromaffin cells possess Ins(1,4,5)P_3- and caffeine- exclusive sensitive Ca^{2+} stores with a degree of overlap between them.

Another possible explanation is that chromaffin cells possess only one store (E.R.?) which expresses both Ins(1,4,5)P_3 and ryanodine-receptors. If the Ins(1,4,5)P_3 receptors are clustered in one pole of this store while the ryanodine receptors are clustered in the other pole, challenge of the store with Ins(1,4,5)P_3 or ryanodine may mobilise only a part of the Ca^{2+} content of the store, without any effect on the rest. This mechanism could explain some of the findings presented in this Chapter. However, results obtained in this study using thapsigargin and ryanodine suggested that Ins(1,4,5)P_3 receptor-expressing stores may be physically different from ryanodine receptor-expressing stores in chromaffin cells (two pool model), indicating a pharmacological differentiation of these stores.

The presence of a ryanodine receptor in chromaffin cells is now well documented (Teraoka et al., 1991; Stauderman and Murawsky, 1991). This receptor is believed to resemble the cardiac type 2 ryanodine receptor, since the type 1 (skeletal type) is only expressed in the cerebellum, and type 3 is thought not to be sensitive to caffeine (Giannini et al., 1992; Meissner, 1994; Pozzan et al., 1994). The effect of

cyclic ADP-ribose, which is believed to activate only type 2 receptors (see Lee, 1993; Berridge, 1993b; Galione, 1994;), was also investigated in permeabilised chromaffin cells. Cyclic ADP-ribose has been shown to stimulate Ca^{2+} mobilisation from the ryanodine-sensitive stores in sea urchin eggs (Dargie et al., 1990; Galione et al., 1991), pituitary cells (Koshiyama et al., 1991) and dorsal root ganglion cells (Currie et al., 1992). Its mobilising action has been reported to be due to its effect on the non-skeletal type of the ryanodine receptor (Meszaros et al., 1993). Their data has shown that cyclic ADP-ribose did not induce any Ca^{2+} release from the skeletal sarcoplasmic reticulum although caffeine can elicit Ca^{2+} release from both. It has also been reported that cyclic ADP-ribose activates the cardiac isoform of the ryanodine receptor channel at low submicromolar $[Ca^{2+}]_i$ levels (Meszaros et al., 1993) suggesting that it may participate in an alternative or synergistic mechanism to the CICR.

It seems likely that cyclic ADP-ribose has a role in Ca^{2+} signalling in some cell types. Whether it functions as a second messenger or has a regulatory effect on calcium signalling is not yet clear. Data in this study suggests that cyclic ADP-ribose does not affect caffeine-induced Ca^{2+} release. The inconsistencies observed between an apparent effect in the first experiments and no effect later is unexplained. However, a negative conclusion seems the only supportable one. Data recently reported suggested that cyclic ADP-ribose may simply be a substitute for ATP (Sitsapesan et al., 1994) indicating that this molecule is unlikely to have a second messenger role. A recent study also demonstrated that cyclic ADP-ribose had no effect on cardiac or skeletal muscle ryanodine receptor, indicating that the effect of cyclic ADP-ribose may occur through different pathways than the ryanodine receptor channels (Fruen et al., 1994).

As discussed previously, phosphorylation of the $Ins(1,4,5)P_3$ receptor has been proposed to have a regulatory role. The effect of phosphorylation on the $Ins(1,4,5)P_3$ receptor stimulated Ca^{2+} release from intracellular stores was studied in chromaffin cells with the use of TPA and forskolin. It has been previously shown that treatment

with TPA cause a stimulation of protein kinase C activity in chromaffin cells (Terbush and Holz, 1986). Experiments in Fig. 5.8 and Fig. 5.9 indicated that TPA and forskolin had no significant effect on the Ins(1,4,5)P_3-induced Ca^{2+} release from permeabilised chromaffin cells, within the limits of these experiments. The effect of phosphorylation is controversial since studies on the cerebellar Ins(1,4,5)P_3 receptor revealed that cAMP-dependent phosphorylation decreased their sensitivity (Supattapone et al., 1988a; Berridge, 1993a; Pozzan et al., 1994) where in peripheral cells (rat hepatocytes) phosphorylation relieves the inhibition occuring under resting conditions (Joseph and Ryan, 1993).

In SH-SY5Y cells pretreatment with forskolin had no effect on Ins(1,4,5)P_3 induced Ca^{2+} release and pretreatment with PKC activators (PDbu: phorbol 12,13-dibutyrate) for 20 minutes or 24 hours did not affect the maximal amount of Ca^{2+} released by Ins(1,4,5)P_3 but significantly increased the EC_{50}, indicating that the effect of PKC was either due to phosphorylation on a separate site of the Ins(1,4,5)P_3 binding site, or to another protein associated with Ca^{2+} release from internal stores (Wojcikiewicz and Nahorski, 1991). Pretreatment with PKC activators (phorbol 12-myristrate 13-acetate) did not appear to have a role in receptor down regulation mediated by muscarinic m3 receptor activation in a later study in SH-SY5Y cells (Wojcikiewicz et al., 1992). Furthermore, forskolin did not activate phosphorylation on the type 1 Ins(1,4,5)P_3 receptor which is present on SH-SY5Y cells in studies on the effect of phosphorylation on down regulation of the Ins(1,4,5)P_3 receptor (Wojcikiewicz et al., 1994). However, no effect of phosphorylation on receptor function has yet been clearly demonstrated.

Molecular studies of the Ins(1,4,5)P_3 receptor revealed gene multiplicity and alternative splicing of the type 1 Ins(1,4,5)P_3 receptor (Mikoshiba et al., 1993; Pozzan et al., 1994). The expression of these splice subtypes is tissue specific and developmentally specific manner indicating that functional differences may occur between the type 1 Ins(1,4,5)P_3 receptor forms. Three of the SII forms are expressed

in the CNS but not in the bovine adrenal gland and other peripheral issues, where the Ins(1,4,5)P_3RSIIABC- is expressed (Nakagawa et al., 1991; Mikoshiba et al., 1993). The SII domains are believed to be related with the phosphorylation sites of the Ins(1,4,5)P_3 receptor since they are located between the two serine residues (amino acids 1588 and 1755), the sites at which the Ins(1,4,5)P_3 receptor is suggested to be phosphorylated. Alternative splicing of these domains may reflect different effects of phosphorylation. The insensitivity of the Ins(1,4,5)P_3 receptor in chromaffin cells to TPA and forskolin probably reflects insensitivity of the SIIABC- subtype (which lacks the SIIABC domain) to phosphorylation and suggests that other mechanisms may be involved in the regulation of the Ins(1,4,5)P_3 receptor in chromaffin cells.

Experiments as presented in Fig. 5.11 and Fig. 5.12 demonstrate that different concentrations of ATP and $[Ca^{2+}]_i$ levels affect the Ins(1,4,5)P_3- and caffeine induced Ca^{2+} release. The effect of ATP seen in this study is not in line with the proposed regulatory role of ATP binding to the Ins(1,4,5)P_3 and ryanodine receptor, indicating that alternative regulatory mechanisms are in operation in chromaffin cells. In the experiments described in this study conditions were the same during $^{45}Ca^{2+}$ indicating that the effect of ATP detected on the Ins(1,4,5)P_3- and caffeine- induced Ca^{2+} mobilisation was probably due to the regulatory action of ATP on the Ins(1,4,5)P_3- and ryanodine- receptor. Findings from this study demonstrated that ATP had a different effect on the Ins(1,4,5)P_3- and caffeine- induced Ca^{2+} release, indicating that chromaffin cells possess Ins(1,4,5)P_3 and ryanodine receptors and therefore Ins(1,4,5)P_3- and caffeine- sensitive Ca^{2+} stores. The possibility that the effect of ATP detected in this study is due to ATP-dependent kinases (such as 3-kinase) cannot be ruled out.

Apart from phosphorylation, Ca^{2+} has a regulatory role on the Ins(1,4,5)P_3 receptor function. Data presented in Fig. 5.12 demonstrates the modulatory effect of $[Ca^{2+}]_i$ on the Ins(1,4,5)P_3 induced Ca^{2+} release. Elevation of $[Ca^{2+}]_i$ levels reduced

the Ins(1,4,5)P_3 response and Ins(1,4,5)P_3 was not able to stimulate Ca^{2+} mobilisation when $[Ca^{2+}]_i$ levels exceeded 1 μM. This data agrees with previous data showing that Ins(1,4,5)P_3 is dependent on cytosolic calcium and displays a bell-shaped curve (Bezprozvanny et al., 1991; Iino and Endo, 1992).

Ca^{2+} release from the ryanodine receptor is activated at micromolar $[Ca^{2+}]_i$ and inhibited in millimolar $[Ca^{2+}]_i$ levels (Bezprozvanny et al., 1991). In the experiments designed in this Chapter 800 nM $[Ca^{2+}]_i$ were used so no effect should be expected due to cytosolic calcium levels on the caffeine-induced Ca^{2+} release. However, the caffeine response was enlarged under these conditions. Since the Ca^{2+} levels during the $^{45}Ca^{2+}$ loading were the same these findings indicate that Ca^{2+} has a regulatory role on the caffeine-induced Ca^{2+} mobilisation via the ryanodine receptor. These results also suggest that chromaffin cells possess two distinct Ca^{2+} stores which are differently regulated by Ca^{2+} and ATP.

Data presented here demonstrates that saponin-permeabilised chromaffin cells possess Ins(1,4,5)P_3- and caffeine- sensitive Ca^{2+} stores, and provides strong evidence that these stores overlap as findings from electrically-permeabilised chromaffin cells presented in Chapter 4 showed. It is possible that chromaffin cells possess Ins(1,4,5)P_3 receptor-expressing stores, ryanodine receptor-expressing stores and stores that express both these receptors. This model suggests a degree of physical overlap between these stores which may account for findings presented here. An alternative explanation is that a degree of functional overlap may exists between these stores. CICR mechanism or second messengers (cADP-ribose?) could be responsible for this communication.

It has been previously suggested that permeabilisation of the cells disrupts the continuity of the endoplasmic reticulum (Renard-Rooney et al., 1993). For this reason the relationship between the intracellular Ca^{2+} stores was next examined using intact chromaffin cells to ascertain whether the findings obtained using permeabilised cells are a true reflection of the relationship between the stores in intact cells.

The following figure 5.13 summarises Ca^{2+} homeostasis in permeabilised chromaffin cells. It demonstrates the characteristics of the Ins(1,4,5)P_3-sensitive and caffeine-sensitive Ca^{2+} stores. It can be seen that Ins(1,4,5)P_3- stimulates the opening of the Ins(1,4,5)P_3 receptor and that this response is decreased by high $[Ca^{2+}]_i$ levels. The effects of the Ca^{2+}-ATPase inhibitor thapsigargin on the Ins(1,4,5)P_3-sensitive store are exhibited. Caffeine is shown to release Ca^{2+} from the caffeine-sensitive Ca^{2+} store and this response can be blocked by ryanodine. The effect of ATP on Ins(1,4,5)P_3- and caffeine-induced Ca^{2+} mobilisation from intracellular Ca^{2+} stores is also illustrated. The possibility that cyclic-ADP-ribose can stimulate Ca^{2+} release from the caffeine-sensitive Ca^{2+} store is also considered. The two Ca^{2+} sensitive stores have been plotted separately although data present in this Chapter using permeabilised chromaffin cells provided strong evidence for a degree of functional overlap.

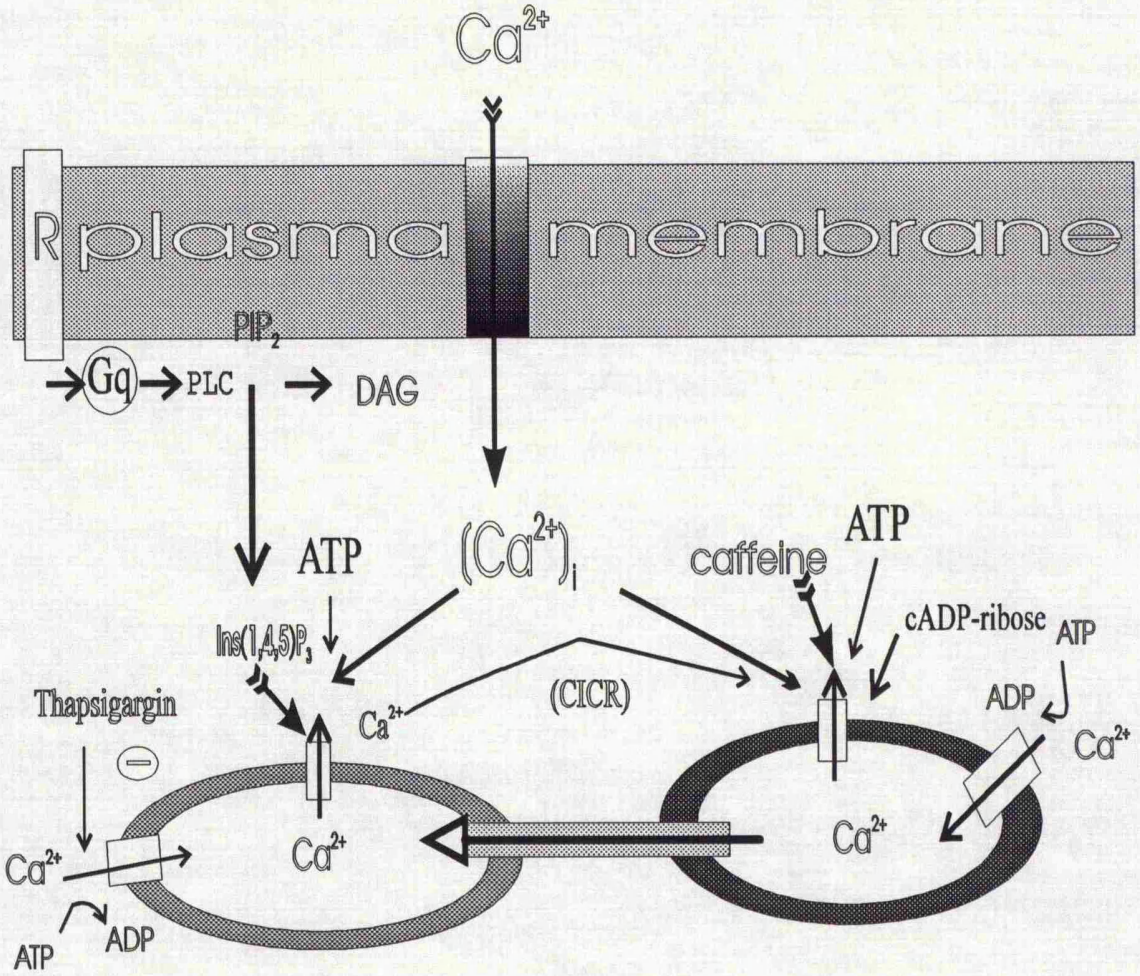


Fig. 5.13. This diagram is a schematic representation of Ca^{2+} homeostasis in permeabilised bovine adrenal chromaffin cells. It also summarises the characteristics of the Ca^{2+} stores within the chromaffin cells. Data from this Chapter confirmed that permeabilised chromaffin cells possess at least two Ca^{2+} stores one sensitive to Ins(1,4,5)P_3 and the other to caffeine. Thapsigargin inhibits the Ca^{2+}-ATPase of the Ins(1,4,5)P_3-sensitive store. The effect of elevated Ca^{2+} levels on the Ins(1,4,5)P_3 receptor and ryanodine receptor is demonstrated. Ryanodine selectively blocks Ca^{2+} release from the caffeine-sensitive store.

CHAPTER 6:

STUDIES ON INTACT BOVINE ADRENAL CHROMAFFIN CELLS.

6.1 INTRODUCTION

A number of studies have demonstrated that depolarisation can activate Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores (Kuba, 1980; Lipscombe et al., 1988, Friel and Tsien, 1992a and b). As discussed previously, Ca^{2+} mobilisation from internal stores has been cited as stimulating secretion in chromaffin cells in some studies but not others. Although some cells possess only Ins(1,4,5)P_3- or ryanodine-sensitive Ca^{2+} stores other cells, including chromaffin cells, possess both (Berridge, 1993a). In PC12 cells the caffeine-sensitive Ca^{2+} store is also sensitive to Ins(1,4,5)P_3 (Zacchetti et al., 1991; Reber et al., 1993; Barry and Cheek, 1994).

Experiments in this Thesis on permeabilised chromaffin cells revealed that these cells possess Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores. Earlier reports suggested that these stores are morphologically and functionally distinct (Burgoyne et al., 1989b; Robinson and Burgoyne, 1991a). However, data from this study also demonstrated that these stores partially overlap as was found in previous reports (Cheek et al., 1991; Liu et al., 1991; Stauderman et al., 1991). The presence of the caffeine-sensitive Ca^{2+} store is important because it implies the presence of the CICR mechanism which has been proposed to be involved in Ca^{2+} oscillations and Ca^{2+} wave propagation (Berridge, 1991 and 1993a). The role of the caffeine-sensitive Ca^{2+} stores in chromaffin cells is still not clear but a number of reports have demonstrated that it can act as a sink for increased cytoplasmic Ca^{2+} levels (Cheek et al., 1990) and a source of Ca^{2+} contributing to Ca^{2+} mobilisation (Stauderman and Murawsky, 1991; Stauderman et al., 1991).

Most of the previous studies examined the relationship between these stores in permeabilised chromaffin cells (Cheek et al., 1991; Liu et al., 1991; Robinson and Burgoyne, 1991a) without using a maximally effective dose of caffeine. Permeabilising the cells may affect the morphology of the cell and disrupt the continuity of the endoplasmic reticulum (Renard-Rooney et al., 1993) leading to misinterpretation. To examine whether this is a real reflection of the nature of the intracellular Ca^{2+} stores

and not a misinterpretation due to the permeabilising procedures, a number of experiments were carried out using both populations of, and single, intact adrenal chromaffin cells.

Another question confronted here was: whether depletion of the Ins(1,4,5)P_3- and the caffeine-sensitive Ca^{2+} stores could stimulate Ca^{2+} entry, as the capacitative entry theory predicts (Putney, 1986 and 1990). According to this model, agonist stimulated store depletion results in Ca^{2+} entry (Putney and Bird, 1993a and b). To this end a fluorimeter was employed to monitor changes in intracellular Ca^{2+} as indicated by the use of the fluorescent probe fura -2 (see Chapter 2, section 7.2). The Ca^{2+}-ATPase inhibitor, thapsigargin, (Thastrup et al., 1989) was used to deplete the Ins(1,4,5)P_3-sensitive stores. Thapsigargin, by inhibiting the Ca^{2+}-ATPase of the Ins(1,4,5)P_3-sensitive store, prevents refilling of the store. Since this store leaks calcium continually, treatment with thapsigargin results in Ins(1,4,5)P_3-sensitive Ca^{2+} store depletion (Thastrup et al., 1990).

Bradykinin and caffeine were used in order to examine the characteristics of the intracellular Ca^{2+} stores in intact single chromaffin cells. Challenge of chromaffin cells with bradykinin results in generation of Ins(1,4,5)P_3 and promotes a transient rise in the intracellular $[Ca^{2+}]_i$ which is biphasic, comprising a release of Ca^{2+} from the intracellular stores and Ca^{2+} entry across the plasma membrane. As previously discussed this Ca^{2+} entry is not believed to be through VSCCs and is thought to be activated by depletion of the intracellular Ca^{2+} stores. The mechanisms by which depletion of the intracellular Ca^{2+} stores regulate Ca^{2+} entry is still unknown (Penner et al., 1993; Putney, 1993; Putney and Bird, 1993a and b). Recent results demonstrated that different agonists induced different patterns of Ca^{2+} entry in chromaffin cells, indicating that these differences may be related to how various stimuli influence cellular function (Cheek et al., 1993a). Furthermore, histamine has been shown to stimulate Ca^{2+} entry which precedes Ca^{2+} mobilization from intracellular stores of chromaffin cells (Cheek et al., 1994b), suggesting that Ca^{2+} entry in response to histamine does not require depletion of the intracellular Ca^{2+} stores. Earlier

electrophysiological studies indicated that an Ins(1,4,5)P_3-gated Ca^{2+} channel exists in chromaffin cells (Mochizuki-Oda et al., 1991a). This channel could be activated by the addition of Ins(1,4,5)P_3 to the cytosolic side of cell attached patches. Therefore, it is possible that Ins(1,4,5)P_3 acts at the chromaffin cell plasma membrane in conjunction with Ca^{2+} stores depletion to activate Ca^{2+} entry. In this Thesis bradykinin and caffeine were used in order to further investigate the relationship between intracellular Ca^{2+} stores and how depletion of these stores activates Ca^{2+} entry.

One experimental approach when studying the relationship of the Ca^{2+} stores is to stimulate the cells with an Ins(1,4,5)P_3 producing agonist and then with caffeine. If the caffeine-sensitive Ca^{2+} store is part of the Ins(1,4,5)P_3-sensitive store the response to caffeine should be affected. The reverse procedure would indicate the effect of the caffeine-sensitive Ca^{2+} store on the Ins(1,4,5)P_3-induced Ca^{2+} release.

6.2 RESULTS

6.2.1 Populations of Intact Cells.

Changes in $[Ca^{2+}]_i$ from intact chromaffin cells in suspension were monitored fluometrically. The fluorescent dye fura-2AM was used to indicate changes in intracellular $[Ca^{2+}]_i$ of intact chromaffin cells. Autofluorescence was corrected before each experiment and F_{max} was determined by adding 1 μM of ionomycin and F_{min} was calibrated by adding sufficient EGTA at the end of each experiment. This calibration method (for more details see Chapter 2 section 7.2) gave reproducible results between different batches of cells.

Studies on intact chromaffin cells in suspension loaded with fura 2-AM demonstrated (Fig. 6.1) that bradykinin (200 nM) in the absence of extracellular calcium resulted in rapid $[Ca^{2+}]_i$ elevation of 203 ± 34 nM (mean ± S.E.M., n=4) which returned to the basal, without any evidence of a sustained phase (see also Table 6.1). This is a clear indication that bradykinin, which is an $Ins(1,4,5)P_3$ generating agonist, evoked Ca^{2+} mobilisation from intracellular Ca^{2+} stores. When bradykinin (100 nM) was added to the Ca^{2+} free medium it stimulated a Ca^{2+} release of 156 ± 23.27 nM (mean ± S.E.M., n=4) above basal, which was not statistically different to the response obtained with 200 nM, $P>0.1$.

When chromaffin cells were challenged with caffeine (50 mM), in a Ca^{2+} free medium, a rapid rise to a peak of $[Ca^{2+}]_i$ (146 nM ± 10.1, mean ± S.E.M., n=4, see Table 6.1 and Fig. 6.2) was detected, without any evidence of a sustained phase, indicating that caffeine evoked Ca^{2+} mobilisation from intracellular stores. The response to caffeine was not found to be significantly different to that of the bradykinin. This data, obtained from population of chromaffin cells, indicates that intact chromaffin cells possess $Ins(1,4,5)P_3$-and caffeine-sensitive Ca^{2+} stores in agreement with the findings from permeabilised chromaffin cells.

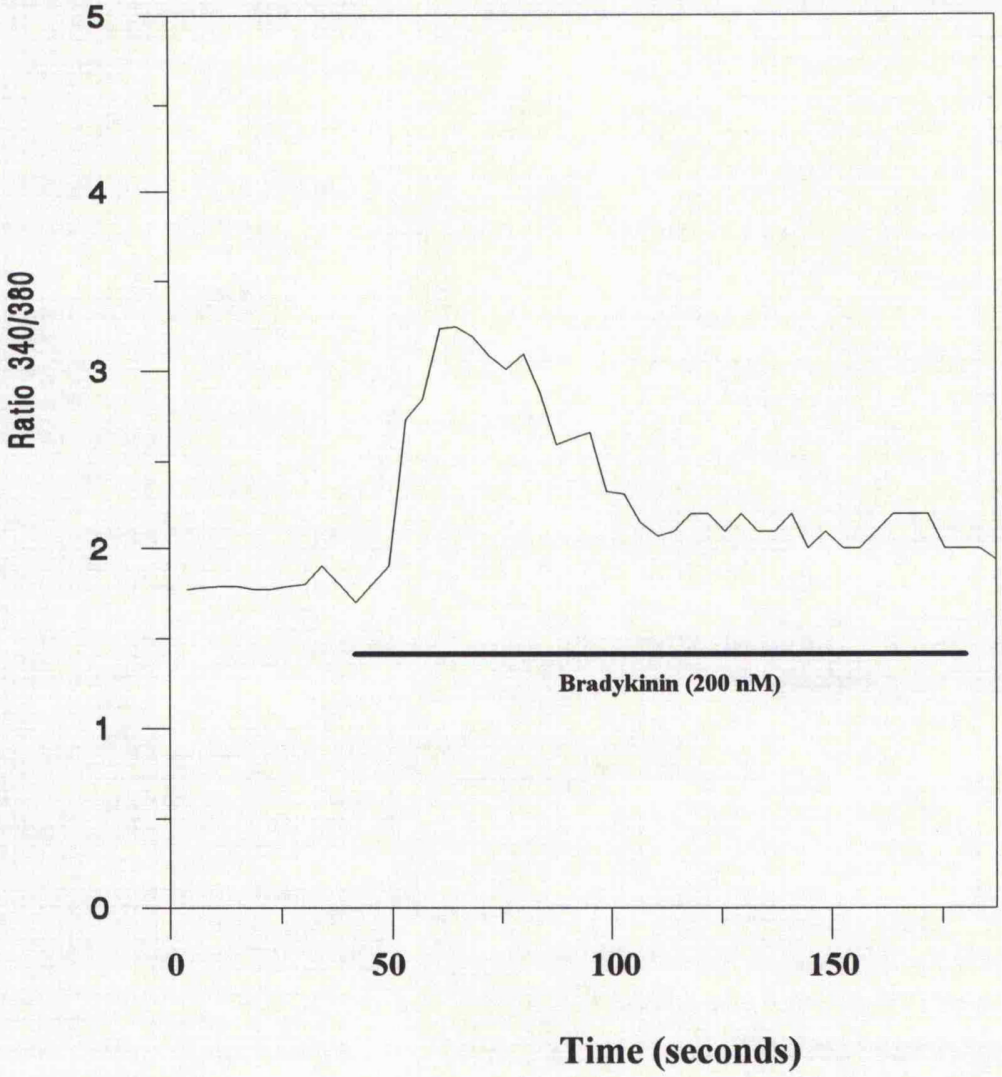


Fig. 6.1. Effect of bradykinin on Ca^{2+} release from populations of chromaffin cells. Intact chromaffin cells in suspension were loaded with fura-2AM. Bradykinin was used at 200 nM. The trace is representative of four experiments and results are expressed as 340/380nm ratio (excitation wavelengths, 3.8s changeover time). Calcium free buffer was used (measured at about 1 μM). Autofluorescence was subtracted in all experiments.

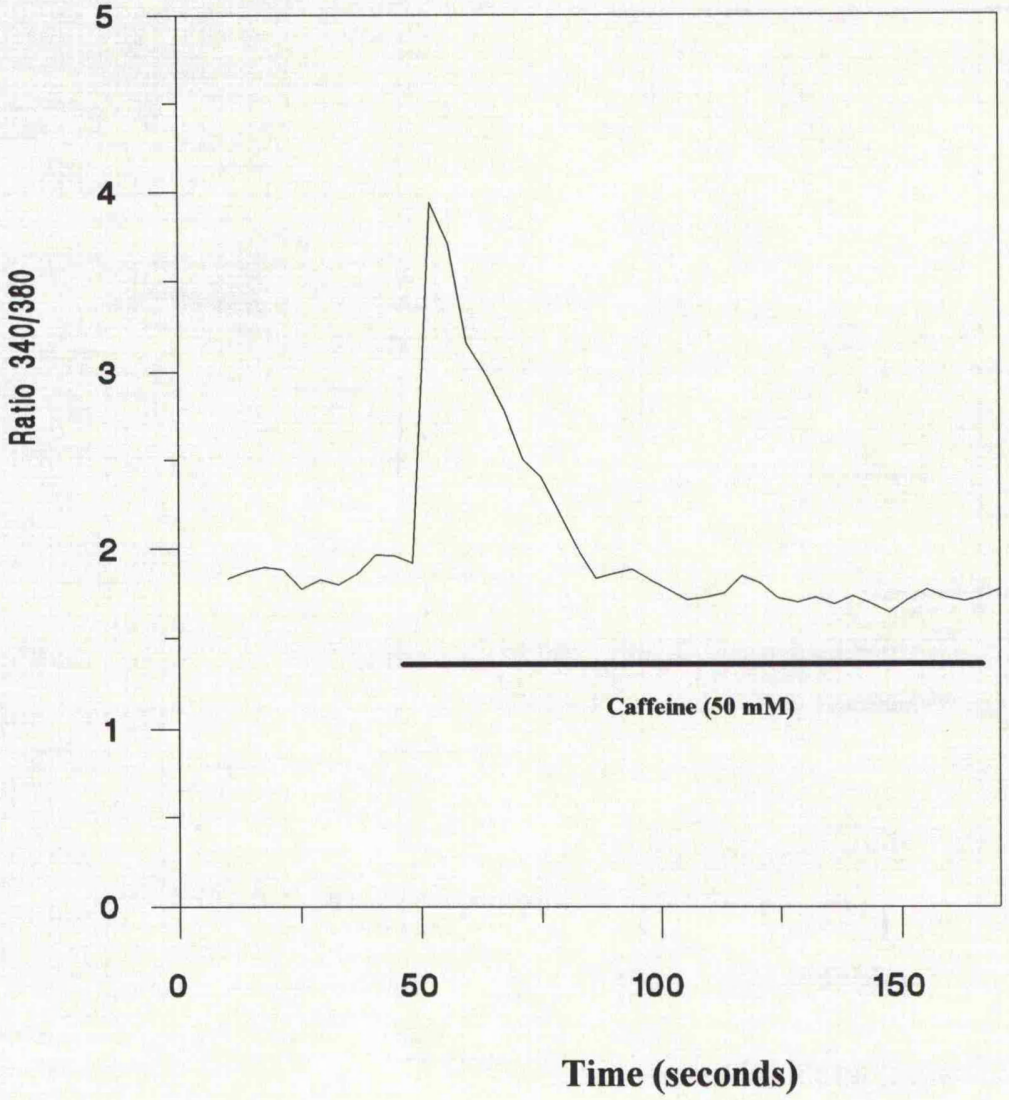


Fig. 6.2. Effect of caffeine (50 mM) on Ca^{2+} release from populations of chromaffin cells. Intact chromaffin cells in suspension were loaded with fura-2AM. Caffeine was used at 50 mM. The trace is representative of three experiments and results are expressed as 340/380nm ratio (excitation wavelengths, 3.8s changeover time). Calcium free buffer was used (measured at about 1 μM). Autofluorescence was subtracted in all experiments.

It has previously been demonstrated that thapsigargin depletes Ca^{2+} from the Ins(1,4,5)P_3-sensitive Ca^{2+} stores in bovine adrenal chromaffin cells (Cheek et al., 1989a; Cheek and Thastrup, 1989; Robinson and Burgoyne, 1991a and b; Robinson et al., 1992). This mobilising effect is due to its ability to inhibit the Ca^{2+}- ATPase responsible for refilling the Ins(1,4,5)P_3-sensitive store (Thastrup et al., 1990).

Challenge of populations of intact chromaffin cells with thapsigargin resulted in a slow $[Ca^{2+}]_i$ elevation (see Fig. 6.3). This slow increase in Ca^{2+} levels was followed by a sustained phase. The peak responses to thapsigargin were different to those of the Ins(1,4,5)P_3 generating agonist, as predicted, due to its Ca^{2+}-ATPase inhibiting action and its inability to release Ca^{2+} directly. Subsequent addition of maximal dose of bradykinin (100 nM) did not result in a statistically significant elevation of $[Ca^{2+}]_i$ above basal (22.3 $\pm$ 12.51 nM, mean $\pm$ S.E.M., n=3, see Table 6.1). This data indicates that bradykinin evoked Ca^{2+} mobilisation from the thapsigargin-sensitive Ca^{2+} store. Pretreatment with thapsigargin significantly reduced the subsequent caffeine (50 mM) response (see Fig. 6.4 and Table 6.1), indicating that depletion of the agonist-sensitive Ca^{2+} stores reduced the amount of Ca^{2+} released from the caffeine-sensitive Ca^{2+} stores. The caffeine response was not abolished providing evidence that intact chromaffin cells possess an exclusive caffeine-sensitive Ca^{2+} store.

Ryanodine has been shown (Chapter 5) to inhibit subsequent caffeine responses and to affect Ins(1,4,5)P_3- induced Ca^{2+} mobilisation from intracellular stores of saponin permeabilised chromaffin cells. Results presented in Fig. 6.5 demonstrate that challenge of intact chromaffin cells with ryanodine (10 μM) inhibits subsequent responses to caffeine (50 mM), indicating that ryanodine depleted the caffeine-sensitive Ca^{2+} stores (see also Table 6.1). This data suggests that caffeine evoked Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores. Pretreatment of cells with ryanodine did not abolish but significantly reduced the effect of subsequent addition of bradykinin (100 nM) providing strong evidence that intact chromaffin cells also possess an exclusive Ins(1,4,5)P_3-sensitive Ca^{2+} store (Fig. 6.6 and Table 6.1). These data suggested firstly that intact chromaffin cells possess a uniquely Ins(1,4,5)P_3-sensitive Ca^{2+} store,

secondly a store which is uniquely caffeine-sensitive and thirdly that functional interactions between these stores also exist.

In order to examine whether depletion of intracellular Ca^{2+} stores stimulates Ca^{2+} influx further experiments were carried out in population of intact chromaffin cells in suspension. Chromaffin cells were loaded with fura 2-AM in the presence of extracellular calcium and then washed with KHB (Ca^{2+} free) buffer. An appropriate amount of EGTA was added to achieve the desirable Ca^{2+} concentration (around 1 μ M). When Ca^{2+} (2 mM) was reintroduced to untreated chromaffin cells a small response was obtained (Fig. 6.7 and Table 6.2). Challenge of chromaffin cells with thapsigargin (1 μM) resulted in depletion of the Ca^{2+} content of the Ins(1,4,5)P_3-sensitive Ca^{2+} store in the absence of extracellular Ca^{2+}. When Ca^{2+} was reintroduced to the Ca^{2+} -free medium a second sustained response was obtained which was significantly larger than that of untreated cells (Fig. 6.8), indicating that depletion of the Ca^{2+} contents of the chromaffin cells Ins(1,4,5)P_3-sensitive stores stimulates Ca^{2+} entry. This data is consistent with the predictions of the capacitative entry model (Putney 1986, 1990).

In Ca^{2+} free conditions caffeine stimulation resulted in a rapid rise of $[Ca^{2+}]_i$ which returned to basal levels within 1 minute. When Ca^{2+} was reintroduced to the Ca^{2+} free medium, a sustained rise was detected similar to that obtained with thapsigargin (see Fig. 6.9 and Table 6.2). This clearly indicates that Ca^{2+} mobilisation from the caffeine-sensitive Ca^{2+} store also results in Ca^{2+} influx. Studies presented here provided strong evidence that Ca^{2+} mobilisation from intracellular stores of population of intact cells activates Ca^{2+} entry across the plasma membrane.

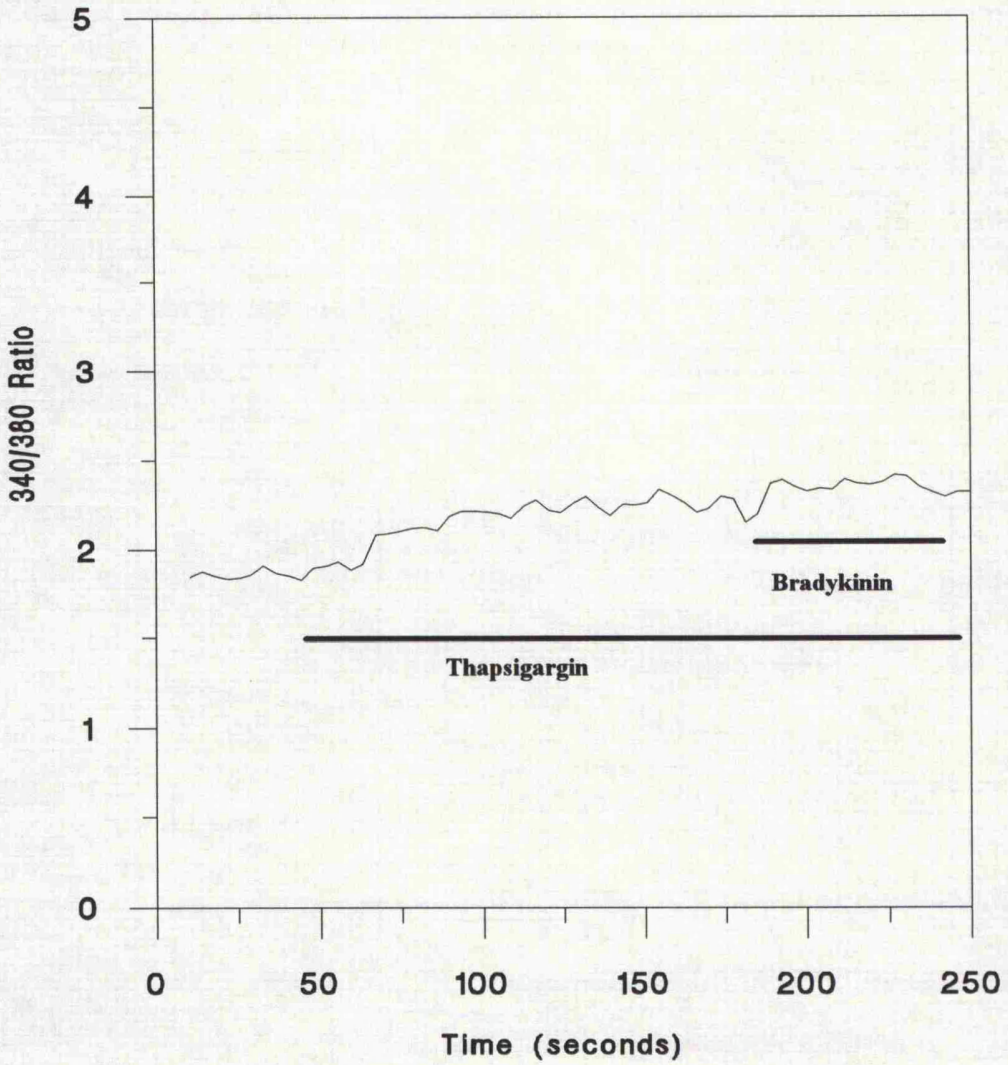


Fig. 6.3. Effect of thapsigargin on bradykinin response. Intact chromaffin cells in suspension were loaded with fura-2AM. Bradykinin was used at 100 nM and thapsigargin at 1 μM. The trace is representative of three experiments and results are expressed as 340/380nm ratio (excitation wavelengths, 3.8s changeover time). Calcium free buffer was used (measured at about 1 μM). Autofluorescence was subtracted in the experiments. Bradykinin stimulation in the absence of thapsigargin resulted in an elevation of $[Ca^{2+}]_i$ of 156 ± 23.27 nM (mean ± S.E.M. of four experiments, see also Fig. 6.1).

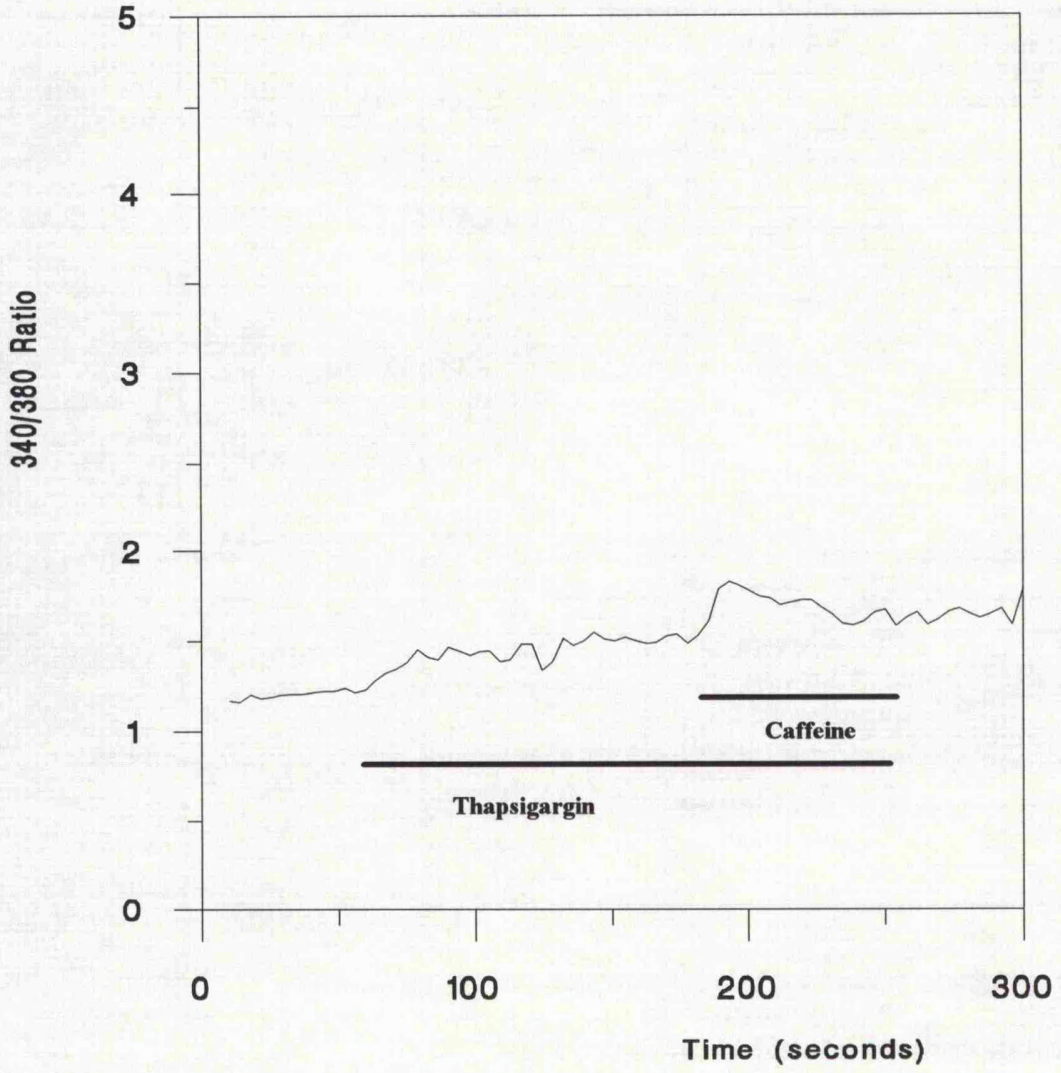


Fig. 6.4. Effect of thapsigargin on caffeine induced Ca^{2+} mobilisation from intracellular stores. Intact chromaffin cells in suspension were loaded with fura-2AM. Caffeine was used at 50 mM and thapsigargin at 1 μM. The trace is representative of four experiments and results are expressed as 340/380nm ratio (excitation wavelengths, 3.8s changeover time). Calcium free buffer was used (measured at about 1 μM). Autofluorescence was subtracted in the experiments. Caffeine stimulation in the absence of thapsigargin resulted in an elevation of $[Ca^{2+}]_i$ of 146 ± 10.14 nM (mean ± S.E.M. of four experiments, see also Fig. 6.2).

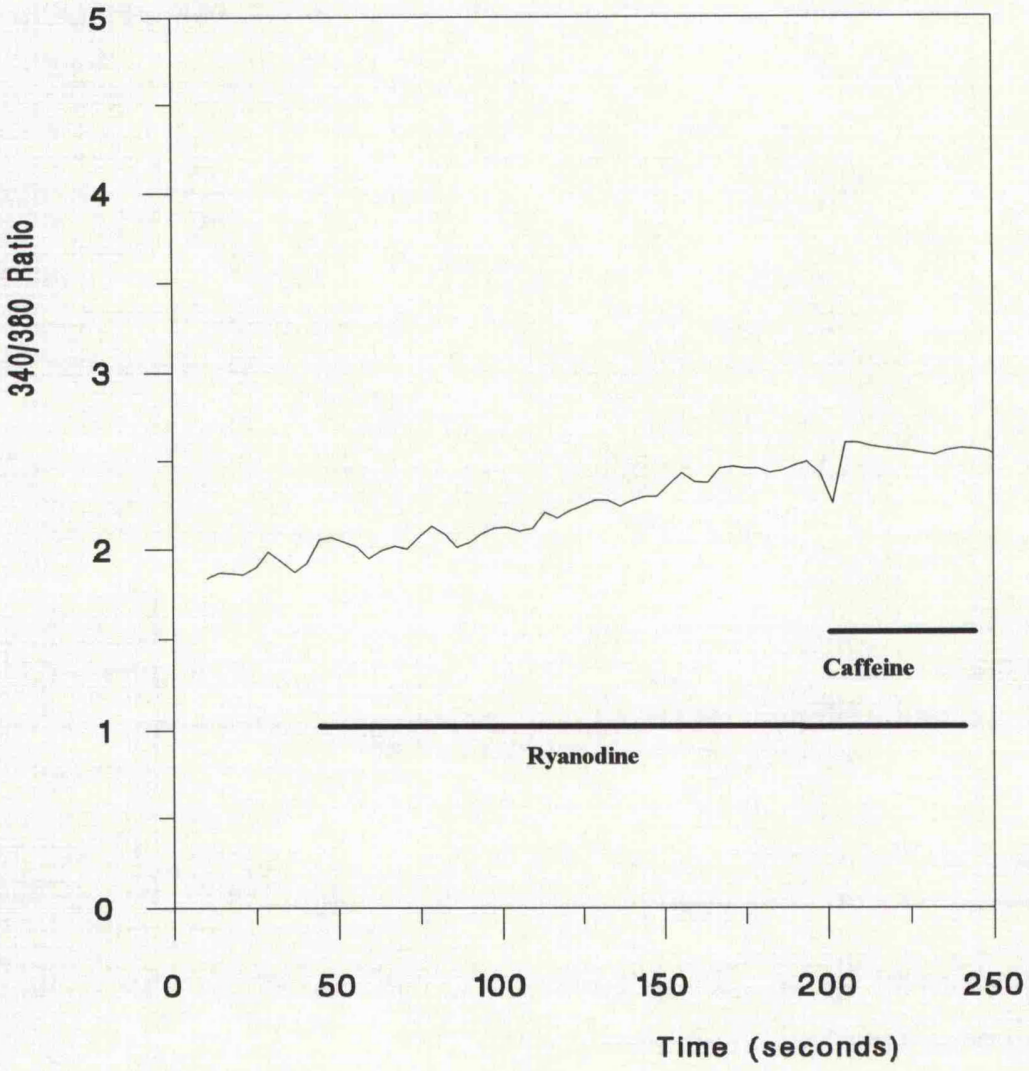


Fig. 6.5. Effect of ryanodine on caffeine response. Intact chromaffin cells in suspension were loaded with fura-2AM. Ryanodine was used at 10 μM and caffeine at 50 mM. The trace is representative of three experiments and results are expressed as 340/380nm ratio (excitation wavelengths, 3.8s changeover time). Calcium free buffer was used (found to be below 1 μM). Autofluorescence was subtracted in the experiments. Caffeine stimulation in the absence of ryanodine resulted in an elevation of $[Ca^{2+}]_i$ of 146 ± 10.14 nM (mean ± S.E.M. of four experiments, see also Fig. 6.2).

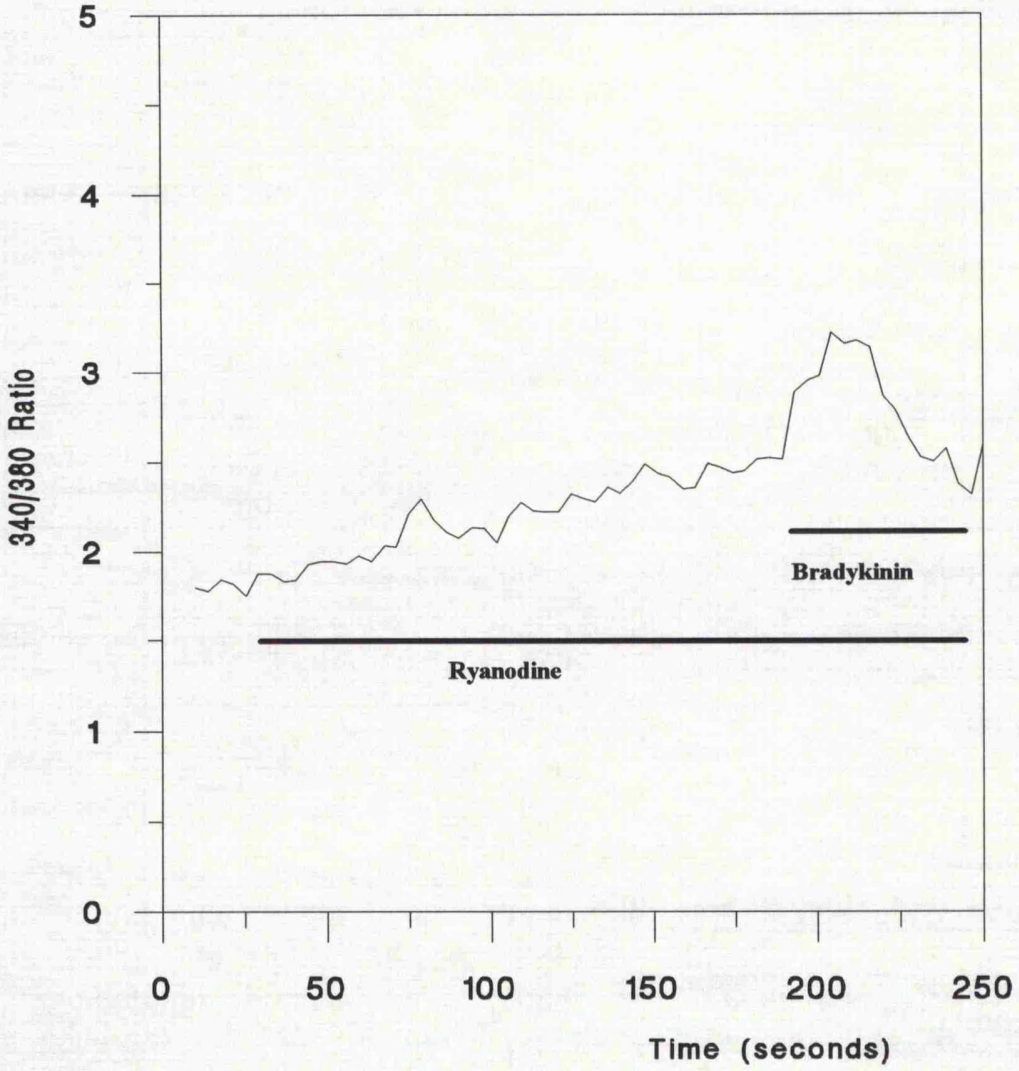


Fig. 6.6. Effect of ryanodine on bradykinin evoked Ca^{2+} release from intracellular stores. Intact chromaffin cells in suspension were loaded with fura-2AM. Ryanodine was used at 10 μM and bradykinin at 100 nM. The trace is representative of four experiments and results are expressed as 340/380nm ratio (excitation wavelengths, 3.8s changeover time). Calcium levels in the buffer were below 1 μM. Bradykinin stimulation in the absence of thapsigargin resulted in an elevation of $[Ca^{2+}]_i$ of 156 ± 23.27 nM (mean ± S.E.M. of four experiments, see also Fig. 6.1). Autofluorescence was subtracted in the experiments.

Table 6.1. Bradykinin and caffeine stimulated mobilisation from intracellular calcium stores and the effect of thapsigargin and ryanodine on the bradykinin and caffeine responses.

		Pretreatments	
Stimulus	**$\Delta[Ca^{2+}]_i$ in nM ± S.E.M.**	**Thapsigargin (1 μM)**	**Ryanodine (10 μM)**
Bradykinin (200 nM)	203 ± 34 * (4)		
Bradykinin (100 nM)	156 ± 23.27 * (4)	22.3 ± 12.51 # (3)	72.8 ± 4.56 * # (4)
Caffeine (50 mM)	146 ± 10.14 * (4)	67.4 ± 8.53 * # (4)	16.7 ± 8.3 # (3)

Chromaffin cells in suspension were loaded with fura- 2AM. Bradykinin was used at 200 or 100 nM and thapsigargin at 1 μM. Caffeine concentration was 50 mM and ryanodine was used at 10 μM. Results are expressed as changes in $[Ca^{2+}]_i$ calibrated from the 340 /380nm ratio as described in Chapter 2. section 7.2. Autofluorescence was subtracted in all experiments. In all the experiments changes in $[Ca^{2+}]_i$ levels for the sustained phase represent changes in the average obtained from eleven points (38s) Changes in $[Ca^{2+}]_i$ levels in response to bradykinin and caffeine represent the peak (average of 3 points: 7.6 seconds). Calcium free solutions were used where the $[Ca^{2+}]_i$ levels were below 1 μM. Basal $[Ca^{2+}]_i$ levels before the 50s were 197 nM ± 22.9 (n=11). Results are mean ± S.E.M. and n= number of experiments (* significant difference above basal, P<0.001), # = smaller than stimuli alone (P<0.001).

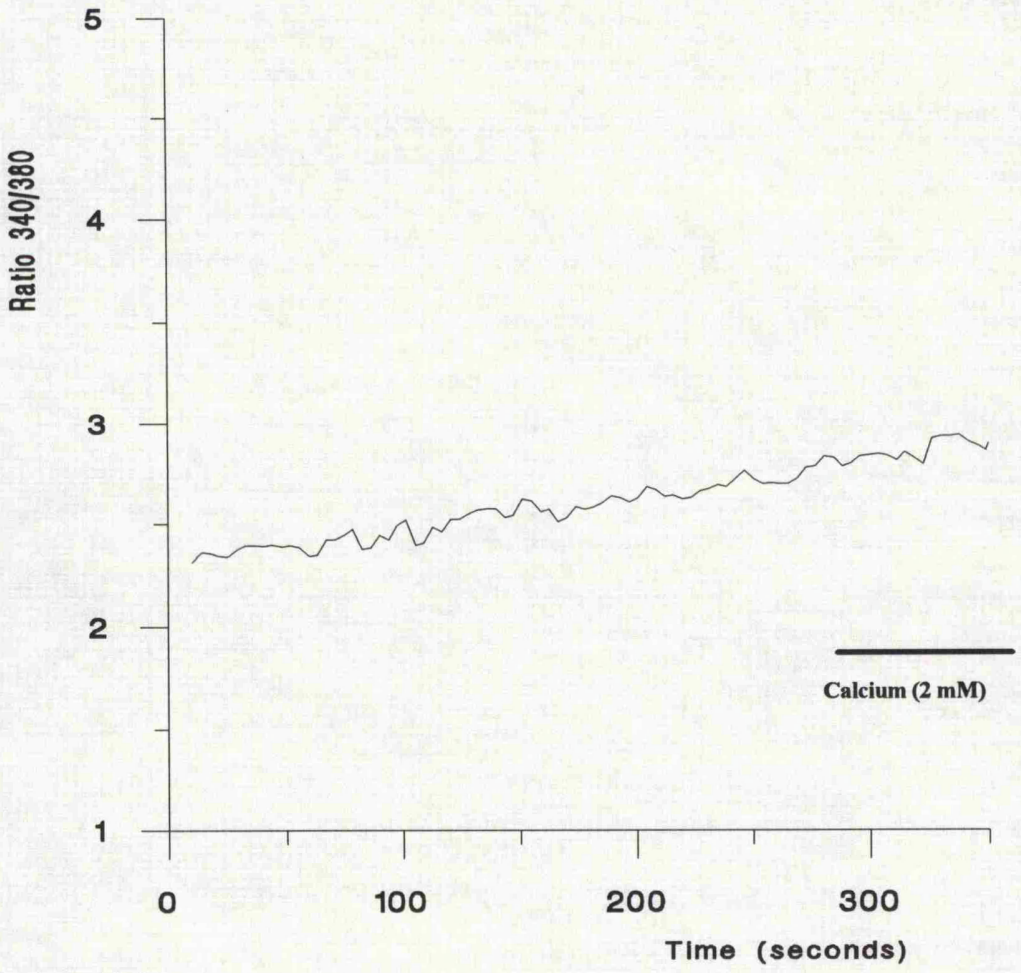


Fig. 6.7. Effect of extracellular Ca^{2+} on $[Ca^{2+}]_i$ levels using population of intact chromaffin cells. Chromaffin cells in suspension were loaded with fura-2AM. The $[Ca^{2+}]_i$ levels of the buffer were below 1 μM. This Fig. illustrates a control experiment where Ca^{2+} (2 mM) was reintroduced to the medium. The trace is representative of 17 experiments.

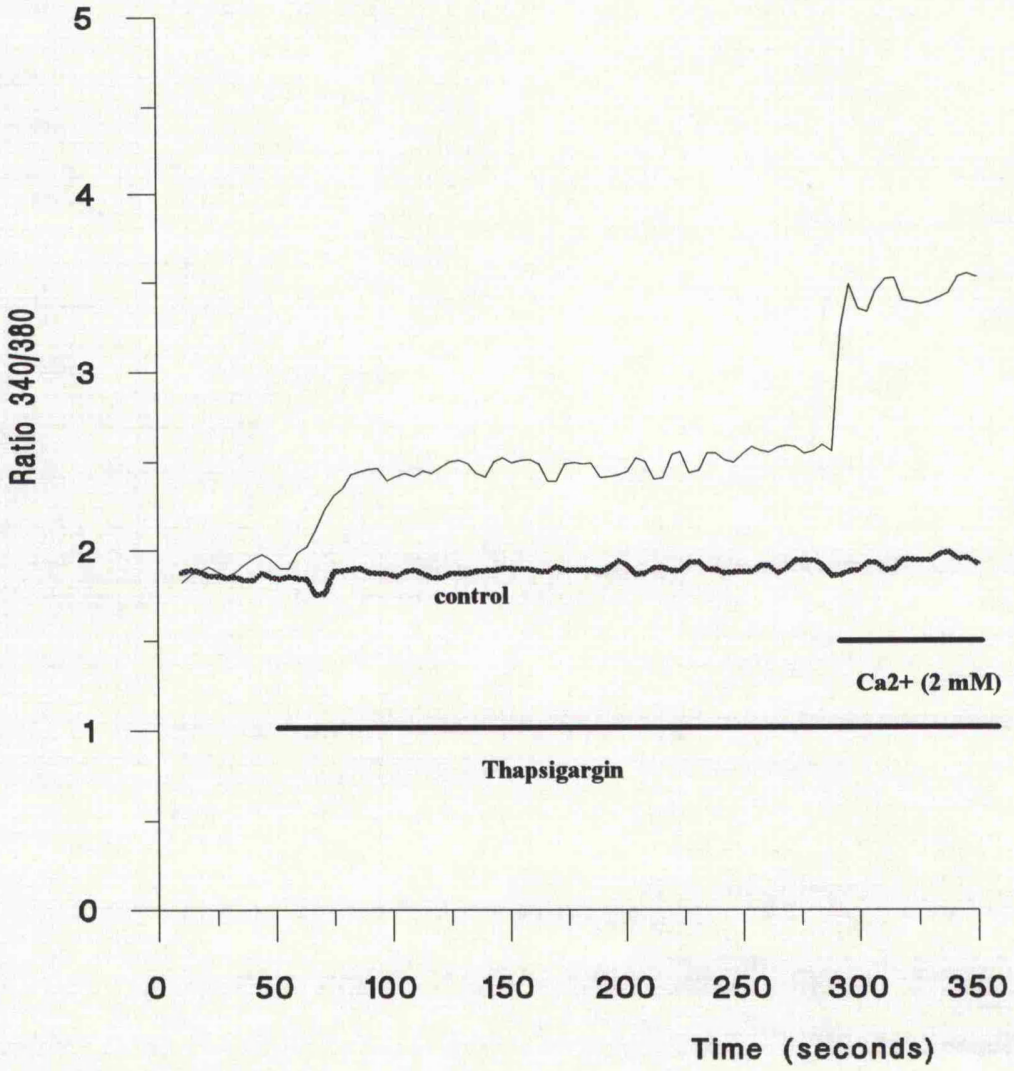


Fig. 6.8. Effect of thapsigargin on Ca^{2+} release from intact chromaffin cells (loaded with fura-2AM) in suspension. Thapsigargin (thin line) elicited a slow sustained Ca^{2+} release in a Ca^{2+}- free medium. (1 μM). When the Ins(1,4,5)P_3-sensitive stores have been depleted with prior addition of thapsigargin, reintroduction of Ca^{2+} to the medium resulted in a second rise in Ca^{2+} levels indicating Ca^{2+} entry. Thapsigargin concentration was 1 μM. Ca^{2+} was added at 2 mM in both cases. The trace is representative of 16 experiments. The trace for the control (thick line) experiment is representative of 17 experiments.

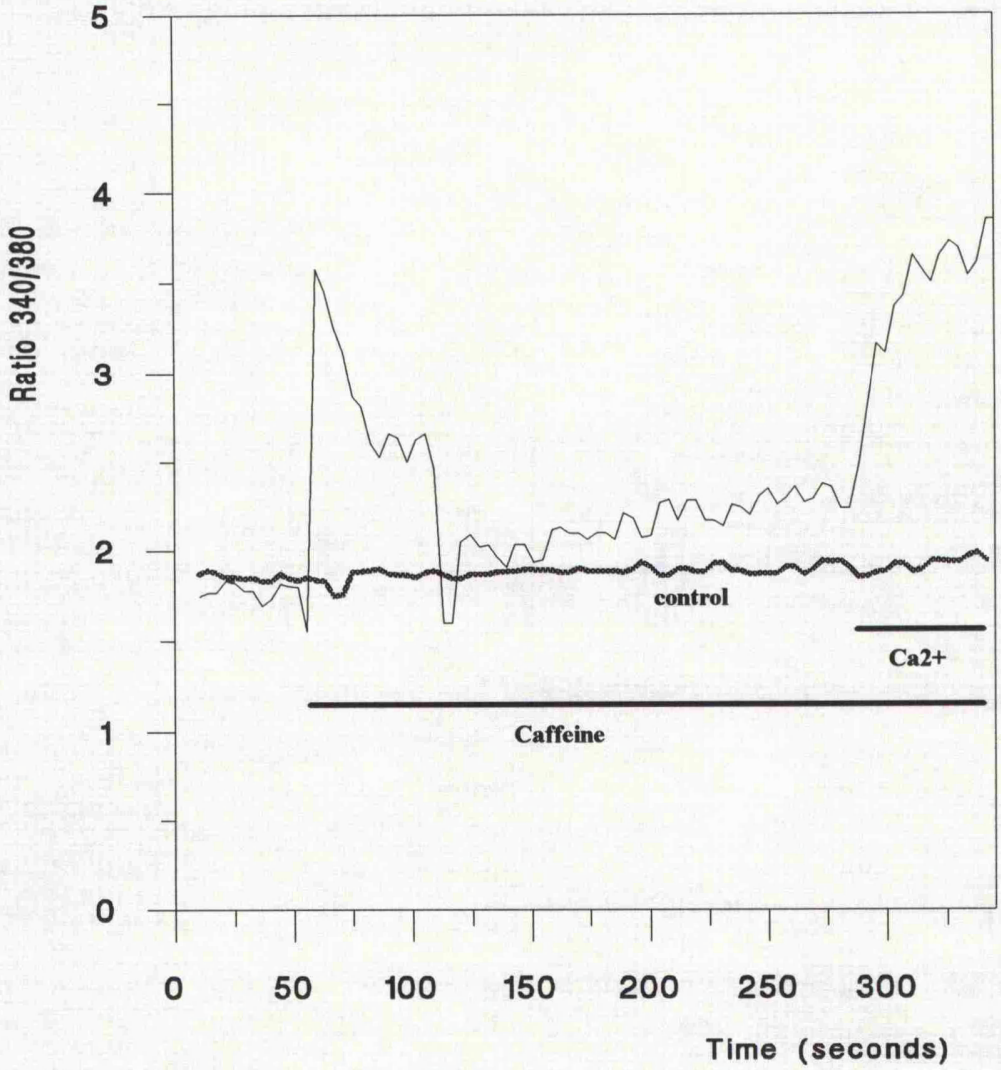


Fig. 6.9. Effect of caffeine on Ca^{2+} release from intact chromaffin cells (loaded with fura 2-AM) in suspension. Addition of caffeine (thin line) resulted in Ca^{2+} release in the Ca^{2+}-free medium (about 1 μM). When Ca^{2+} was reintroduced into the medium after prior addition of caffeine a second response was obtained. Caffeine concentration was 50 mM. Ca^{2+} was added to give a final $[Ca^{2+}]_i$concentration of 2 mM in both cases. The trace is representative of 4 experiments. The trace for the control experiment (thick line) is representative of 17 experiments.

Table 6.2. Thapsigargin and caffeine induced changes in $[Ca^{2+}]_i$. Effect of Ca^{2+} (2 mM) when the agonist store has been depleted (a) and the caffeine-sensitive store has been depleted (b).

Stimulus	**$\Delta[Ca^{2+}]_i$ in nM ± S.E.M. at 50 secs**	**$\Delta[Ca^{2+}]_i$ in nM ± S.E.M. at 290 secs**	**n**
(a)			
control	17.88 ± 3.80	69.72 ± 13.22	11
thapsigargin (1 μM)	76.59 ± 9.42*	171.43 ± 13.15*	16
(b)			
control	11.41 ± 3.83	84.84 ± 19.76	6
caffeine (50 mM)	146 ± 10.14*	237.5 ± 14.63*	4

Intact chromaffin cells in suspension were loaded with fura-2AM. Thapsigargin (1 μM) or caffeine (50 mM) were added at 50s. Thapsigargin addition at 50s stimulated a rise of $[Ca^{2+}]_i$ above basal. Caffeine stimulated a rapid peak elevation in $[Ca^{2+}]_i$. Addition of calcium (2 mM) at 290s, after the stores had been depleted, evoked a significantly different above basal ($P<0.001$) elevation in $[Ca^{2+}]_i$ levels. In all the experiments changes in $[Ca^{2+}]_i$ levels represent changes in the average obtained from eleven points (38s) before and after the addition of stimuli, except for caffeine where data represents the peak (3 points: 7.6 seconds). Calcium free solutions (+ EGTA if necessary) were used where the Ca^{2+} levels were below 1 μM. Basal $[Ca^{2+}]_i$ levels before the 50s were 197 nM ± 22.9 (n=11). Results are mean ± S.E.M. and n= number of experiments. *= significant above control ($P< 0.001$).

6.2.2 Single Cell Experiments

In order to verify the previous findings a number of experiments were carried out using single cells (prepared as described in Chapter 2, section 9). Changes in the $[Ca^{2+}]_i$ levels were monitored by using a Photon Technology International (P.T.I.) Deltascan system (as described in Chapter 2, section 9.1).

Bradykinin (1 μM) in the absence of extracellular Ca^{2+} resulted in an increase of $[Ca^{2+}]_i$ (ratio: 340/380 4.31 ± 0.48 above basal, mean ± S.E.M., n=7). This increase was transient, returning to basal level within two minutes (Fig. 6.10). In the presence of external calcium (2 mM) bradykinin provoked rapid peak elevations of $[Ca^{2+}]_i$ levels (4.45 ± 0.28 above basal, mean ± S.E.M., n=6) which then declined to a maintained elevated plateau (ratio 340/380: 1.11 ± 0.09 above basal, mean ± S.E.M., n=6, see also Fig. 6.11 and Table 6.3). There was no statistical difference between the maximal response evoked by bradykinin in the presence, or absence, of extracellular Ca^{2+}. The biphasic pattern of response to bradykinin in the presence of extracellular Ca^{2+} indicates that Ca^{2+} release from the Ins(1,4,5)P_3-sensitive stores is followed by Ca^{2+} influx though the plasma membrane. Similar results have been reported with other Ins(1,4,5)P_3 generating stimuli in chromaffin cells (Stauderman et al., 1990; Cheek et al., 1993a).

Challenge of single cells with caffeine (50 mM) resulted in Ca^{2+} mobilisation from internal stores confirming the presence of caffeine-sensitive Ca^{2+} stores. In the absence of extracellular Ca^{2+} caffeine resulted in a transient Ca^{2+} peak (ratio 340/380: 4.61 ± 0.64 above basal, mean ± S.E.M. n=7, see also Table 6.2) and no significant elevated plateau phase was detected (Fig. 6.12). In the presence of external Ca^{2+} (2 mM) caffeine resulted in a transient elevation of $[Ca^{2+}]_i$ (ratio 340/380: 3.98 ± 0.21 above basal mean ± S.E.M., n=4, Fig. 6.13) followed by a sustained phase (0.85 ± 0.09 above basal, mean ± S.E.M, n=4, Table 6.3). Maximal responses evoked by caffeine were not statistically different in the presence, or absence, of extracellular Ca^{2+}. This data provides strong evidence that Ca^{2+} mobilisation from the caffeine-

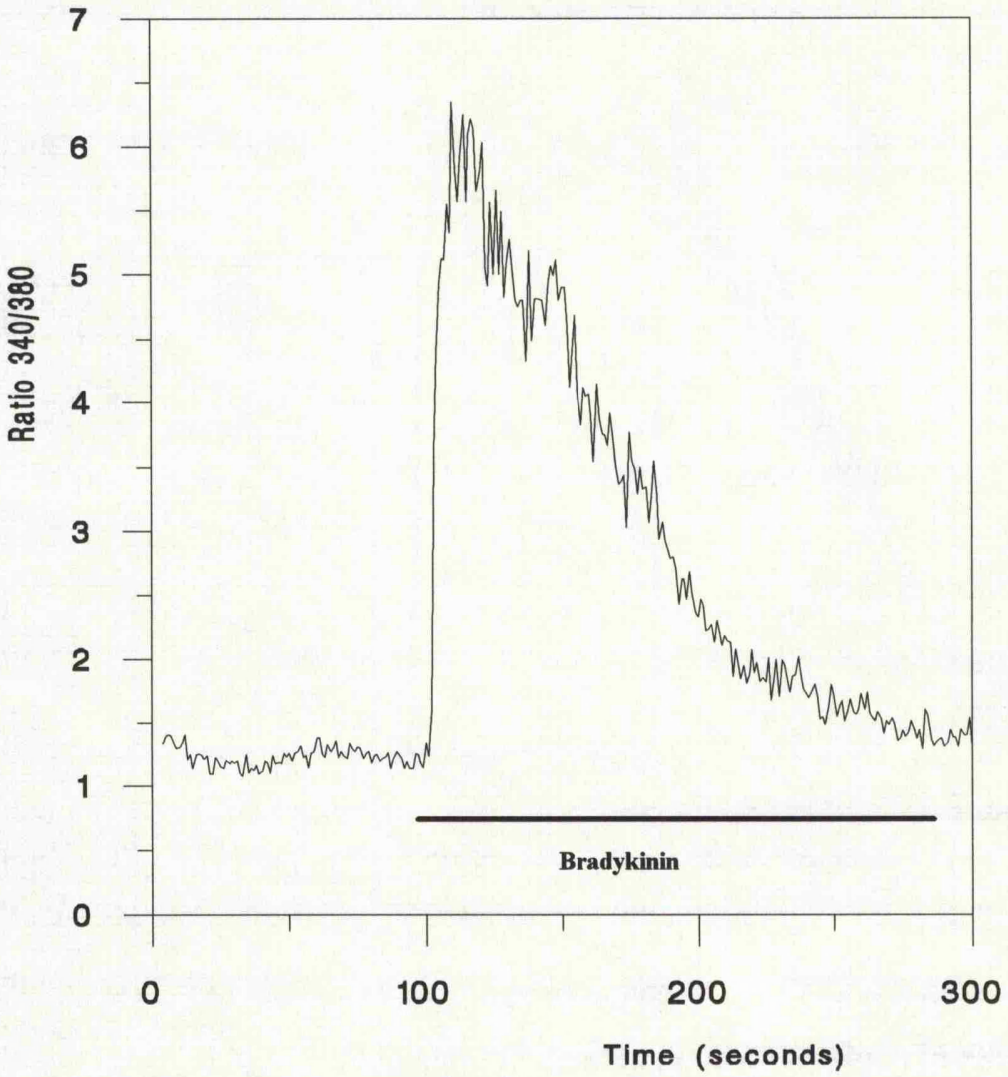


Fig. 6.10. Effect of bradykinin on Ca^{2+} release from single chromaffin cells in the absence of extracellular Ca^{2+}. Single chromaffin cells were loaded with fura-2AM for 20 minutes. Calcium free buffer was used (KHB: Krebs/Henseleit buffer) and the Ca^{2+} levels were measured at about 1 μM. The trace is representative of seven experiments and results are expressed as 340/380 ratio (excitation wavelengths, frequency of cycling= 1 per second). The background value in autofluorescence for unloaded cells was subtracted in all experiments.

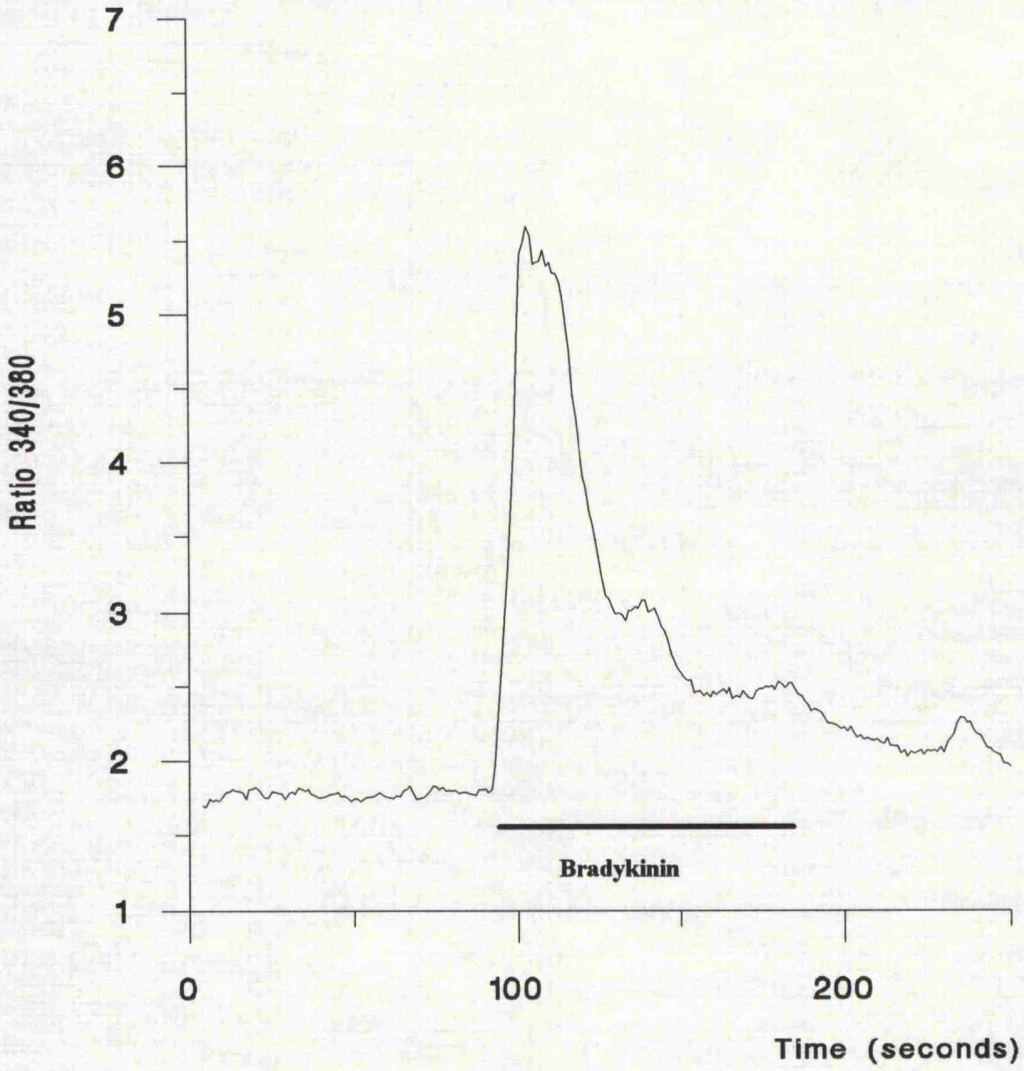


Fig. 6.11. Effect of bradykinin on Ca^{2+} release from single chromaffin cells in the presence of extracellular Ca^{2+}. Single chromaffin cells were loaded with fura-2AM for 20 minutes. Ca^{2+} concentration in the KHB buffer used was 2 mM. The trace is representative of five experiments and results are expressed as 340/380 ratio (excitation wavelengths, frequency of cycling= 1 per second). The background value in autofluorescence for unloaded cells was subtracted in all experiments.

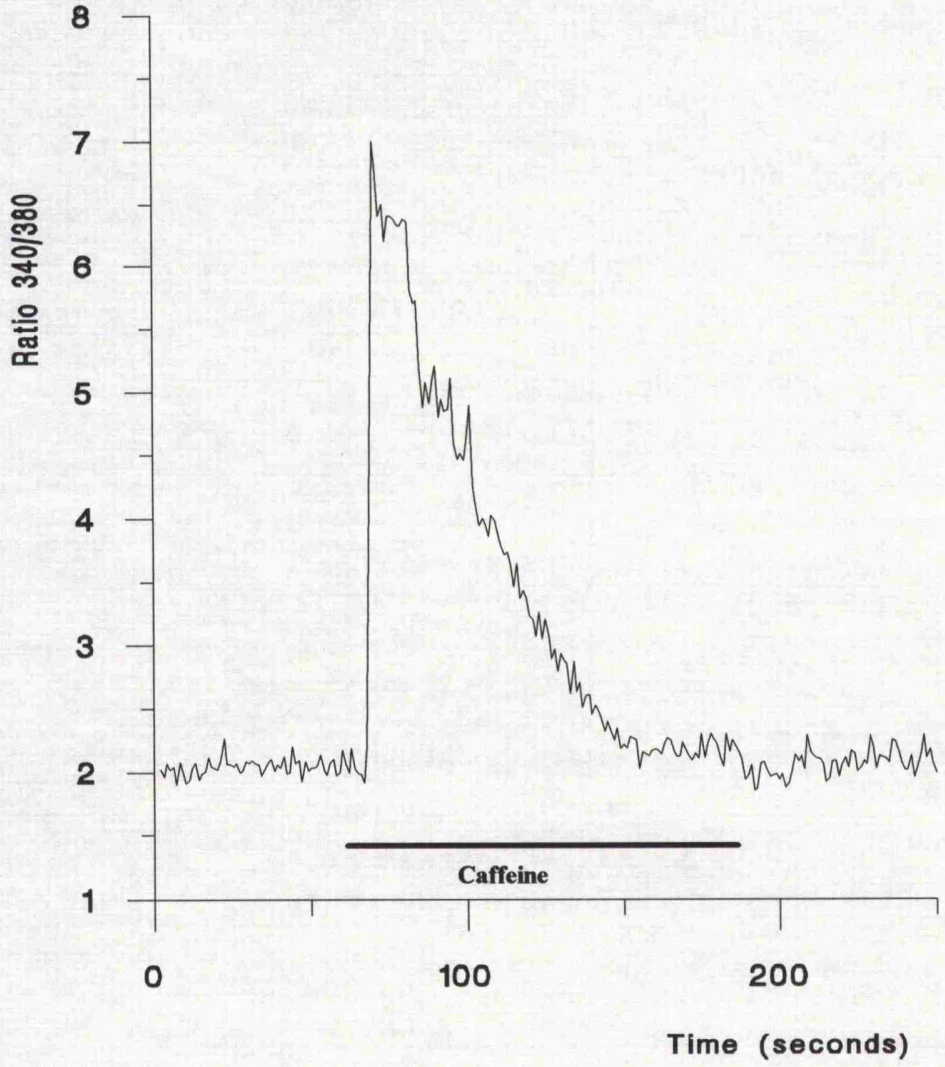


Fig. 6.12. Effect of caffeine (50 mM) on Ca^{2+} release from single chromaffin cells in the absence of extracellular Ca^{2+}. Single chromaffin cells were loaded with fura-2AM for 20 minutes. Calcium free buffer was used (KHB: Krebs/Henseleit buffer) and the Ca^{2+} levels were measured at about 1 μM. The trace is representative of seven experiments and results are expressed as 340/380 ratio (excitation wavelengths, frequency of cycling= 1 per second). The background value in autofluorescence for unloaded cells was subtracted in all experiments.

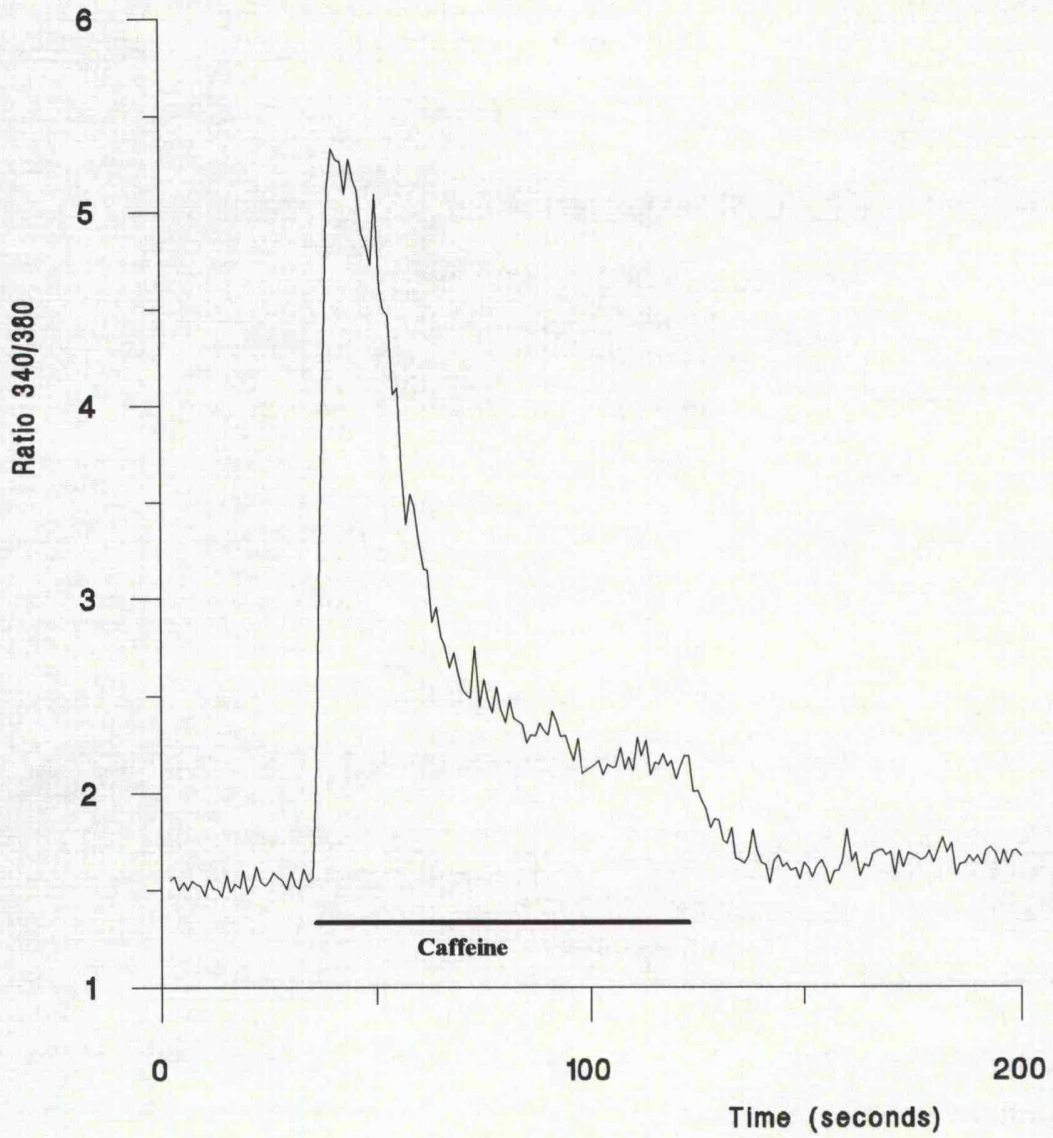


Fig. 6.13. Effect of caffeine on Ca^{2+} release from single chromaffin cells in the presence of extracellular Ca^{2+}. Single chromaffin cells were loaded with fura-2AM for 20 minutes. Ca^{2+} concentration in the KHB buffer used was 2 mM. The trace is representative of four experiments and results are expressed as 340/380 ratio (excitation wavelengths, frequency of cycling= 1 per second). The background value in autofluorescence for unloaded cells was subtracted in all experiments.

Table 6.3. Effect of bradykinin and caffeine on Ca^{2+} mobilisation from intact single chromaffin cells in the presence (a), or absence (b), of extracellular Ca^{2+}.

(a)			
Stimuli	**Peak amplitude Changes in 340/380 Ratio**	**Sustained Phase**	**n**
Bradykinin (1 μM)	4.45 ± 0.28	1.11 ± 0.08	6
Caffeine (50 mM)	3.98 ± 0.2	0.85 ± 0.1	4
(b)			
Bradykinin (1 μM)	4.31 ± 0.48	No	7
Caffeine (50 mM)	4.61 ± 0.64	No	7

Single adrenal chromaffin cells loaded with fura- 2AM were challenged with bradykinin (1 μM) and caffeine (50 mM), in the presence, or absence, of extracellular Ca^{2+} (2 mM). Results are expressed as differences in 340/380 ratio (excitation wavelengths) above basal. Basal 340/380 ratio was 1.58 ± 0.07 (mean ± S.E.M., n=11) and 1.61 ± 0.08 (mean ± S.E.M., n=17). in the presence, or absence, of extracellular Ca^{2+} respectively. Results shown as mean ± S.E.M., n= number of experiments. All responses were significantly above basal ($P<0.001$). No significant difference was found under any of the above conditions. The background value in autofluorescence for unloaded cells was subtracted in all experiments.

sensitive Ca^{2+} stores stimulates Ca^{2+} entry in single bovine adrenal chromaffin cells, confirming results (previously described) obtained from populations of chromaffin cells. Although bradykinin and caffeine produced different patterns of Ca^{2+} transients there was no significant differences between the maximal increases of $[Ca^{2+}]_i$ levels (see Table 6.3).

The effect of thapsigargin is illustrated in Fig. 6.14 and Fig. 6.15. In the absence of extracellular Ca^{2+} thapsigargin evoked an increase in Ca^{2+} levels (340/380 ratio: 1.16 $\pm$ 0.12, mean $\pm$ S.E.M., n=6) which was followed by a sustained phase, similar to that obtained using populations of intact cells. Bradykinin after prior pretreatment of chromaffin cells with thapsigargin for five minutes, resulted in significantly reduced Ca^{2+} mobilisation from intracellular stores (340/380 ratio: 1.46 $\pm$ 0.40, mean $\pm$ S.E.M., n=3, see also Fig. 6.14 and Table 6.4). When bradykinin was applied after chromaffin cells were challenged for 8 minutes with thapsigargin the response was markedly reduced to the point where it was barely detectable (348/380 ratio: 0.34 $\pm$ 0.09, mean $\pm$ S.E.M., n=3, see Fig. 6.15). These results indicate that pretreatment with thapsigargin for 8 minutes caused depletion of the agonist-sensitive Ca^{2+} stores.

Although the relationship between the Ins(1,4,5)P_3-sensitive and caffeine-sensitive Ca^{2+} stores in chromaffin cells has been extensively researched, only a limited number of studies have used intact single cells (Stauderman and Murawsky, 1991; Stauderman et al., 1991). However, in the previous studies only 10 mM of caffeine were used which, as demonstrated here and by others (Cheek et al., 1993b and 1994a), is not the maximally effective dose. A recent study in PC12 cells (Zacchetti et al., 1991) indicated that maximally effective concentrations of caffeine (40 mM, as determined by their experiments) completely prevented responsiveness to bradykinin.

In order to investigate the relationship between the intracellular Ca^{2+} stores in chromaffin cells maximal concentrations (1 μM) of bradykinin (an Ins(1,4,5)P_3-generating agonist) and caffeine were used. When bradykinin was applied, after prior addition of caffeine, it evoked a transient elevation in Ca^{2+} levels resulting in a

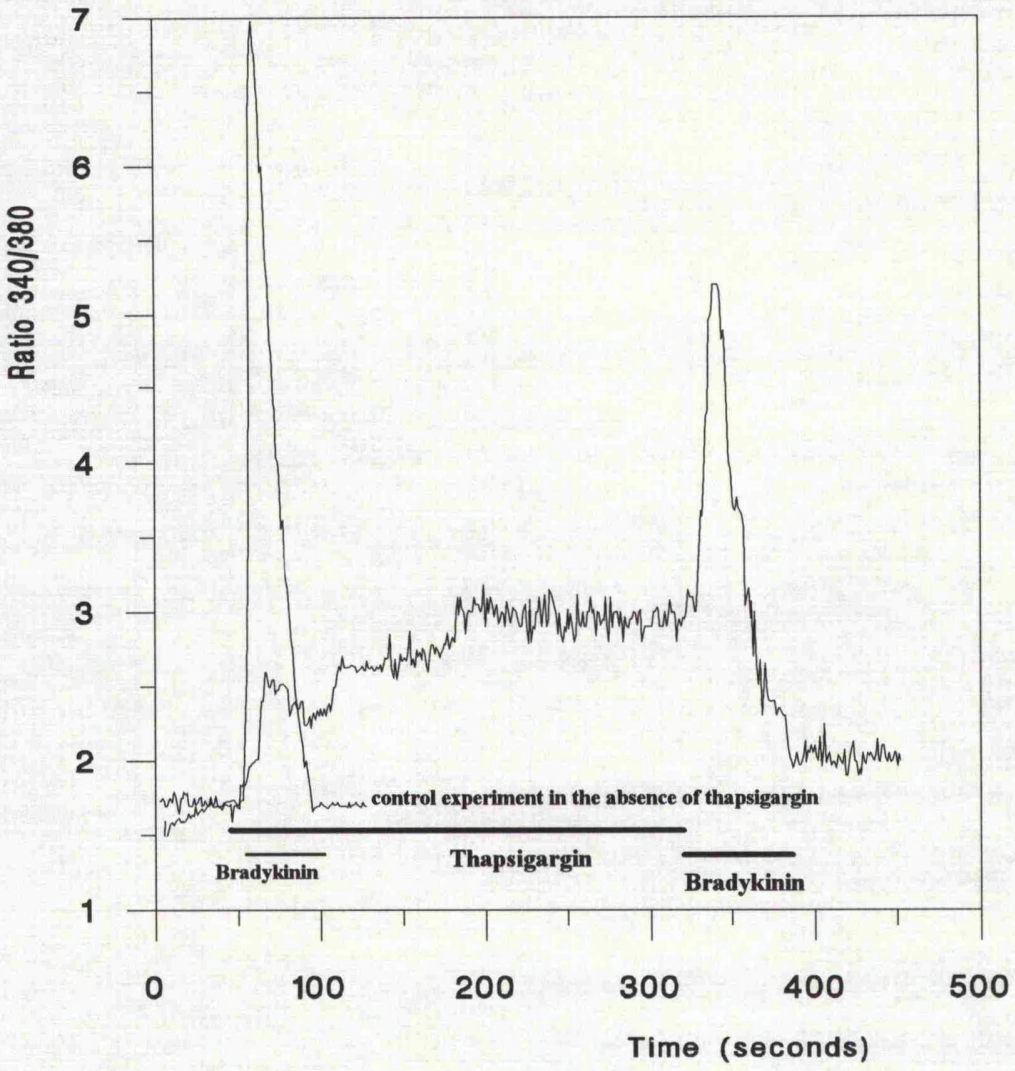


Fig. 6.14. Effect of thapsigargin (5 minutes) on bradykinin evoked Ca^{2+} release from single chromaffin cells in the absence of extracellular Ca^{2+}. Single chromaffin cells were loaded with fura -2AM for 20 minutes. KHB free Ca^{2+} buffer was used. Chromaffin cells were challenged with thapsigargin (1 μM) for five minutes. Bradykinin concentration was 1 μM. The trace is representative of three experiments and results are expressed as 340/380 ratio. The background value in autofluorescence for unloaded cells was subtracted in all experiments. The trace for the control experiment shows a typical bradykinin response in the absence of thapsigargin.

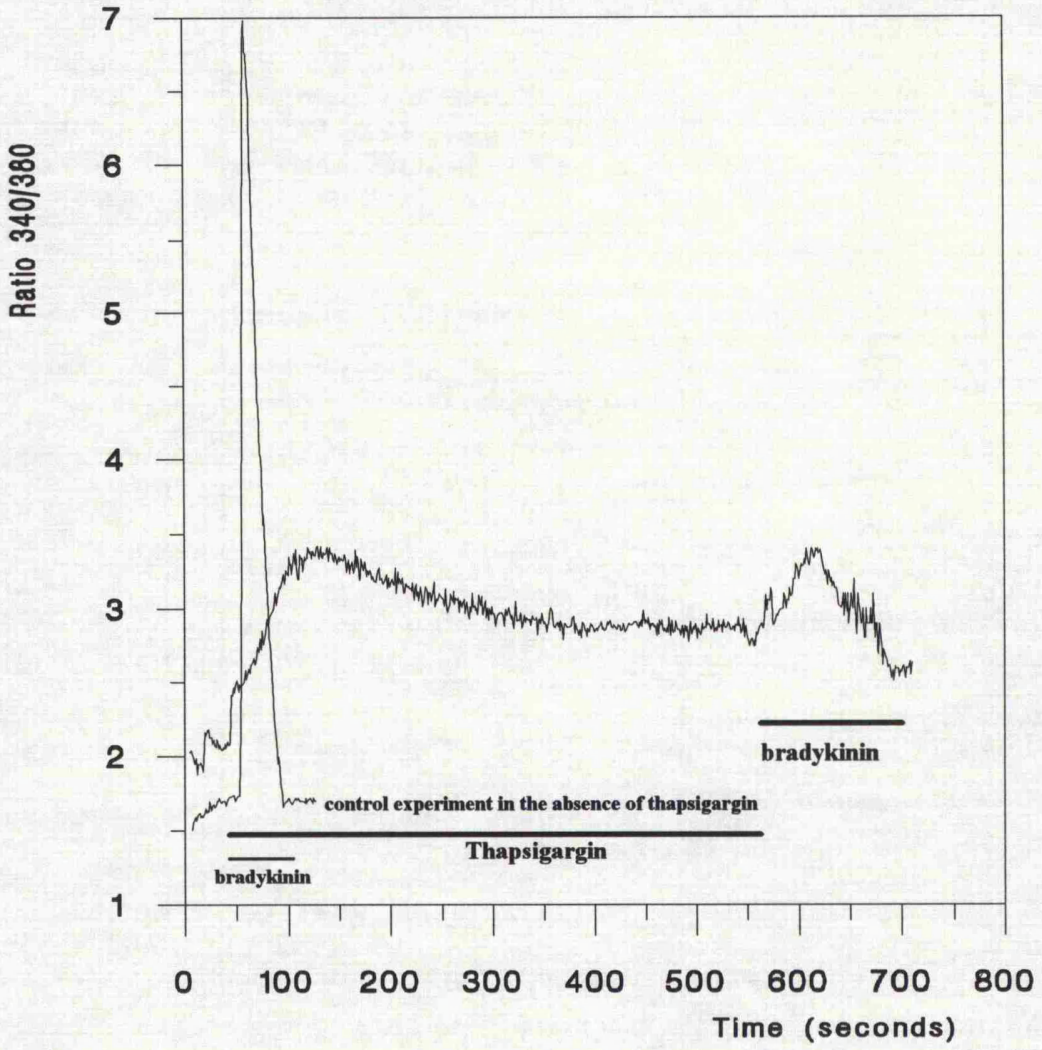


Fig. 6.15. Effect of thapsigargin (8 minutes) on bradykinin induced Ca^{2+} mobilisation from single permeabilised chromaffin cells. Single chromaffin cells were loaded with fura -2AM for 20 minutes. KHB free Ca^{2+} buffer was used. Chromaffin cells were challenged with thapsigargin (1 μM) for eight minutes. Bradykinin concentration was 1 μM. The trace is representative of three experiments and results are expressed as 340/380 ratio. The background value in autofluorescence for unloaded cells was subtracted in all experiments. The trace for the control experiment shows a typical bradykinin response in the absence of thapsigargin.

Table 6.4. Effect of thapsigargin on bradykinin induced Ca^{2+} mobilisation from single chromaffin cells.

Condition	**Peak amplitude Changes in 340/380 Ratio**	**n**
Bradykinin (1 μM)	4.31 ± 0.48	7
Thapsigargin (1 μM)	1.16 ± 0.12	6
Bradykinin (1 μM) after thapsigargin (1 μM) pretreatment for five minutes	1.46 ± 0.40	3
Bradykinin (1 μM) after thapsigargin (1 μM) pretreatment for eight minutes	0.34 ± 0.09	3

Single adrenal chromaffin cells loaded with fura- 2AM were challenged with bradykinin (1 μM) and thapsigargin (1 μM) in the absence of extracellular Ca^{2+}. Results are expressed as differences in 340/380 ratio (excitation wavelengths) above basal. Results shown as mean ± S.E.M., n= number of experiments. All responses were significantly above basal ($P<0.001$). The background value in autofluorescence for unloaded cells was subtracted in all experiments.

maximal response of 3.92 ± 0.15 above basal (ratio 340/380, mean ± S.E.M., n=3) which was not statistically different to that in the absence of caffeine. The sustained phase which followed the transient Ca^{2+} elevation was also not affected (0.99 ± 0.15 above basal, mean ± S.E.M., n=3, see also Table 6.5 and Fig. 6.16). In the presence of extracellular Ca^{2+} caffeine evoked Ca^{2+} mobilisation, after prior addition of bradykinin, which was not significantly different (ratio 340/380, 3.73 ± 0.34 above basal, mean ± S.E.M., n=6) to that obtained in the absence of bradykinin (3.98 ± 0.2 above basal, mean ± S.E.M., n=4, see also Table 6.5 and Fig. 6.17). The transient elevation in Ca^{2+} levels was followed by a sustained phase which was also not affected (0.96 ± 0.04 above basal mean ± S.E.M. n=6). These results demonstrate that in the presence of extracellular Ca^{2+} depletion of the bradykinin- and caffeine-sensitive Ca^{2+} stores resulted in rapid Ca^{2+} influx across the chromaffin cell plasma membrane. Depletion of the bradykinin-sensitive Ca^{2+} store did not affect subsequent caffeine response and vice versa, indicating that the Ca^{2+} influx was adequate to refill the intracellular Ca^{2+} stores, in the presence of 2 mM extracellular Ca^{2+}.

The same protocol was carried out in the absence of extracellular Ca^{2+}. In order to avoid any contribution of Ca^{2+} entry across the plasma membrane Ca^{2+} free buffers were used. If the $[Ca^{2+}]_i$ levels were high, EGTA was added to achieve the desirable Ca^{2+} concentration (below 1 μM). As described above bradykinin and caffeine in the absence of extracellular Ca^{2+} resulted in transient increases in Ca^{2+} levels (4.31 ± 0.48 and 4.61 ± 0.64, above basal respectively) with no indication of a sustained phase. Single adrenal chromaffin cells were challenged firstly with caffeine and then with bradykinin (Fig. 6.18). Bradykinin-evoked Ca^{2+} mobilisation was not abolished after prior addition of a maximally effective concentration of caffeine indicating that intact single adrenal chromaffin cells possess an exclusive Ins(1,4,5)P_3-sensitive Ca^{2+} store (Fig. 6.18). Bradykinin response though was found to be significantly reduced after prior addition of caffeine (1.4 ± 0.69 above basal, mean ± S.E.M., n=6, see also Table 6.6). Single adrenal chromaffin cells were then challenged firstly with bradykinin and then with caffeine (Fig. 6.19). Pretreatment with bradykinin

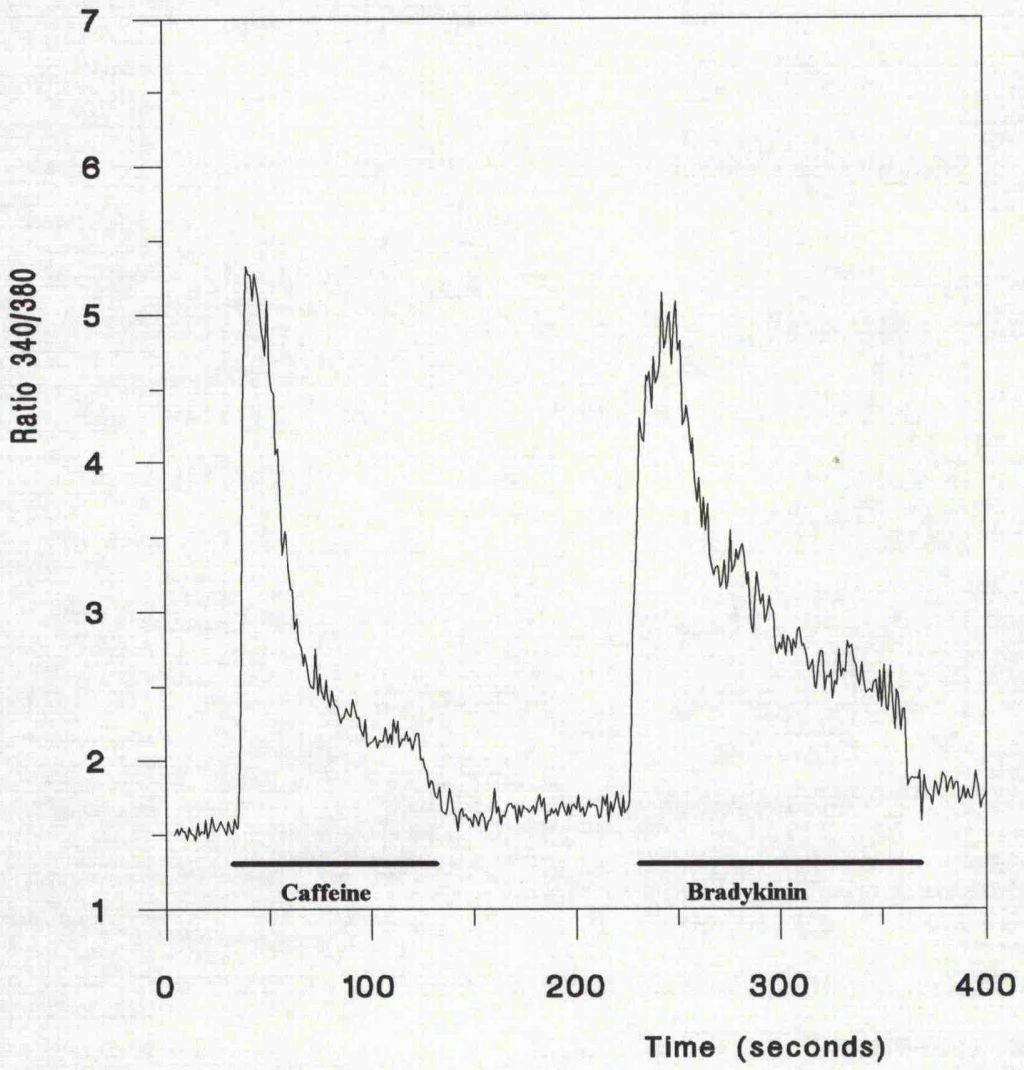


Fig. 6.16. Effect of caffeine on bradykinin evoked Ca^{2+} release from single chromaffin cells in the presence of extracellular Ca^{2+}. Single chromaffin cells were loaded with fura-2AM for 20 minutes. Ca^{2+} concentration in the KHB buffer used was 2 mM. The trace is representative of six experiments and results are expressed as 340/380 ratio (excitation wavelengths, frequency of cycling= 1 per second). The background value in autofluorescence for unloaded cells was subtracted in all experiments.

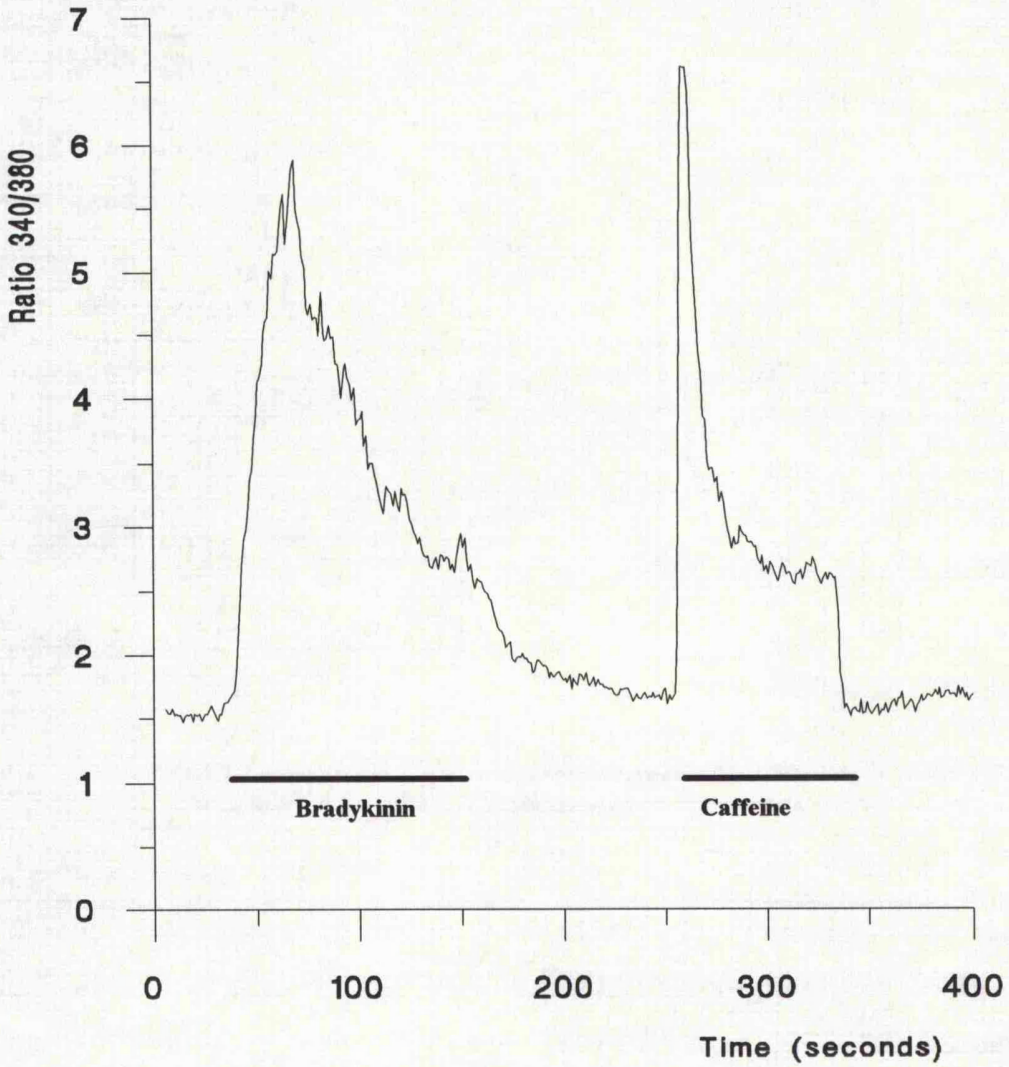


Fig. 6.17. Effect of bradykinin on caffeine evoked Ca^{2+} mobilisation from single chromaffin cells in the presence of extracellular Ca^{2+}. Single chromaffin cells were loaded with fura-2AM for 20 minutes. Ca^{2+} concentration in the KHB buffer used was 2 mM. The trace is representative of six experiments and results are expressed as 340/380 ratio (excitation wavelengths, frequency of cycling= 1 per second). The background value in autofluorescence for unloaded cells was subtracted in all experiments.

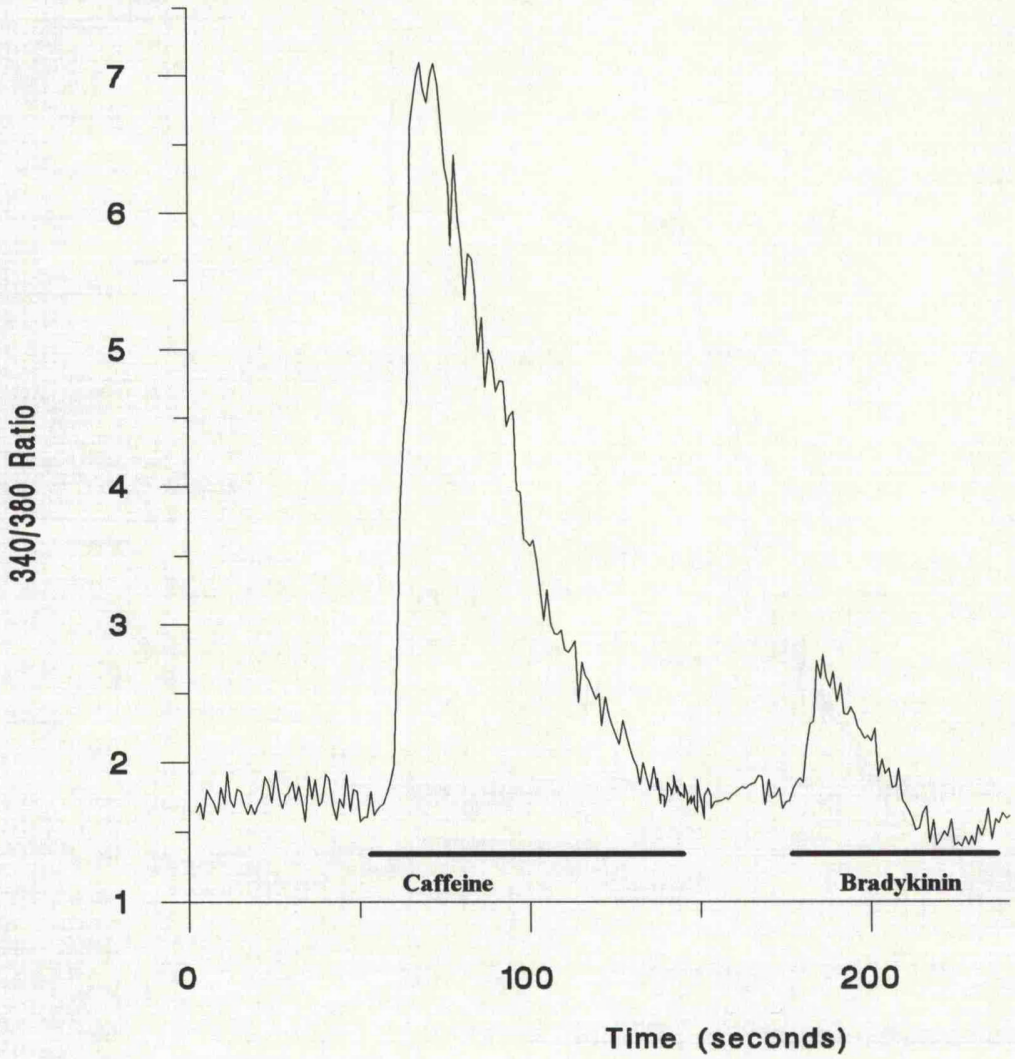


Fig. 6.18. Effect of caffeine on bradykinin induced Ca^{2+} release from single chromaffin cells in the absence of extracellular Ca^{2+}. Single chromaffin cells were loaded with fura-2AM for 20 minutes. Calcium free buffer was used (KHB: Krebs/Henseleit buffer) and the Ca^{2+} levels were measured at about 1 μM. The trace is representative of nine experiments and results are expressed as 340/380 ratio (excitation wavelengths, frequency of cycling= 1 per second). The background value in autofluorescence for unloaded cells was subtracted in all experiments.

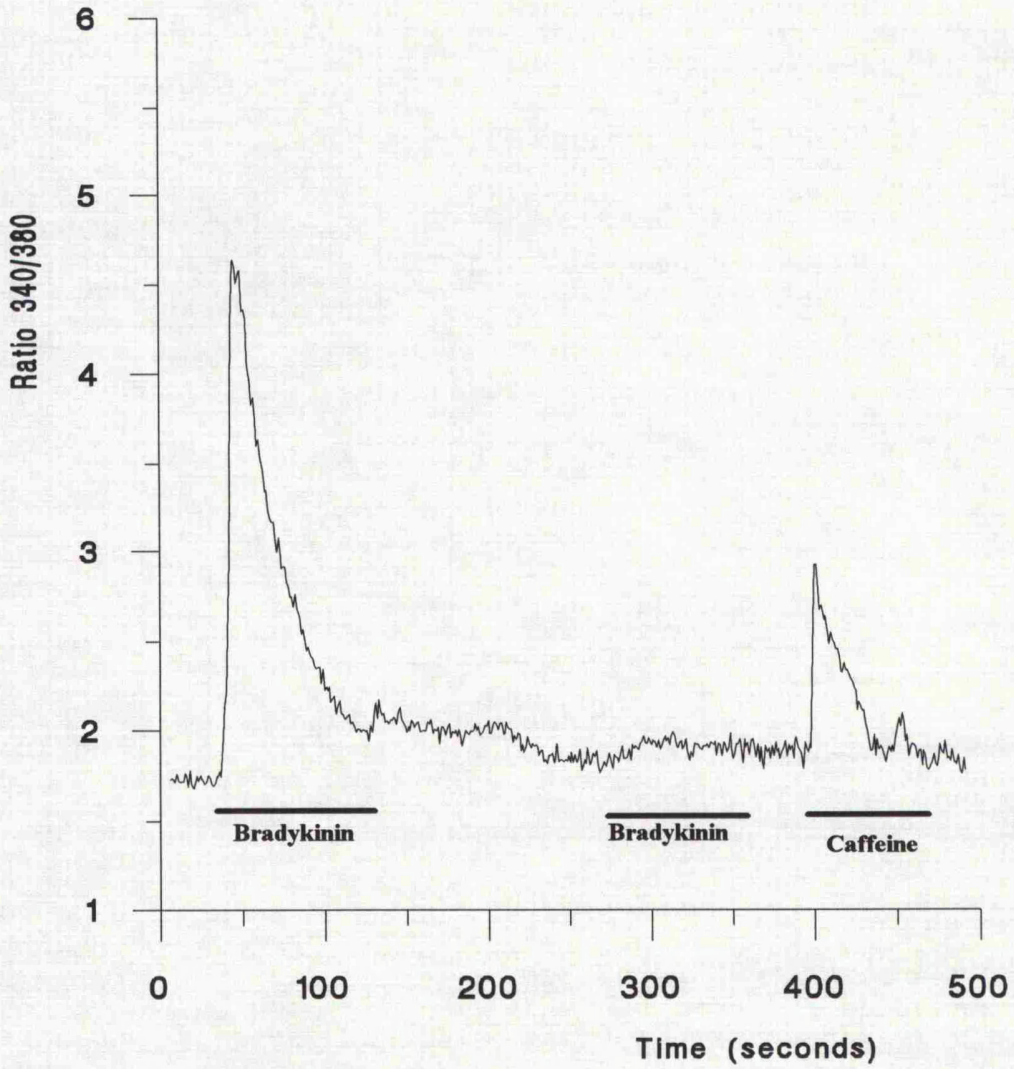


Fig. 6.19. Effect of bradykinin on caffeine induced Ca^{2+} mobilisation from single chromaffin cells in the absence of extracellular Ca^{2+}. Single chromaffin cells were loaded with fura-2AM for 20 minutes. Calcium free buffer was used (KHB: Krebs/Henseleit buffer) and the Ca^{2+} levels were measured at about 1 μM. The trace is representative of nine experiments and results are expressed as 340/380 ratio (excitation wavelengths, frequency of cycling= 1 per second). The background value in autofluorescence for unloaded cells was subtracted in all experiments.

Table 6.5. Effect of bradykinin and caffeine on Ca^{2+} mobilisation from intact single chromaffin cells in the presence of extracellular Ca^{2+} (2 mM).

Stimuli	**Peak amplitude Changes in 340/380 Ratio**	**Sustained Phase**	**n**
Bradykinin (1 μM)	4.45 ± 0.28	1.11 ± 0.08	6
Bradykinin (1 μM) after Caffeine (50 mM)	4.20 ± 0.88	0.99 ± 0.07	3
Caffeine (50 mM)	3.98 ± 0.2	0.85 ± 0.1	4
Caffeine (50 mM) after Bradykinin (1 μM)	3.73 ± 0.34	0.96 ± 0.04	6

Single adrenal chromaffin cells loaded with fura- 2AM were challenged with bradykinin (1 μM) and caffeine (50 mM), in the presence of extracellular Ca^{2+} (2 mM). Results are expressed as differences in 340/380 ratio (excitation wavelengths) above basal. Basal 340/380 ratio was 1.58 ± 0.07 (mean ± S.E.M., n=11) in the presence of extracellular Ca^{2+}. Results shown as mean ± S.E.M., n= number of experiments. All responses were significantly above basal ($P<0.001$). No significant effect was found under any of the above conditions. The background value in autofluorescence for unloaded cells was subtracted in all experiments.

Table 6.6. Effect of bradykinin and caffeine on Ca^{2+} mobilisation from intact single chromaffin cells in the absence of extracellular Ca^{2+}.

Stimuli	Peak amplitude Changes in 340/380 Ratio	Sustained Phase	Duration of the transient in seconds
Bradykinin (1 μM)	4.31 ± 0.48 (7)	No	103 ± 9.8 (11)
Bradykinin (1 μM) after caffeine (50 mM)	1.40 ± 0.69 * (6)	No	73.62 ± 5.38 ∇ (9)
Caffeine (50 mM)	4.61 ± 0.64 (7)	No	76.22 ± 10.2 # (9)
Caffeine (50 mM) after bradykinin (1 μM)	1.75 ± 0.39 * (6)	No	52.11 ± 5.9 ∇ (9)

Single adrenal chromaffin cells loaded with fura-2AM were challenged with bradykinin (1 μM) and caffeine (50 mM), in the absence of extracellular Ca^{2+} (less than 1 μM). Results are expressed as differences in 340/380 ratio (excitation wavelengths) above basal. Basal 340/380 ratio in the absence of extracellular Ca^{2+} was 1.61 ± 0.08 (mean ± S.E.M., n=17). Results shown as mean ± S.E.M., n= number of experiments. All responses were significantly above basal ($P<0.001$). * = smaller than stimuli alone ($P<0.001$). The duration of the transients evoked by bradykinin was significantly longer (# : $P<0.01$) than for caffeine. ∇ = smaller than stimuli alone ($P<0.01$). The background value in autofluorescence for unloaded cells was subtracted in all experiments.

significantly reduced subsequent caffeine response (1.75 $\pm$ 0.39 above basal, mean $\pm$ S.E.M., n=7, see also Table 6.6). The caffeine response was not abolished even when the Ins(1,4,5)P_3-sensitive Ca^{2+} stores had been depleted demonstrating that intact chromaffin cells possess a caffeine-exclusive sensitive Ca^{2+} store. These results clearly demonstrate that agonist evoked Ca^{2+} mobilisation was markedly diminished after caffeine pretreatment and vice versa providing strong evidence that intracellular Ca^{2+} stores in chromaffin cells do functionally overlap.

Challenge of chromaffin cells in the absence of extracellular Ca^{2+} with caffeine resulted in a transient elevation in Ca^{2+} levels which returned to the original basal levels within 76.22 $\pm$ 10.2 seconds (mean $\pm$ S.E.M., n= 9, see also Table 6.6). Bradykinin also elicited a transient elevation in the Ca^{2+} levels which was significantly prolonged 103.2 $\pm$ 9.8 seconds (mean $\pm$ S.E.M., n= 11). These two stimuli evoked different patterns of Ca^{2+} transients which are probably consistent with the existence of different intracellular Ca^{2+} stores. The maximal response to bradykinin and caffeine was not found to be significantly different (Table 6.6). When the caffeine releasable Ca^{2+} store had been depleted the transient evoked by bradykinin was found to be significantly reduced 73.62 $\pm$ 5.38 seconds (mean $\pm$ S.E.M., n=9). Depletion of the agonist-sensitive Ca^{2+} store also affected the transient evoked by caffeine which, under these conditions, was present for 52.11 $\pm$ 5.9 seconds (mean $\pm$ S.E.M, n=9). These results are also consistent with the existence of distinct Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores in chromaffin cells, indicating that Ca^{2+} released from one store could be sequestered into the other.

As illustrated in most of the Figures presented in this Chapter, bradykinin elicited more prolonged transients, where caffeine resulted in single transients in most of the cells examined. Furthermore, bradykinin produced two discernible but overlapping transients in only three chromaffin cells (For example see Fig. 6.20). The maximal increase in Ca^{2+} levels for these three cells was lower compare to the others. It is noticeable that although these cells display different patterns of Ca^{2+} transients evoked by bradykinin, subsequent addition of caffeine resulted in Ca^{2+} mobilisation

from intracellular stores. Closer examination of the cells used in the experiments described here (n= 58) did not reveal any other significant variations in the patterns of Ca^{2+} transients among the cells.

Although depletion of one Ca^{2+} store may affect the filling state of the other results presented here clearly indicate that depletion of Ins(1,4,5)P_3-sensitive Ca^{2+} store significantly reduced caffeine responses and vice versa proving strong evidences that Ins(1,4,5)P_3- and caffeine- sensitive Ca^{2+} stores do partially overlap in single chromaffin cells. The degree of overlap, previously detected using permeabilised chromaffin cells, is shown to be a real reflection of the nature of the intracellular Ca^{2+} stores in intact single bovine adrenal chromaffin cells.

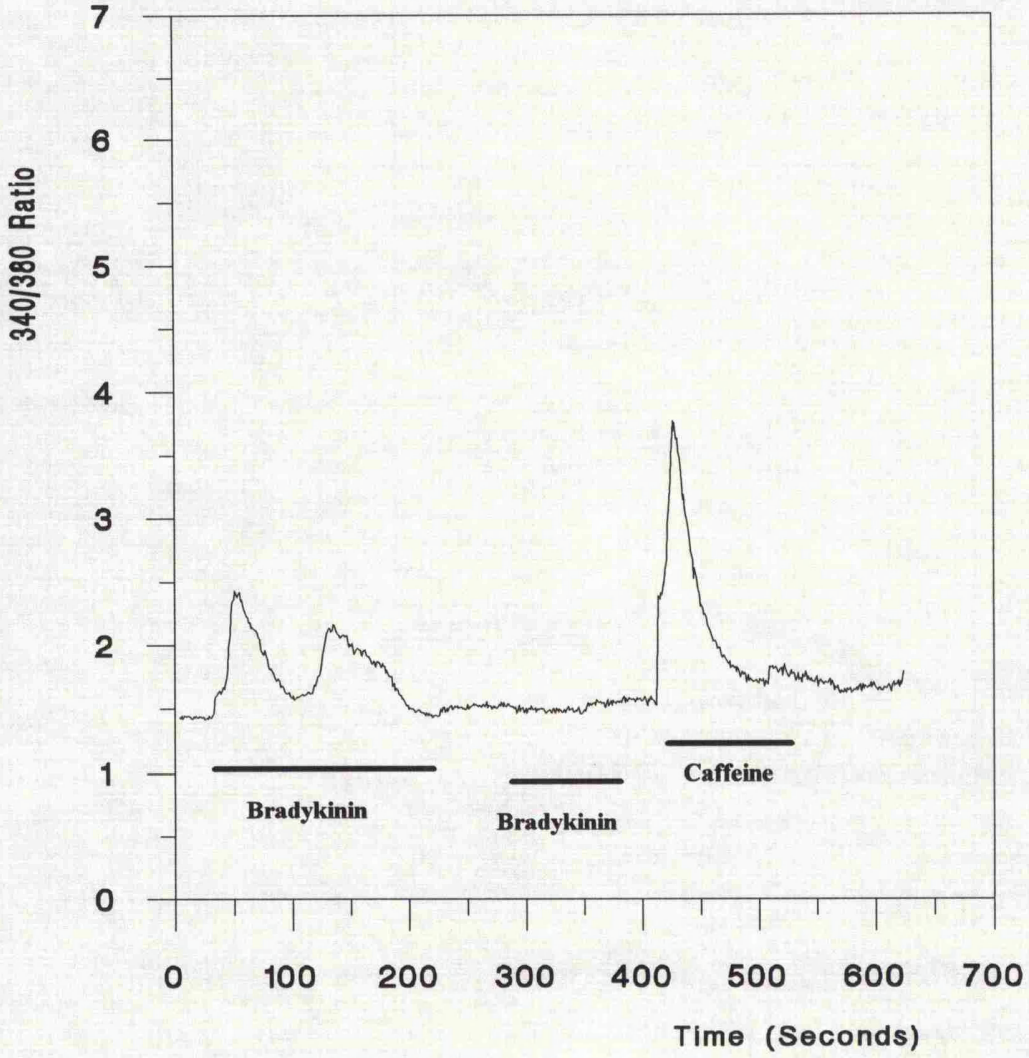


Fig. 6.20. Bradykinin evoked Ca^{2+} mobilisation from intracellular stores of single chromaffin cells in the absence of extracellular Ca^{2+}.

Single chromaffin cells were loaded with fura-2AM for 20 minutes. Ca^{2+} concentration in the KHB buffer used was below 1 μM. The trace is representative of three experiments and results are expressed as 340/380 ratio (excitation wavelengths, frequency of cycling= 1 per second). The background value in autofluorescence for unloaded cells was subtracted in all experiments.

6.3 DISCUSSION

The characteristics of the intracellular Ca^{2+} stores were studied in previous Chapters using permeabilised chromaffin cells. Experiments in this Chapter on populations of intact chromaffin cells also demonstrate that bradykinin and caffeine evoked Ca^{2+} mobilisation from intracellular Ca^{2+} stores in the absence of extracellular Ca^{2+}. Thapsigargin pretreatment abolishes responses to bradykinin but not to caffeine, consistent with the existence of two intracellular stores in intact chromaffin cells. Findings from this study also suggest that depletion of the agonist-sensitive Ca^{2+} store affects the amount of Ca^{2+} released from the ryanodine-sensitive Ca^{2+} stores.

The caffeine response was completely abolished after prior treatment with ryanodine indicating that caffeine evoked Ca^{2+} mobilisation from ryanodine-sensitive Ca^{2+} stores (ryanodine receptor expressing stores). Results presented here also indicate that pretreatment with ryanodine reduced the amount of Ca^{2+} released by a subsequent addition of bradykinin providing strong evidence that there is a functional interaction between Ins(1,4,5)P_3- and caffeine- sensitive Ca^{2+} stores. These findings indicate either that some stores possess Ins(1,4,5)P_3 and ryanodine receptors together or that the Ca^{2+} released from the Ins(1,4,5)P_3-sensitive Ca^{2+} stores activates a CICR mechanism which stimulates Ca^{2+} mobilisation from the ryanodine-sensitive Ca^{2+} stores.

The results from populations of intact chromaffin cells indicate that thapsigargin pretreatment inhibits a subsequent response to bradykinin but not to caffeine and ryanodine pretreatment abolishes responses to caffeine but not to bradykinin, consistent with the existence of two independent intracellular Ca^{2+} stores. These findings also demonstrate that bradykinin is acting indirectly through the Ins(1,4,5)P_3 receptor in chromaffin cells. Depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} store affected subsequent responses to caffeine and vice versa, providing strong evidence that a degree of overlap between these stores exists in chromaffin cells. Findings from populations of intact chromaffin cells also indicate that findings from

previous Chapters (where permeabilised chromaffin cells were used) were a true reflection of the nature of the intracellular stores and not a misinterpretation due to the permeabilising procedures used.

The nature of these stores was further examined here using populations of intact cells and intact single chromaffin cells. Bradykinin and caffeine elicited different patterns of Ca^{2+} transients indicating that different Ca^{2+} releasing mechanisms may be present in these stores. Challenge of chromaffin cells with caffeine resulted in single transients where bradykinin produced more prolonged transients, and in only three cells elicited two discernible but overlapping transients.

In the presence of extracellular Ca^{2+}, pretreatment with caffeine did not affect the amount of Ca^{2+} released by a subsequent addition of bradykinin (1 μM). Prior challenge with the Ins(1,4,5)P_3 generating agonist, bradykinin, had no effect on subsequent responses to caffeine. These findings suggest that Ca^{2+} was rapidly sequestered into the intracellular stores in the presence of extracellular Ca^{2+}.

When the extracellular Ca^{2+} was removed to avoid any contribution of Ca^{2+} entry across the chromaffin cell plasma membrane, pretreatment with bradykinin reduced subsequent responses to caffeine and vice-versa. However, both of the stimuli evoked Ca^{2+} mobilisation after prior addition of the other agent. These findings clearly demonstrate that depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} store did not abolish Ca^{2+} mobilisation from the caffeine-sensitive Ca^{2+} store and depletion of the caffeine-sensitive Ca^{2+} store did not abolish Ins(1,4,5)P_3- induced Ca^{2+} release. This data is in agreement with previous findings (Chapter 4 and Chapter 5) from permeabilised chromaffin cells and indicates the existence of Ins(1,4,5)P_3-exclusive sensitive, caffeine-exclusive sensitive Ca^{2+} stores and also the presence of stores which are sensitive to both Ins(1,4,5)P_3 and caffeine.

At the same time another study on chromaffin cells demonstrated that caffeine (10 mM) response was abolished after pretreatment of single chromaffin cells with histamine, and markedly reduced (>90%) after pretretmeant with angiotensin II (Stauderman et al., 1991), indicating that either histamine and angiotensin II evoked

Ca^{2+} mobilisation from Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores or that the submaximal caffeine dose used was insufficient to evoke further Ca^{2+} release. Their data also showed that caffeine inhibited subsequent responses to agonists by >90%. Results presented here indicate that bradykinin reduced subsequent response to caffeine by 65%. Pretreatmeant with caffeine reduced the amount of Ca^{2+} mobilised by subsequent application of bradykinin by 70%.

The differences between the caffeine concentrations used (50 mM in this study, 10 mM Stauderman et al., 1991) could explain the effect of the agonist on the caffeine induced Ca^{2+} release, since 10 mM of caffeine were probably insufficient to completely discharge all the Ca^{2+} content of the caffeine-sensitive Ca^{2+} store. However, these differences cannot explain the greater effect of caffeine on the agonist response observed in their study. These inconsistencies between results presented here and their data (Stauderman et al., 1991) also indicate that the effect of caffeine is independent of the indirect inhibition of Ins(1,4,5)P_3 formation (as discussed previously) since higher concentrations of caffeine had less effect on agonist induced Ca^{2+} mobilisation than lower ones. Recent data from PC12 cells demonstrated that maximal dose of caffeine did not inhibit agonist induced phosphoinositide hydrolysis (Barry and Cheek, 1994) suggesting that this role of caffeine is unlikely to reflect the inhibition of agonist induced Ca^{2+} release.

Thapsigargin pretreatment abolishes responses to bradykinin indicating that bradykinin evoked Ca^{2+} mobilisation from the Ins(1,4,5)P_3-sensitive Ca^{2+} stores, since caffeine was still able to stimulate Ca^{2+} release. Results presented here are in agreement with previous studies indicating that challenge of chromaffin cells with thapsigargin for 3 or 5.5 minutes did not completely deplete the Ins(1,4,5)P_3-sensitive Ca^{2+} stores (Cheek et al., 1989a; Cheek and Thastrup, 1989). Their data also demonstrated that application of thapsigargin for 14 minutes inhibited methacholine responses.

Previous reports in PC12 cells demonstrated evidence for the existence of one pool sensitive to both Ins(1,4,5)P_3 and caffeine (Zacchetti et al., 1991). Recent data

indicates that different PC12 subclones contain two pools; one exclusive Ins(1,4,5)P_3-sensitive and another sensitive to both Ins(1,4,5)P_3 and caffeine (Reber et al., 1993; Barry and Cheek, 1994). Results from this study suggest that intact single chromaffin cells possess stores that are uniquely Ins(1,4,5)P_3-sensitive, stores uniquely sensitive to caffeine, and stores that are sensitive to both. It is clear then that the relationship between Ins(1,4,5)P_3- and CICR- sensitive Ca^{2+} stores may vary in different cell types, reflecting heterogeneity of Ca^{2+} stores and differences in Ins(1,4,5)P_3 and ryanodine receptors expressed. Furthermore, there is evidence demonstrating differential immunohistochemical localisation of Ins(1,4,5)P_3- and ryanodine-sensitive Ca^{2+} release channels in rat brain (Sharp et al., 1993a and b), indicating that these stores have distinct roles in controlling intracellular Ca^{2+} levels.

Previous reports demonstrated that pretreatment of single adrenal chromaffin cells with ryanodine (10 μM) or ryanodine + caffeine (10 mM) inhibited subsequent responses to histamine by 50% and 80%, respectively (Stauderman and Murawsky, 1991). Their data also showed that ryanodine produced a use-dependent inhibition of subsequent caffeine- and histamine-evoked Ca^{2+} responses. This use-dependent action of ryanodine may account for the above findings. When ryanodine + caffeine were added together this prevented refilling of the all the caffeine releasable Ca^{2+} stores decreasing by 80% the histamine responses. It is possible that ryanodine alone prevented refilling of the caffeine-exclusive Ca^{2+} stores but it was unable to prevent refilling of the Ins(1,4,5)P_3- and caffeine- sensitive Ca^{2+} stores in the presence of histamine, therefore resulting in only 50% inhibition. A possible explanation is that in the presence of histamine, Ca^{2+} was mobilised from the Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores lowering the levels of intraluminal Ca^{2+} content of this store, which may prevent the opening of the ryanodine receptor channel. However, no evidence for a use-dependent action of ryanodine were found in this Thesis.

Findings presented in this Chapter demonstrate the existence of Ins(1,4,5)P_3- and caffeine- sensitive Ca^{2+} stores in populations and single intact chromaffin cells, in agreement with the two pool model described in permeabilised adrenal chromaffin cells

(This Thesis, Cheek et al., 1991; Liu et al., 1991; Robinson and Burgoyne, 1991a; Stauderman et al., 1991). There is also evidence suggesting that these stores are differentially localised within adrenal chromaffin cells (Burgoyne et al., 1989b). Results presented here, using bradykinin as an Ins(1,4,5)P_3 generating agonist, demonstrate that depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores affects the ryanodine-sensitive Ca^{2+} stores, providing strong evidence for functional interactions between the two pools, indicative of a degree of overlap between Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores.

These findings support a multiple Ca^{2+} stores model where ryanodine-sensitive Ca^{2+} stores play an important role. A significant part of the bradykinin-induced Ca^{2+} mobilisation comes from the ryanodine-sensitive Ca^{2+} stores and vice-versa. The mechanisms by which this interaction occurs are not yet clear. One explanation is that some intracellular Ca^{2+} stores possess both Ins(1,4,5)P_3 and ryanodine receptors where others possess either Ins(1,4,5)P_3 or ryanodine receptors. An alternative possibility is that Ca^{2+} released from the Ins(1,4,5)P_3-sensitive Ca^{2+} stores activates CICR from closely located ryanodine-sensitive Ca^{2+} stores. Further experiments are required in order to investigate the mechanisms by which bradykinin activates Ca^{2+} release from the ryanodine-sensitive Ca^{2+} stores.

6.3.1 Ca^{2+} Release Activated Ca^{2+} Entry

It has been demonstrated in many cell types and chromaffin cells, without being clearly understood, that agonist induced Ca^{2+} signals stimulate both Ca^{2+} release from intracellular stores and external Ca^{2+} influx (O'Sullivan et al., 1989; Cheek et al., 1993a). As previously discussed, chromaffin cells also possess various types of VSCCs. However, although these channels are responsible for the major part of Ca^{2+} entry in chromaffin cells leading to secretion there is evidence that Ca^{2+} entry in response to bradykinin is not mediated through these channels (Owen et al., 1989a and b). Furthermore, histamine and angiotensin II stimulate Ca^{2+} entry through a voltage

independent pathway in chromaffin cells (Bunn and Boyd, 1992; Cheek et al., 1993a). Previous reports also indicated that thapsigargin was able to activate Ca^{2+} entry (Cheek and Thastrup, 1989; Robinson et al., 1992), indicating the existence of a capacitative entry mechanism in these cells.

Various possible mechanisms have been offered to explain these phenomena such as: Ca^{2+} entry could be activated by a receptor-linked channel, a second messenger operated channel controlled by Ca^{2+}, Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 alone or combined together, or by the capacitative entry theory (Putney, 1990; see also Penner et al., 1993; Putney and Bird, 1993b). A large number of investigators have implicated Ins(1,4,5)P_3 as having a role in Ca^{2+} entry following receptor activation (Kuno and Gardner, 1987; Penner et al., 1988; Mochizuki-Oda et al., 1991a).

The other proposed model (previously discussed) is the capacitative entry theory which proposes that depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} store is the signal that stimulates Ca^{2+} entry. It has been previously shown that thapsigargin causes depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores in chromaffin cells (Cheek and Thastrup, 1989; Cheek et al, 1989; Robinson and Burgoyne, 1991a and b). In order to examine whether depletion of the intracellular Ca^{2+} stores could stimulate Ca^{2+} entry thapsigargin, a Ca^{2+}-ATPase inhibitor of the Ins(1,4,5)P_3-sensitive store, was used to empty this store and caffeine to deplete the caffeine-sensitive Ca^{2+} store.

Findings from this Chapter indicated that thapsigargin and caffeine were able to elicit Ca^{2+} release from intracellular Ca^{2+} stores in the absence of extracellular Ca^{2+}. Thapsigargin induced mobilization of the Ca^{2+} content of the Ins(1,4,5)P_3-sensitive Ca^{2+} store and caffeine was able to release Ca^{2+} from the caffeine-sensitive store when applied in a Ca^{2+} free medium. These results indicated that thapsigargin and caffeine were able to completely or partially deplete the Ins(1,4,5)P_3-sensitive and the caffeine-sensitive Ca^{2+} stores respectively. When extracellular Ca^{2+} was reintroduced into the Ca^{2+} free medium a second response could be detected suggesting that calcium entry was stimulated in the presence of extracellular Ca^{2+}. These results indicate that

depletion of the Ins(1,4,5)P_3-sensitive and caffeine-sensitive Ca^{2+} pools stimulated Ca^{2+} entry.

Similar results were obtained from intact single chromaffin cells. Both bradykinin and caffeine elicited transient elevations of Ca^{2+} followed by a sustained phase in the presence of extracellular Ca^{2+}. There was no evidence of sustained phase when the extracellular Ca^{2+} was removed indicating that Ca^{2+} release from intracellular stores activates Ca^{2+} influx across the chromaffin cells plasma membrane. Furthermore, when extracellular Ca^{2+} was present prior challenge with bradykinin or caffeine did not have any significant effect on subsequent responses to the other stimuli, indicating that Ca^{2+} entry was responsible for refilling the depleted internal Ca^{2+} stores.

Results presented in this study indicate that it is depletion of the calcium content of the intracellular Ca^{2+} stores that stimulates Ca^{2+} influx. It is still not clear how thapsigargin and caffeine stimulate Ca^{2+} entry. There is evidence that sub-maximal doses of caffeine did not appear to stimulate sustained Ca^{2+} entry (Cheek et al., 1990). The possibility that increased Ca^{2+} levels (see Chapter 1) could activate the opening of the second messenger operated Ca^{2+} channels (SMOC's) cannot be ruled out in chromaffin cells although depletion of the Ca^{2+} stores by ionomycin, which is able to release Ca^{2+} from the internal Ca^{2+} stores (Morgan and Jacob, 1994), did not lead to activation of Ca^{2+} entry in chromaffin cells (Stauderman and Pruss, 1989).

The data presented here agrees with the capacitative theory model showing that depletion of the Ins(1,4,5)P_3-sensitive stores can stimulate Ca^{2+} influx. This data also agrees with data from other cell types showing that emptying of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores can stimulate Ca^{2+} entry (see Berridge, 1993a and Pozzan et al., 1994). The nature of the signal which mediates capacitative Ca^{2+} entry after store depletion is not yet known. Depletion of internal Ca^{2+} stores has been proposed to cause conformational changes in the organelle which are transmitted to the plasma membrane via the cytoskeleton (Putney and Bird, 1993b). The possible involvement of another second messenger such as cytochrome P-450 (Alvarez et al., 1992; Garcia et

al., 1992; Montero et al., 1992) and cGMP in pancreatic acinar cells (Pandol and Schoeffield-Payne, 1990; Bahnson et al, 1993) cannot be ruled out. The involvement of the calcium influx factor (CIF) is also a possibility (Randriamampita and Tsien, 1993). This factor was isolated from extracts of lymphocytes after the intracellular stores had been depleted by a Ca^{2+} mobilising agonist. At the same time studies on *Xenopus* oocytes, of which the intracellular stores had been depleted (Parekh et al, 1993), revealed the presence of a soluble second messenger which activates Ca^{2+} entry.

This factor has been correlated (Clapham, 1993) with the calcium release-activated current (Icrac) which has been recently characterised (Hoth and Penner, 1992; Zweifach and Lewis, 1993). This current is highly selective for calcium, not inhibited by heparin and mediated by voltage-independent conductance. The hypothesis that CIF represents a diffusible second messenger which regulates capacitative Ca^{2+} entry has a weak point. It has been demonstrated that CIF can move across the cell plasma membrane and stimulate adjacent cells (Clapham, 1993). For what reason a second messenger (if CIF is one) needs to be permeable to the plasma membrane remains a mystery.

Very recent data demonstrated that Ca^{2+} entry in response to histamine precedes Ca^{2+} mobilisation from intracellular Ca^{2+} stores in chromaffin cells (Cheek et al., 1994b). Their data also shown that depletion of the ryanodine-sensitive Ca^{2+} stores almost abolished histamine-evoked Ca^{2+} release but did not affect the ability of histamine to activate Ca^{2+} entry, indicating that histamine-induced Ca^{2+} entry and Ca^{2+} release from the ryanodine-sensitive Ca^{2+} stores can be dissociated. Furthermore, histamine-induced Ca^{2+} influx was abolished after prior treatment with heparin suggesting the involvement of either G-proteins or inositol phosphates. These findings lead them to the conclusion that Ca^{2+} entry in response to histamine does not require store depletion in chromaffin cells.

It is possible that hormone-activated Ca^{2+} entry also requires the action of $Ins(1,4,5)P_3$ at the chromaffin cells plasma membrane. Recent evidence demonstrates

the existence of Ins(1,4,5)P_3 receptors located in the plasma membrane of lymphocytes (Khan et al., 1992a and b). Ins(1,4,5)P_3-sensitive Ca^{2+} channels also appear to exist in olfactory cells (Resterpo et al., 1990; Fadool et al., 1992) and on the plasma membrane of other cell types (Sharp et al., 1992; Fujimoto et al., 1992; McDonald et al., 1993). According to this evidence it was suggested that Ins(1,4,5)P_3 could stimulate Ca^{2+} entry through the plasma membrane Ins(1,4,5)P_3-receptors. A similar role has been proposed for Ins(1,3,4,5)P_4 (Irvine and Moore, 1987; Morris et al., 1987; Changya et al., 1989; Petersen, 1989). An Ins(1,3,4,5)P_4 receptor operated Ca^{2+} channel has been identified in endothelial cells (Lückhoff and Clapham, 1992).

There is evidence indicating the existence of an Ins(1,4,5)P_3 activated Ca^{2+} channel in the plasma membrane of chromaffin cells (Mochizuki-Oda et al., 1991a). This action of Ins(1,4,5)P_3 could be due to Ins(1,4,5)P_3 receptors located at the plasma membrane of chromaffin cells. Another possible explanation is that binding of the Ins(1,4,5)P_3 to the Ins(1,4,5)P_3 receptors expressed on the intracellular Ca^{2+} stores causes conformational changes in the receptor which are physically transmitted to the plasma membrane through the cytoskeleton (Putney and Bird, 1993b) or by direct coupling (Irvine, 1991 and 1992). However, findings in this Chapter demonstrated that depletion of the caffeine-sensitive Ca^{2+} stores activates Ca^{2+} entry across the chromaffin cell plasma membrane, indicating that all the intracellular Ca^{2+} stores are linked to the activation of Ca^{2+} entry. Therefore, these results may suggest that depletion of the intracellular Ca^{2+} stores in conjunction with the Ins(1,4,5)P_3 proposed role at the plasma membrane of chromaffin cells control Ca^{2+} entry.

The mechanism which activates Ca^{2+} entry after caffeine induced Ca^{2+} release presented in this study is even more difficult to interpret. This study demonstrates that chromaffin cells possess Ins(1,4,5)P_3- and caffeine- sensitive Ca^{2+} stores. Experiments presented in this Chapter and Chapter 5 also suggest a large degree of overlap between the two stores, in agreement with previous studies (Liu et al., 1991; Stauderman et al., 1991). Caffeine evoked Ca^{2+} mobilisation from an Ins(1,4,5)P_3-sensitive Ca^{2+} store in chromaffin cells. Depletion of this store which is sensitive to both Ins(1,4,5)P_3 and

caffeine (and shares characteristics with both stores) could stimulate Ca^{2+} entry according to the capacitative Ca^{2+} influx theory. It is also possible that caffeine, by mobilising Ca^{2+} from the caffeine-sensitive Ca^{2+} stores, activates Ca^{2+} release from the Ins(1,4,5)P_3-sensitive Ca^{2+} stores by a mechanism similar to the CICR. Furthermore, this partial depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores may be responsible for the Ca^{2+} entry detected in response to caffeine. Another explanation is that the filling state of the caffeine-sensitive store may also regulate Ca^{2+} entry in chromaffin cells by a mechanism similar to those proposed for the Ins(1,4,5)P_3-sensitive Ca^{2+} stores. Further investigation would be required in order to examine this is a reflection of the properties of the caffeine-exclusive-sensitive Ca^{2+} stores (possessing ryanodine receptors only) or of the stores which are sensitive both to Ins(1,4,5)P_3 and caffeine (expressing both Ins(1,4,5)P_3 and ryanodine receptors).

CHAPTER 7:

CONCLUSIONS AND FUTURE WORK

7.1 MAIN CONCLUSIONS

In this study I have attempted to characterise the role of intracellular Ca^{2+} stores in bovine adrenal chromaffin cells in order to increase our knowledge of the mechanisms involved in Ca^{2+} signalling, and the relationship between Ca^{2+} regulation and stimulus secretion coupling in these cells. The importance of these mechanisms could be widespread, shedding light on Ca^{2+} signalling pathways in a wide variety of other excitable cells.

At the same time as this study, other groups have also examined the characteristics of intracellular Ca^{2+} stores in chromaffin cells. Results from digitonin-permeabilised chromaffin cells (Robinson and Burgoyne, 1991a; Cheek et al., 1991) indicate the existence of two distinct Ca^{2+} stores, one Ins(1,4,5)P_3-sensitive, and the other caffeine-sensitive. Robinson and Burgoyne's results suggested that Ins(1,4,5)P_3-evoked Ca^{2+} mobilisation had no effect on subsequent responses to caffeine (10 mM). Thapsigargin pretreatment abolished responses to Ins(1,4,5)P_3 but not to caffeine, whereas ryanodine selectively blocked caffeine-induced Ca^{2+} release without affecting the Ins(1,4,5)P_3-induced Ca^{2+} release. These findings led them to the suggestion that chromaffin cells possess two distinct nonoverlapping Ca^{2+} stores sensitive to either Ins(1,4,5)P_3 or caffeine. The other report (Cheek et al., 1991) using high doses of Ins(1,4,5)P_3 (10-40 μM) and caffeine (10-40 mM), involving the use of pyrophosphate to increase the Ca^{2+} buffering of the stores, also concluded that the caffeine-sensitive Ca^{2+} store is largely distinct from the Ins(1,4,5)P_3-sensitive Ca^{2+} store but also suggested that a degree of overlap between these stores may exist.

A third report using α- toxin permeabilised cells apparently demonstrated that there is a large degree of overlap between these stores (Stauderman et al., 1991), indicating that three stores are present in chromaffin cells: one exclusive Ins(1,4,5)P_3-sensitive, a second exclusive caffeine-sensitive and a third sensitive to both. These stores represent about 20%, 55% and 25% of total Ca^{2+} released by Ins(1,4,5)P_3 and

caffeine (10 mM) respectively. Although these studies obtained significantly different results they are consistent with the two pool model.

In this Thesis, the role of intracellular Ca^{2+} stores was first examined using both electrically, and saponin permeabilised chromaffin cells. The relationship between these stores was initially examined in electrically permeabilised cells. Pretreatment with high doses of caffeine did not abolish subsequent responses to Ins(1,4,5)P_3 indicating that an exclusive Ins(1,4,5)P_3-sensitive Ca^{2+} store may be present in chromaffin cells. Poorly metabolised Ins(1,4,5)P_3 synthetic analogues such as Ins(1,4,5)PS_3 and Ins(4,5)PS_2, were used in order to deplete the Ins(1,4,5)P_3-sensitive Ca^{2+} stores in chromaffin cells. Although the Ins(1,4,5)P_3-sensitive Ca^{2+} store had been depleted caffeine was still able to evoke Ca^{2+} mobilisation, consistent with the presence of an exclusive caffeine-sensitive Ca^{2+} store. Findings from these experiments also indicated that the Ins(1,4,5)P_3 response was significantly reduced after prior treatment with caffeine providing evidence that these stores may functionally overlap. The experiments on electrically permeabilised cells also provided evidence for the presence of two distinct intracellular Ca^{2+} stores in chromaffin cells and also suggested that these stores may partially overlap.

To evaluate the Ca^{2+}- selective electrode method a number of experiments were carried out using human neuroblastoma SH-SY5Y cells. The characteristics of the Ins(1,4,5)P_3 responses are well studied in these cells. The fact that Ins(1,4,5)P_3 was able to release almost 70-80% of total mobilizable Ca^{2+}, indicated that 20-30% of the ionomycin response was from an Ins(1,4,5)P_3-insensitive store. Findings from this Thesis indicated that caffeine was also able to stimulate Ca^{2+} mobilisation from these cells. To my knowledge this is the first report demonstrating the existence of caffeine-sensitive Ca^{2+} stores in SH-SY5Y cells, indicating that CICR mechanisms may also be present in these cells. Recent studies characterised the type of the ryanodine receptor expressed in SH-SY5Y cells and indicated that it resembles the type 2 cardiac form (J. Mackrill, personal communication). Further investigation is required in order to

examine the relationship between Ins(1,4,5)P_3- and ryanodine-sensitive Ca^{2+} stores in these cells. The use of poorly metabolised Ins(1,4,5)P_3 analogues could prove a key tool in experimentally testing this relationship in SH-SY5Y cells and other cell types.

Another method was developed in order to further examine the characteristics of the intracellular Ca^{2+} stores in permeabilised chromaffin cells. According to this method saponin-permeabilised chromaffin cells can be used to examine the mechanisms by which Ins(1,4,5)P_3- and caffeine-induce Ca^{2+} release from intracellular Ca^{2+} stores in chromaffin cells. The experiments on saponin-permeabilised chromaffin cells also suggest that two distinct intracellular Ca^{2+} stores are present in chromaffin cells that could be distinguished pharmacologically. The first store was found to be sensitive to Ins(1,4,5)P_3 which stimulated Ca^{2+} mobilisation with an EC_{50} of 619 nM. The second store was sensitive to caffeine which released Ca^{2+} with an EC_{50} of 16.59 mM. The responses to Ins(1,4,5)P_3/caffeine when added simultaneously were larger than each of these agonists when added alone, consistent with the two pool model. Furthermore, the response to Ins(1,4,5)P_3/caffeine was smaller than the product of Ins(1,4,5)P_3 + caffeine when added alone, indicating that a degree of overlap between these stores exist in chromaffin cells.

Pretreatment with thapsigargin abolished the effect of subsequent addition of Ins(1,4,5)P_3, indicating that the Ins(1,4,5)P_3-sensitive Ca^{2+} stores had been depleted. However, it did not inhibit subsequent caffeine response providing evidence that chromaffin cells possess caffeine exclusive Ca^{2+} stores. These results may also suggest that Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores possess different types of Ca^{2+}-ATPases (SERCA).

Ryanodine pretreatment was found to inhibit the caffeine response and to significantly reduce the Ins(1,4,5)P_3-evoked Ca^{2+} mobilisation, indicating that Ins(1,4,5)P_3-induced Ca^{2+} release was followed by Ca^{2+} induced Ca^{2+} release (CICR) from ryanodine-sensitive Ca^{2+} stores in saponin permeabilised chromaffin cells. Ryanodine does not inhibit the Ins(1,4,5)P_3 receptor, indicating that it is a selective

pharmacological tool (Ehrlich et al., 1994). This data also provided evidence for the existence of an exclusive Ins(1,4,5)P_3-sensitive Ca^{2+} store in chromaffin cells.

Further evidence, which would be difficult to explain in terms of one homogenous Ca^{2+} store, came from the study on the effect of ATP and elevated $[Ca^{2+}]_i$ levels on the Ins(1,4,5)P_3 and caffeine induced Ca^{2+} release. Data from this study revealed that ATP and Ca^{2+} regulate Ins(1,4,5)P_3 and caffeine responses in a different manner. This may be indicative of the differences between the Ins(1,4,5)P_3 and ryanodine receptor in chromaffin cells and so reflecting the existence of two distinct Ca^{2+} stores. The physiological significance of these findings is not yet clear. In the present study, complexity in the mechanisms that modulate Ins(1,4,5)P_3 and ryanodine receptors was suggested. Furthermore, no evidence for a regulatory role of phosphorylation on the Ins(1,4,5)P_3- induced Ca^{2+} release was found. Neither cAMP dependent protein kinase nor PKC seem to influence the Ins(1,4,5)P_3 receptor within the limits of these experiments. How Ins(1,4,5)P_3 and ryanodine receptors are regulated in chromaffin cells remains to be fully illustrated.

The experiments performed on permeabilised chromaffin cells in this study suggested that two distinct intracellular Ca^{2+} stores are present in these cells and evidence for a degree of functional overlap was also found. However, no clear evidence suggesting overlap between the two stores was found in previous studies using digitonin permeabilised chromaffin cells (Robinson and Burgoyne, 1991a) whereas similar results to those presented here were reported from studies using α-toxin permeabilised cells (Stauderman et al., 1991). Differences in the permeabilising protocols could account for variances between these studies. It is possible that digitonin permeabilisation disrupts the morphology of the endoplasmic reticulum (see Renard-Rooney et al., 1993) causing fragmentation which obstructs characteristics of the Ins(1,4,5)P_3 and caffeine-sensitive Ca^{2+} stores which can be detected using saponin or α-toxin as permeabilising agents. To examine whether findings from permeabilised cells reflect the nature of the intracellular stores in intact cells, a number

of experiments was carried out using populations of intact chromaffin cells loaded with fura2-AM.

Bradykinin (an Ins(1,4,5)P_3 generating agonist) and caffeine were able to stimulate Ca^{2+} release from intracellular Ca^{2+} stores. The amount of Ca^{2+} released in response to these stimuli was not found to be significantly different, indicating that the size of the Ins(1,4,5)P_3 and caffeine releasable pools is similar. Pretreatment with ryanodine abolished subsequent additions of caffeine, indicating that caffeine releases Ca^{2+} from a ryanodine-sensitive Ca^{2+} store and that ryanodine, by locking the ryanodine receptor in an open state, prevents Ca^{2+} accumulation by the store. When chromaffin cells, which had already had their caffeine-sensitive Ca^{2+} stores depleted, were challenged with bradykinin a significantly reduced response was obtained. This data suggests that bradykinin stimulation activated a CICR mechanism which evoked Ca^{2+} release from the caffeine-sensitive Ca^{2+} stores, or that some intracellular Ca^{2+} stores possess Ins(1,4,5)P_3 and ryanodine receptors together. It is also possible that bradykinin activates another second messenger, such as cyclic ADP-ribose, which stimulates Ca^{2+} mobilisation from the ryanodine-sensitive Ca^{2+} stores. As previously discussed, there is evidence indicating that caffeine inhibited Ins(1,4,5)P_3-induced Ca^{2+} release in *Xenopus* oocytes and cerebellar microsomes (Parker and Ivorra, 1991; Brown et al., 1992). Recent data suggested that this inhibitory effect of caffeine was due to a direct action of caffeine on the Ins(1,4,5)P_3 receptor (Bezprozvanny et al., 1994) and also indicated that complete inhibition was observed with 10 mM caffeine. This action of caffeine could also provide an alternative explanation for the effect of caffeine on the bradykinin response observed in this Thesis. The possibility that caffeine or caffeine-induced Ca^{2+} release down regulates the bradykinin receptor cannot be ruled out. A recent report demonstrated that carbachol stimulation abolished the elevation of Ca^{2+} by bradykinin and the bradykinin-mediated Ins(1,4,5)P_3 elevation in SH-SY5Y cells (Willars and Nahorski, 1995). Furthermore, this data suggested that depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores may account for the reduced

Ins(1,4,5)P_3 responses to bradykinin due to lack of Ca^{2+} feedforward activation of PLC. Therefore, it is possible that caffeine by depleting part of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores resulted in a smaller elevation of Ins(1,4,5)P_3 in response to bradykinin. This mechanism could also be responsible for the reduced agonist response after prior treatment with caffeine, as observed in chromaffin cells.

Findings in this study also demonstrated that bradykinin response was abolished after prior treatment with thapsigargin suggesting that the agonist-sensitive Ca^{2+} store had been depleted. Subsequent addition of caffeine resulted in Ca^{2+} mobilisation indicating the presence of a unique caffeine-sensitive Ca^{2+} store in chromaffin cells. However, under these conditions the caffeine response was significantly reduced, suggesting either that there are some stores expressing both Ins(1,4,5)P_3 and ryanodine receptors, or that the effect of thapsigargin on the Ca^{2+}-ATPase is not that specific for the Ins(1,4,5)P_3-sensitive Ca^{2+} store.

All of the earlier studies in chromaffin cells considered thapsigargin as a selective inhibitor of the Ca^{2+}-ATPase responsible for the refilling of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores. Furthermore, no effect of thapsigargin pretreatment on the caffeine-induced Ca^{2+} release had been previously reported in chromaffin cells. In the present study it is demonstrated that thapsigargin affected Ca^{2+} accumulation in caffeine-sensitive Ca^{2+} stores in chromaffin cells. This raises doubts over the specificity of thapsigargin for the Ins(1,4,5)P_3-sensitive Ca^{2+} store Ca^{2+}-ATPase and suggests that ryanodine-sensitive pools in chromaffin cells may also use Ca^{2+}-ATPase isoforms which are thapsigargin sensitive. Similar results have been recently reported in other cell types (see Pozzan et al., 1994). These findings are also consistent with the two store model. The Ins(1,4,5)P_3-sensitive Ca^{2+} stores in the experiments described in this study appears to leak Ca^{2+} rapidly where the caffeine-sensitive Ca^{2+} stores do so more slowly. Therefore differences in the nature of the intracellular stores may be responsible for the supposed selective action of thapsigargin seen before. Hence, in the future thapsigargin should be used with caution as a specific inhibitor of the Ca^{2+}-

ATPase responsible for the refilling of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores in chromaffin cells.

Using epifluorescence microscopy with fura 2 loaded single chromaffin cells, bradykinin has been also found to activate the CICR mechanism. Pretreatment with caffeine significantly reduced subsequent responses to bradykinin and vice-versa. The inability of caffeine pretreatment to completely inhibit bradykinin responses contribute to evidence indicating that an exclusive Ins(1,4,5)P_3-sensitive Ca^{2+} store is present in intact single chromaffin cells. Bradykinin stimulation appears to activate both Ins(1,4,5)P_3 induced Ca^{2+} release and CICR. However, bradykinin pretreatment did not abolish caffeine response although the CICR appeared to be active under these conditions. Two mechanisms can be offered to explain these findings.

The first is related to the localisation of the Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores within the chromaffin cells. It has been previously shown that Ins(1,4,5)P_3-sensitive Ca^{2+} stores are located at one pole of the cell, whereas the caffeine-sensitive Ca^{2+} stores are diffusely distributed (Burgoyne et al., 1989b). It is possible that the Ca^{2+} released in response to bradykinin, under conditions where low extracellular Ca^{2+} levels were applied, was adequate to stimulate CICR from neighbouring caffeine-sensitive Ca^{2+} stores but not from the more distant ones.

The other possible explanation is that heterogeneity between caffeine-sensitive Ca^{2+} stores may exist which probably involves different intracellular Ca^{2+} requirements in order for the CICR mechanism to occur. Bradykinin stimulation resulted in Ca^{2+} mobilisation from Ins(1,4,5)P_3-sensitive stores, which under the conditions studied here, may activate CICR from caffeine-sensitive Ca^{2+} stores which require lower Ca^{2+} levels to respond. The stores with higher Ca^{2+} requirements may be activated when Ca^{2+} entry across the plasma membrane occurs providing chromaffin cells with a mechanism to respond in situations where large amounts of Ca^{2+} enters the cell as in response to more potent secretagogues or depolarising stimuli. Spatial localisation and heterogeneity of the caffeine-sensitive Ca^{2+} stores may

together also be responsible for the effect of bradykinin on caffeine responses detected in this study.

Bradykinin has been found to activate Ca^{2+} entry. However, previous results indicated that this does not occur via L- or N- types of VSCCs (Owen et al., 1989a and b). It remains possible that other types of VSCCs recently discovered in the plasma membrane of chromaffin cells, such as P-type (Gandia et al., 1993; Albillos et al., 1993) or Q-type channels (Lopez et al., 1994), may be activated by bradykinin resulting in Ca^{2+} entry. Caffeine in the present study was also able to stimulate Ca^{2+} entry, suggesting that all the intracellular Ca^{2+} stores are equally linked to the activation of Ca^{2+} influx. These findings demonstrate that the mechanism by which Ca^{2+} released from intracellular stores activates Ca^{2+} influx is not a unique characteristic of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores but is also present on the ryanodine-sensitive Ca^{2+} stores. Extracellular Ca^{2+} entry is the source responsible for refilling the depleted Ca^{2+} stores after termination of receptor activation. The signal which activates Ca^{2+} entry after depletion of the caffeine-sensitive Ca^{2+} stores is not known. It is possible that theories offered in order to explain the nature of this signal after depletion of Ins(1,4,5)P_3-sensitive Ca^{2+} stores may also apply for the ryanodine-sensitive Ca^{2+} stores. A soluble second messenger such as Ca^{2+}, metabolites of P-450 (Alvarez et al., 1992; Garcia et al., 1992; Montero et al., 1992), cGMP (Pandol and Schoeffield-Payne, 1990; Bahnson et al., 1993), and CIF (Randriamampita and Tsien, 1993) are suitable candidates. Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Irvine, 1991 and 1992; Irvine and Cullen, 1993) may be involved and so explain part of the Ca^{2+} entry after depletion of Ins(1,4,5)P_3-sensitive Ca^{2+} stores in some cell types. The action of these two second messengers cannot however be part of the mechanisms which activate Ca^{2+} entry after depletion of the ryanodine-sensitive Ca^{2+} stores. Conformational changes transmitted to the plasma membrane via the cytoskeleton should also be considered.

The present study reveals the existence of two non identical Ca^{2+} stores; one sensitive to Ins(1,4,5)P_3 and the other sensitive to caffeine. Remarkable similarities between the characteristics of these stores exist in chromaffin cells. They are both regulated by cytoplasmic Ca^{2+} and ATP. The Ca^{2+}-ATPase responsible for the refilling of both stores appears to be sensitive to thapsigargin, although the caffeine-sensitive Ca^{2+} stores were previously considered not to be sensitive to thapsigargin in chromaffin cells. Depletion of the stores activates Ca^{2+} influx across the plasma membrane of chromaffin cells. Very recent studies demonstrate 'quantal' Ca^{2+} release from caffeine-sensitive Ca^{2+} stores in chromaffin cells (Cheek et al., 1993b and 1994a). Their findings suggested heterogeneity between the caffeine-sensitive Ca^{2+} stores.

Recent reports investigated the effect of 2,5-di-(tert-butyl)-1,4-benzohydroquinone (tBHQ), which is another Ca^{2+}-ATPase inhibitor, on Ca^{2+} mobilisation from intracellular stores (Robinson and Burgoyne, 1991b; Robinson et al., 1992). Their data suggested that treatment of the cells with tBHQ before either angiotensin II or caffeine, had no effect (or only a slight effect) on Ca^{2+} released by these two stimuli and vice versa. However, the tBHQ-sensitive store appeared to be a subset of the thapsigargin-sensitive stores, since thapsigargin pretreatment inhibited Ca^{2+} mobilisation from the tBHQ stores. They suggested that chromaffin cells possess a third intracellular Ca^{2+} store which is tBHQ sensitive but they did not describe any degree of overlap between this store and the Ins(1,4,5)P_3- or caffeine-sensitive Ca^{2+} stores. Previous reports from this group did not detect any functional overlap between these two stores. If heterogeneity between caffeine-sensitive pools is hypothesised it would be interesting to examine whether the effect of tBHQ is due to ryanodine-sensitive Ca^{2+} stores that are more accessible to the CICR mechanisms induced by Ca^{2+} mobilisation from the Ins(1,4,5)P_3-sensitive Ca^{2+} stores. However, the possibility that the Ca^{2+}-ATPase responsible for the refilling of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores is also sensitive to tBHQ cannot be excluded and, as such, requires further investigation.

The major part of the exocytotic process in chromaffin cells is due to Ca^{2+} entry across the plasma membrane (Burgoyne, 1991). However, this study indicated that bradykinin, which stimulates Ca^{2+} mobilisation from Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores, was able to evoke secretion which was partially independent of the presence of extracellular Ca^{2+}. These findings suggest that intracellular Ca^{2+} stores are involved in stimulus-secretion coupling. Depletion of the intracellular Ca^{2+} stores activated Ca^{2+} entry, indicating that Ca^{2+} mobilisation and Ca^{2+} influx into the cell could be important in triggering secretion. In the absence of extracellular Ca^{2+}, agonist-induced Ca^{2+} release from internal stores may be responsible for the small amount of catecholamine secretion detected. In the presence of extracellular Ca^{2+}, capacitative Ca^{2+} entry may account for the increased bradykinin-evoked catecholamine secretion observed. However, the magnitude of catecholamine secretion in response to bradykinin was found to be smaller than that of nicotine (a depolarising stimulus). Recent studies (Augustine and Neher, 1992) indicated that depolarisation of the chromaffin cells resulted in a burst of secretory activity, while Ca^{2+} perfusion from a patch-pipette resulted in a slower onset of secretion. The rate and the magnitude of Ca^{2+} entry via the Ca^{2+} release-activated Ca^{2+} entry channels, compared to that of the VSCCs, and the localisation of both these channels in relation to secretory granules, seem likely explanations for these differences.

The physical location of intracellular stores cannot explain differences in the potency of different secretagogues as previously described (Mochizuki-Oda et al., 1991a and b; Cheek et al., 1993a). How different stimuli give rise to different patterns of Ca^{2+} mobilisation and Ca^{2+} entry (Cheek et al., 1993a) requires further investigation. Do these differences reflect pharmacologically differentiable stores which, by virtue of their physical location and release properties, account for some tasks rather than others? Is the capacitative Ca^{2+} entry the only mechanism activating Ca^{2+} influx through a voltage-independent pathway in chromaffin cells or do other mechanisms exist which may be related to the characteristics of the different agonists

receptors? It is also possible that the localisation of these receptors on the plasma membrane may account for these differences. Agonists that stimulate diffusely distributed receptors could evoke Ca^{2+} mobilisation from a larger number of accessible Ins(1,4,5)P_3-sensitive Ca^{2+} stores activating more CICR pools resulting in Ca^{2+} entry from Ca^{2+} channels across the plasma membrane. Furthermore, agonists that stimulate receptors clustered in one spatial localised area of the plasma membrane may evoke Ca^{2+} release from a limited number of Ins(1,4,5)P_3-sensitive Ca^{2+} stores which activates CICR from neighbouring ryanodine-sensitive pools leading to a spatial localised Ca^{2+} entry. Earlier results indicated that VSCCs are clustered on frog presynaptic synapses (Robitaille et al., 1990 and 1993).

It also possible that hormones such as histamine induced Ca^{2+} entry which precedes Ca^{2+} release from the intracellular Ca^{2+} stores as recently described in chromaffin cells (Cheek et al., 1994b). The physiological importance of these findings remains obscure. Their data suggested that this Ca^{2+} entry was not achieved via the activation of receptor-operated Ca^{2+} channels (ROCs), indicating that this entry could be mediated via G-proteins activation or inositol phosphates production. Various mechanisms have been proposed (and discussed previously) in order to explain this action of Ins(1,4,5)P_3, such as Ins(1,4,5)P_3 receptors located at the plasma membrane or that conformational changes due to the binding of Ins(1,4,5)P_3 to the Ins(1,4,5)P_3 receptor are transmitted to the plasma membrane either by direct coupling or via the cytoskeleton. However, results from this Thesis also demonstrated that depletion of the Ca^{2+} content of the intracellular Ca^{2+} stores also activated Ca^{2+} entry. The possibility that the exclusive Ins(1,4,5)P_3-sensitive Ca^{2+} stores are localised near by the plasma membrane (as proposed by Cheek et al., 1994b) and therefore their depletion can activate Ca^{2+} entry, appears unlikely since evidence presented here indicated that depletion of the caffeine-sensitive Ca^{2+} stores also activated Ca^{2+} influx. The more likely explanation is that Ca^{2+} entry in chromaffin cells is controlled by an

undetermined yet action of Ins(1,4,5)P_3 or G-proteins in conjunction with Ins(1,4,5)P_3- and caffeine-sensitive Ca^{2+} stores depletion

A model for Ca^{2+} stores in bovine adrenal chromaffin cells has been proposed as a consequence of findings in this study (Fig. 7.1). This model suggests the existence of two distinct Ins(1,4,5)P_3 and ryanodine receptor expressing stores, which are distinguishable on the basis of their pharmacological properties. The Ins(1,4,5)P_3 receptor can be activated by Ins(1,4,5)P_3 and Ins(1,4,5)P_3 synthetic analogues, and the ryanodine receptor can be stimulated by caffeine. Cytosolic Ca^{2+} levels and ATP can regulate responses to Ins(1,4,5)P_3 and caffeine but in a different manner. The Ca^{2+}-ATPase responsible for refilling the stores are sensitive to thapsigargin. Depletion of the intracellular Ca^{2+} stores also activates Ca^{2+} entry through a plasma membrane Ca^{2+} channel. The stores have been drawn separately although there is evidence in permeabilised and intact chromaffin cells suggests that there is a degree of functional overlap. In this model the CICR mechanism has been offered to explain this degree of overlap. This Figure illustrates a basic working model to outline the relationship between the intracellular Ca^{2+} stores in chromaffin cells that can be experimentally tested and modified to produce of a more complicated model, and so depict the complexity of current findings.

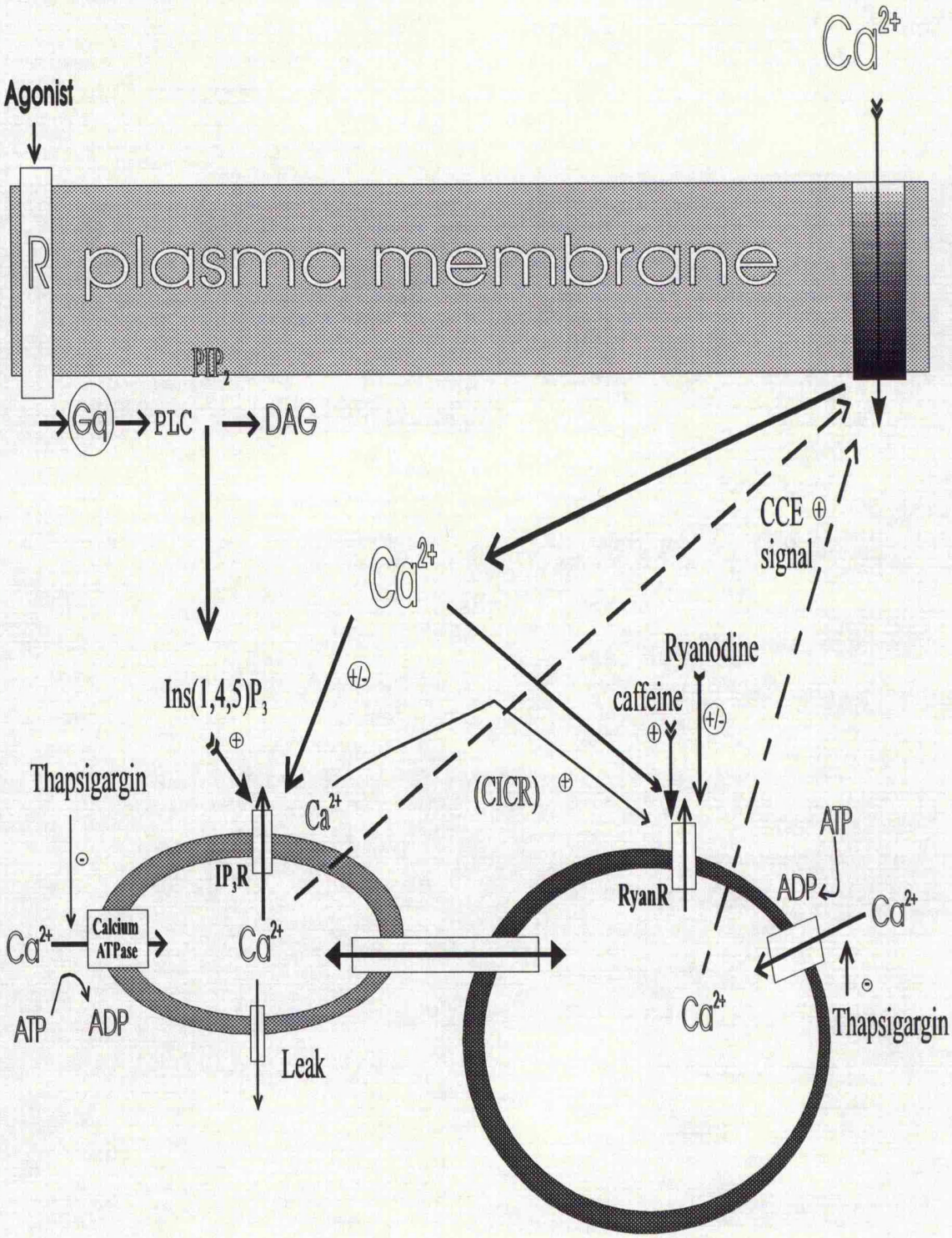


Fig. 7.1. Intracellular Ca^{2+} stores in bovine adrenal chromaffin cells.

7.2 FUTURE EXPERIMENTAL APPROACHES

This study investigated the nature of the intracellular Ca^{2+} stores in permeabilised and intact chromaffin cells. The present findings suggest that bradykinin was able to stimulate Ca^{2+} mobilisation from Ins(1,4,5)P_3-sensitive Ca^{2+} stores followed by Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores provide some evidence for the physiological role of the intracellular Ca^{2+} pools. Bradykinin evoked catecholamine secretion was not abolished in the absence of extracellular Ca^{2+} indicating that intracellular stores do not act only as a 'sink' of Ca^{2+}, buffering elevated Ca^{2+} levels following stimulation of chromaffin cell, but may also have an important role in triggering secretion. Mn^{2+} quench of fura 2 fluorescence has been demonstrated in other cell types and chromaffin cells to be a reliable method of assessing divalent cation-permeant channel activation. This technique can be used to examine the effect of agents used here such as thapsigargin and ryanodine in depolarising stimuli (such as K^+) activated Ca^{2+} entry in order to investigate the effect of depletion of the intracellular Ca^{2+} stores in depolarising stimuli responses.

A number of modulators of Ca^{2+} stores not used in this study, such as the Ins(1,4,5)P_3 receptor blocker heparin, the Ca^{2+}-ATPase inhibitors such as tBHQ (Moore et al., 1987; Witcome et al., 1992), and cyclopiazonic acid (Seidler et al., 1989), in addition with those used here such as poorly metabolised Ins(1,4,5)P_3 analogues, ryanodine, and thapsigargin might prove useful in further characterising the intracellular Ca^{2+} stores and differentiating on a pharmacological basis. However, recent data demonstrated that heparin activated the ryanodine receptor Ca^{2+} channels that had been incorporated into bilayers, indicating that it is not as specific as it was originally thought (Bezprozvanny et al., 1993). Furthermore, thapsigargin appeared to block VSCCs in adrenal glomerulosa cells (Rossier et al., 1993), indicating that it has to be used with some caution. A novel Ca^{2+} pump inhibitor (bis-phenol) has been recently reported (Brown et al., 1994) which was found to be more potent than

thapsigargin and tBHQ, but also inhibits plasma membrane Ca^{2+}-ATPases. The effect of this inhibitor on the Ca^{2+}-ATPases of intracellular Ca^{2+} stores in chromaffin cells requires investigation in order to further examine the characteristics of these stores.

The experimental approach described in this Thesis measuring $^{45}Ca^{2+}$ release from saponin-permeabilised chromaffin cells provides us with a technique that measures Ca^{2+} mobilisation directly. This technique can also be very useful in studies concerning the specificity of Ca^{2+}-ATPase inhibitors on different Ca^{2+} pools. The effect of various modulators of the Ins(1,4,5)P_3 and caffeine induced Ca^{2+} release could also be examined. Synthetic Ins(1,4,5)P_3 analogues such as Ins(4,5)PS_2 and Ins(1,4,5)PS_3 used here and others recently described (see Nahorski and Potter, 1992; Safrany et al., 1994) could provide a key tool in future studies testing the relationship between intracellular Ca^{2+} stores in other cell types.

Depletion of ryanodine-sensitive Ca^{2+} stores in this study appeared to activate Ca^{2+} entry across the chromaffin cell plasma membrane. These findings could be tested using Mn^{2+} quench of fura-2 fluorescence using single chromaffin cells and also other cell types. The nature of the signal by which Ca^{2+} depletion of Ins(1,4,5)P_3- and ryanodine-sensitive stores activates Ca^{2+} entry requires further investigation.

Immunocytochemistry studies and electron microscopy might be useful to examine the distribution of the Ins(1,4,5)P_3 and ryanodine receptors in chromaffin cells. It would also be interesting to examine the distribution of agonists receptors on single chromaffin cells, but unfortunately no such experiments can be performed at this moment. These experiments could provide us with some explanations as to where differences in the receptor location or heterogeneity of intracellular stores account for the variable patterns of agonist induced Ca^{2+} mobilisation and Ca^{2+} entry in bovine adrenal chromaffin cells. The localisation of Ins(1,4,5)P_3 and ryanodine receptors within the cell (and on the plasma membrane in the case of Ins(1,4,5)P_3 receptors) may provide us with important findings about the physical location of these stores within the cell and increase our knowledge of Ca^{2+} signalling in chromaffin cells.

Chromaffin cells provide a useful and reliable model for the study of mechanisms of Ca^{2+} regulation. They possess a variety of mechanisms that elevate $[Ca^{2+}]_i$ and can be used as permeabilised or intact populations or single cells. Patch-clamp techniques, microinjection and biochemical studies can also performed on chromaffin cells. They also provide a very useful model for studies on stimulus-secretion coupling. It appears that chromaffin cells are ideal for studies such as the present one, in which the relationship between intracellular Ca^{2+} stores and their role in secretion are examined. Whether neuronally-derived adrenal chromaffin cells represent a good model for neurons or not remains ambiguous. However, studies on these cells have been significant valuable in elucidating postsynaptic events involved in stimulus-secretion coupling. Mechanisms that operate in these cells control secretion from the adrenal medulla and may be important on maintaining homeostasis in the autonomic nervous system.

APPENDIX 1: MATERIALS

All tissue culture media was obtained from Gibco LTD., Middlesex, UK.

"Primaria" 24 well plates were from Becton Dickinson, Oxford, UK.

Radiochemicals from Amersham International PLC, Aylesbury, UK.(unless otherwise indicated).

[^{3}H]-inositol from New England Nuclear, Stevenage, UK.

Ins(1,4,5)P_3 was obtained from Amersham International PLC, Aylesbury, UK.

Fura-2 and Fura-2 AM was from Calbiochem La Jolla, CA, USA.

$^{45}CaCl_2$ was from Amersham International PLC, Aylesbury, UK.

Bradykinin was from Cambridge Research Biochemicals, Cambridge, UK.

All other chemicals and general laboratory reagents were obtained from Sigma, Poole, UK.

APPENDIX 2: ABBREVIATIONS

ATP:	Adenosine 5'- triphosphate
BSS:	Balanced Salt Solution
$[Ca^{2+}]$:	Free Ca^{2+} concentration
$[Ca^{2+}]_i$:	Free intracellular Ca^{2+} concentration
CAFF:	Caffeine
cADP-R:	Cyclic adenosine diphosphate ribose
CICR:	Calcium-induced calcium release
DAG:	Diacylglycerol
DMEM:	Dulbeccos Modiefied Eagles Medium
EDTA:	Ethylenediaminetetraacetic Acid
EGTA:	Ethyleneglycol-bis-(aminoethyl ester)-N-N-N'-N'-tetraacetic acid
G-protein:	Guanosine nucleotide binding protein
HEPES:	N-[-2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HPLC:	High Pressure Liguid Chromatography
$Ins(1,4,5)P_3$:	Inositol- 1,4,5- trisphosphate
$Ins(1,3,4,5)P_4$:	Inositol- 1,3,4,5- tetrakisphosphate
$Ins(4,5)PS_2$:	Inositol- 4,5- bisphosphorothioate
$Ins(1,4,5)PS_3$:	Inositol-1,4,5-trisphosphorothioate
$[Na]_i$:	Free intracellular sodium concentration
PKC:	Protein kinase C
PLC:	Phospholipase C
PLD:	Phospholipase D
PtdIns:	Phosphatidyl inositol
$PtdIns4,5P_2$:	Phosphatidyl inositol 4,5-bisphosphate
Quin-2:	2-{[2-bis-(carboxymethyl)-amino-5-methylphenoxyl]-methyl}-6-methoxy 8-bis-(carboxymethyl)-aminoquinoline
ROCs:	Receptor operated Ca^{2+} channels

RYAN:	Ryanodine
SMOCs:	Second messenger operated Ca^{2+} channels
TG:	Thapsigargin
TPA:	12-O-tetradecanoylphorbol 13-acetate
VSCCs:	Voltage sensitive Ca^{2+} channels

APPENDIX 3: COMPOSITION OF BUFFERS

1) Lockes Solution

Lockes Solution consisted of the following ingredients :

concentration	chemical name	chemical formula
154 mM	sodium chloride	NaCl
5.6 mM	potassium chloride	KCl
5.6 mM	glucose	$C_6H_{12}O_6$
5.0 mM	Hepes	$C_8H_{18}N_2O_4S$

pH to 7.4 with 5 M sodium hydroxide (NaOH).

2) Balanced Salt Solution (BSS)

concentration	chemical name	chemical formula
125 mM	sodium chloride	$NaCl$
30 mM	Hepes	$C_8H_{18}N_2O_4S$
16.2 mM	sodium hydrogen carbonate	$NaHCO_3$
5.5 mM	glucose	$C_6H_{12}O_6$
5.4 mM	potassium chloride	KCl
1.0 mM	sodium dihydrogen orthophosphate	KH_2PO_4
0.8 mM	magnesium sulphate hexahydrate	$MgSO_4.6H_2O$
1.8 mM	calcium chloride dihydrate if required.	$CaCl_2.2H_20$

pH to 7.4 with 5 M sodium hydroxide (NaOH).

3) Intracellular-Like Buffer (I.C.B.)

concentration	chemical name	chemical formula
120 mM	potassium chloride	KCl
20 mM	Hepes (free acid)	$C_8H_{18}N_2O_4S$
6.0 mM	magnesium chloride	$MgCl_2$
5.0 mM	sodium succinate	$(CH_2COONa)_2 \cdot 6H_20$
2.0 mM	potassium dihydrogen orthophosphate	KH_2PO_4
5.0 mM	ATP (Adenosine 5'-trisphosphate disodium-salt). After addition of ATP the pH was adjusted to 6.9 with 20% (w/v) KOH.	

An appropriate aliquot of EGTA (10 mM/ 20 mM KOH\pH 7.2) was added to drive the free Ca^{2+} concentration between 100-200 nM.

4) Krebs/Heseleit Solution

concentration	chemical name	chemical formula
118.6 mM	sodium chloride	$NaCl$
10.0 mM	Hepes (free acid)	$C_8H_{18}N_2O_4S$
4.2 mM	sodium hydrogen carbonate	$NaHCO_3$
11.7 mM	glucose	$C_6H_{12}O_6$
1.2 mM	magnesium sulphate hexahydrate	$MgSO_4.6H_2O$
1.2 mM	potassium dihydrogen orthophosphate	KH_2PO_4
4.7 mM	potassium chloride	KCl
2.0 mM	calcium chloride dihydrate	$CaCl_2.2H_20$

pH to 7.4 with 1 M sodium hydroxide (NaOH).

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