Lack of receptor selective effects of either RGS2, RGS3 or RGS4 on muscarinic M_3- and gonadotropin-releasing hormone-receptor-mediated signalling through $G\alpha_{q/11}$

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Abstract

Termination of signalling by G-protein-coupled receptors requires inactivation of the Gα-subunits of heterotrimeric G-proteins and the re-association of Gα- and Gβγ-subunits. Inactivation of Gα-subunits is achieved by the hydrolysis of bound GTP by an intrinsic GTPase activity, which is considerably enhanced by GTPase activating proteins. Regulators of G-protein signalling (RGS) proteins are a large family of GTPase activating proteins, many of which have structures indicating roles beyond GTPase activating protein activity and suggesting that the identity of the RGS protein recruited may also be critical to other aspects of signalling. There is some evidence of selective effects of RGS proteins against different G-protein-coupled receptors coupling to the same signalling pathways and growing evidence of physical interactions between RGS proteins and G-protein-coupled receptors. However, it is unclear as to how common such interactions are and the circumstances under which they are functionally relevant. Here we have examined potential selectivity of RGS2, 3 and 4 against signalling mediated by $G\alpha_{q/11}$-coupled muscarinic M_3 receptors and gonadotropin-releasing hormone in an immortalised mouse pituitary cell line. Despite major structural differences between these two receptor types and agonist-dependent phosphorylation of the muscarinic M_3- but not gonadotropin-releasing hormone-receptor, signalling by both receptors was similarly inhibited by expression of either RGS2 or RGS3, whereas RGS4 has little effect. Thus, at least in these circumstances, RGS protein-dependent inhibition of signalling is not influenced by the nature of the G-protein-coupled receptor through which the signalling is mediated.

Index words: RGS proteins; GPCR; calcium; phosphoinositides; G-proteins

1. Introduction

Activated G-protein-coupled receptors promote the loss of GDP from α-subunits of heterotrimeric G-proteins and the subsequent binding of GTP. These GTP-bound α-subunits dissociate from their βγ-subunits, thereby allowing interactions with effector molecules. Bound GTP is subsequently hydrolysed by a GTPase activity intrinsic to the α-subunit, which promotes re-association with βγ-subunits and the termination of signalling. The α-subunits have a relatively low rate of intrinsic GTPase activity but this is markedly enhanced by GTPase activating proteins, which are needed to evoke the rate of signal termination required under physiological conditions. Although an effector molecule of $G\alpha_{q/11}$, phospholipase Cβ1, can act as a GTPase activating protein (Berstein et al., 1992), regulators of G-protein signalling (RGS) proteins are a large family of proteins with considerable GTPase activating activity toward the α-subunits of heterotrimeric G-proteins (Siderovski et al., 2005; Willars, 2006). Some RGS proteins can also compete with effector molecules for binding to Gα-subunits (Scheschonka et al., 2000; Anger et al., 2004; Garzon et al., 2005a) or act as guanine nucleotide dissociation inhibitors (Kimple et al., 2001; Traver et al., 2004), suggesting they may regulate signalling through mechanisms other than enhancing GTPase activity.

In their simplest form, RGS proteins contain an RGS domain of approximately 120 amino acids and relatively little else. However, many contain N- and/or C-terminal sequences, which in some instances contain motifs often associated with protein-protein interactions. Indeed, interactions of RGS proteins with other cellular proteins have been established, and these may influence the subcellular distribution of RGS proteins and cell signalling events (Siderovski et al., 2005; Willars, 2006). As RGS proteins not only terminate signalling but may influence the nature of the signalling events, this suggests a need for selectivity in the type of RGS protein recruited. Such selectivity could be enforced by the cellular and

subcellular expression and distribution patterns and also the specificity of interactions with Gα-subunits (Willars, 2006). There is also some evidence that the receptor may directly contribute to specificity. This was initially suggested in studies demonstrating that addition of purified RGS1, 4 or 16 inhibited signalling by $G\alpha_{q/11}$-coupled muscarinic receptors more effectively than that mediated by cholecystokinin receptors coupled to the same G-proteins (Xu et al., 1999). Other examples of specificity exist and direct interactions of RGS proteins with fragments of G-protein-coupled receptors, specifically the C-terminal tail and third intracellular loop, have been demonstrated (Snow et al., 1998; Bernstein et al., 2004). However, the role of receptors in the specificity of RGS protein recruitment and action is far from clear. Here we have employed two G-protein-coupled receptors of very different structures, but which typically couple to the same Gα-subunit family to further assess the role of the receptor in dictating the specificity of RGS protein function. We have used the muscarinic M_3 receptor and gonadotropin releasing-hormone receptor and determined the impact of members of the B/R4 RGS sub-family (RGS2, 3 and 4) on signalling. Both these receptors couple to $G\alpha_{q/11}$, however, the gonadotropin-releasing hormone receptor has a short third intracellular loop (49 amino acids) compared to the muscarinic M_3 receptor (240 amino acids). Furthermore, whilst the muscarinic M_3 receptor has a C-terminal tail (predicted 43 amino acids), mammalian gonadotropin-releasing hormone receptors are essentially tail-less making them structurally atypical G-protein-coupled receptors. As a consequence of such structural differences the gonadotropin-releasing hormone receptor is not phosphorylated following agonist occupation and is resistant to the associated acute desensitisation (Willars et al., 1999), which is in contrast to the majority of G-protein-coupled receptors, including the muscarinic M_3 receptor (Tobin et al., 1993).

2. Materials and Methods

2.1 Materials

Tissue culture reagents and media were supplied by Invitrogen (Paisley, U.K.). Cell culture plastic-ware was from NUNC (Roskilde, Denmark). Unless stated otherwise, reagents were supplied by Sigma Aldrich (Poole, Dorset, U.K.), Merck (Darmstadt, Germany) or BDH Laboratory Supplies (Poole, U.K.).

2.2 DNA constructs

Plasmids containing full-length constructs encoding human RGS2 (L13463), human RGS3 (U27655) and rat RGS4 (U27767) were gifts from Dr. C. Doupnik (University of South Florida, Tampa, FL, U.S.A.). The plasmids for RGS2 and RGS3 in the mammalian expression vector pRcCMV (Invitrogen, Paisley, U.K.) were originally from Dr. K. Druey and Dr. J. Kehrl (National Institute of Health, Bethesda, MD, U.S.A.), whilst the plasmid for RGS4 in the mammalian expression vector pcDNA3.1 (Invitrogen, Paisley, U.K.) originated from Dr. H. Lester (California Institute of Technology, Pasadena, CA, U.S.A.). The plasmid containing a constitutively active $G\alpha_q$ mutant (Q209L) (CA-$G\alpha_q$) originated from Dr. J. Hepler (Emory University, Antland, GA, U.S.A.) and was kindly donated by Dr. S. Heximer (University of Washington, St. Louis, MO, U.S.A.). The biosensor vectors containing the fusion construct between either enhanced green fluorescent protein (eGFP) or enhanced yellow fluorescent protein (eYFP) and the pleckstrin homology (PH) domain of phospholipase C (PLC) δ1 (eGFP-$PH_{PLC\delta1}$ and eYFP-$PH_{PLC\delta1}$ respectively) were kindly provided by Professor T. Meyer (Stamford University, CA, U.S.A.) as was the eGFP-tagged $C1_2$ domain of protein kinase Cγ (eGFP-PKC$\gamma C1_2$). To generate *myc*- and (cyan fluorescent protein-) CFP-tagged RGS protein constructs (RGS-*myc* and RGS-CFP respectively), RGS 2, 3 and 4 were amplified by PCR from their original vectors to incorporate KpnI and XhoI (*myc*-tag) or KpnI and AgeI (CFP-

tag) restriction sites. The resulting PCR fragments were then column purified (Qiagen, Crawley, U.K.) and sub-cloned into either pcDNA3.1/*myc*-His (Invitrogen, Paisley, U.K.) or pECFP-N1 (Clontech, CA, U.S.A.). Expression of these constructs results in the generation of C-terminal epitope-tagged RGS proteins.

2.3 Cell culture and transfection

The αT3-1 gonadotrope cell line was originally a gift from Dr. P. Mellon (University of California, San Diego, CA, U.S.A.). In the current study we used a clonal cell line (αT3-1/M_3) derived from this, which expresses the recombinant human muscarinic M_3 receptor (~350 fmol mg protein^{-1}; Willars et al., 1998). Cells were cultured in Dulbecco's modified Eagle's medium with Glutamax-1, supplemented with foetal calf serum (10% v/v), penicillin (50 IU ml^{-1}) and streptomycin (50µg ml^{-1}) at 37°C in a humidified atmosphere of 5% CO_2 in air. For experiments, cells were harvested from tissue culture flasks and used for membrane preparation (see below) or re-seeded into 6-well multi-dishes containing 25mm diameter borosilicate glass coverslips coated with 0.01% (w/v) poly-D-lysine. Where expression of recombinant proteins was required, cells were cultured for a further two days before being transiently transfected with the appropriate DNA constructs using Lipofectamine according to the manufacturer's instructions (Invitrogen, Paisley, U.K.). Transfections used 0.5µg of biosensor cDNA either alone or with 1.5µg of the RGS cDNA in each well of the 6-well multi-dish. Where required, for experiments using either eGFP-$PH_{PLC\delta1}$ or eGFP-PKC$\gamma C1_2$, cells were co-transfected with RGS-*myc* constructs whereas for experiments using eYFP-$PH_{PLC\delta1}$, cells were co-transfected with RGS-CFP constructs.

2.4 Live-cell Ca^{2+} and biosensor imaging

For imaging of intracellular Ca^{2+}, cells cultured on coverslips were loaded with 2μM fluo-3-acetoxymethyl ester (fluo-3-AM; TEF labs Austin, TX, U.S.A.) in Krebs-HEPES buffer (KHB) (composition (mM, unless otherwise stated): HEPES 10; $NaHCO_3$ 4.2; D-glucose 11.7; $MgSO_4.7H_2O$ 1.18; KH_2PO_4 1.18; KCl 4.69; NaCl 118; $CaCl_2.2H_2O$ 1.29; 0.01% w/v bovine serum albumin, pH 7.4) for 45min at 20ºC followed by a further 45min incubation in KHB to allow de-esterification of the indicator. Coverslips were then mounted in a chamber onto the stage of an Olympus inverted microscope. Chamber volume was maintained at 0.5ml and was perfused at 5ml min^{-1} with KHB containing test reagents as required. Bath temperature was maintained at 37ºC using a Peltier device. Using either an Olympus FV500 or a PerkinElmer Ultra*VIEW* confocal microscopes, cells were excited with a 488nm laser line and emitted light collected above 510nm. Confocal images were collected by either PMT (FV500) or cooled CCD camera (Ultra*VIEW*) at a rate of approximately one frame per second. Regions of interest were highlighted in individual cells and cytosolic fluorescence expressed as the change in fluorescence relative to the average of that in the period preceding agonist application as an index of the intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$).

PLC activity in single cells was monitored using fluorescent biosensors. At rest eGFP-$PH_{PLC\delta1}$ and eYFP-$PH_{PLC\delta1}$ are localised to the plasma membrane, as the PH domain binds with high affinity and selectivity to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (Stauffer et al., 1998; Nash et al., 2001). However, on agonist stimulation, Ins(1,4,5)P_3 is produced which binds to the PH domain with high affinity, thereby displacing the biosensors from the membrane and resulting in a cytosolic localisation proportional to PLC activation (Nash et al., 2001). In contrast, eGFP-PKC$\gamma C1_2$ has a cytosolic localisation under resting conditions but upon agonist stimulation and diacylglycerol production it is recruited to the plasma membrane (Oancea et al., 1998; Oancea and Meyer, 1998). For imaging of the

biosensors, coverslips on which cells had been cultured and transfected were mounted in a chamber onto the stage of an Olympus inverted microscope as above. Confocal imaging of the eGFP-tagged biosensors was monitored using either an Olympus FV500 or a PerkinElmer Ultra*VIEW* confocal microscope as described above. Images of eYFP-$PH_{PLC\delta1}$ were collected at approximately one frame per second using an Olympus FV500 with an excitation wavelength of 514nm and emission collected at 535-565nm. Changes in cytosolic fluorescence are expressed as eGFP or eYFP fluorescence/basal fluorescence (F/F_0 or (F/F_0)-1 to provide a basal subtraction; eGFP-$PH_{PLC\delta1}$ and eYFP-$PH_{PLC\delta1}$) or (F_0/F) (eGFP-PKC$\gamma C1_2$). In experiments using eYFP-$PH_{PLC\delta1}$, where cells were co-transfected with RGS-CFP constructs, CFP was detected with excitation and emission wavelengths of 458nm and 480-495nm respectively.

2.5 Immunostaining of RGS-*myc* proteins

RGS-*myc* proteins were immunostained as previously described (Tovey et al., 2001; Tovey and Willars, 2004). Briefly, cells were fixed with 4% paraformaldehyde in PBS for 30min and permeabilised in PBS containing 0.2% Triton-X100. Cells were then incubated with an anti-*myc* polyclonal antibody ((New England Biolabs, MA, U.S.A.); 1:100 in PBS with 3% BSA) overnight at 4°C. Detection of primary antibody was performed by an anti-rabbit FITC conjugated secondary antibody (Vector Laboratories, Peterborough, U.K.; 1:250 in PBS containing 10% normal goat serum). Coverslips were mounted onto microscope slides using VECTASHIELD (Vector Laboratories, Peterborough, U.K.). FITC labelling was visualised using either an Olympus FV500 or a PerkinElmer Ultra*VIEW* confocal microscope with excitation at 488nm and emitted light collected above 510nm.

2.6 Determination of G-protein activation

Membrane preparation. Confluent monolayers of cells were harvested from 175cm^2 flasks with PBS, collected by centrifugation (200g, 5min, 4^{o}C) and the pellet homogenised (Polytron, ~20s) in lysis buffer (composition (mM) HEPES, 10; EDTA, 10; pH 7.4). This suspension was centrifuged (30000g, 15min, 4^{o}C) and the final pellet homogenised in freezing buffer (composition (mM) HEPES, 10; EDTA, 0.1; pH 7.4). Protein concentration was adjusted to 1mg ml^{-1} and stored at –20^{o}C until use. *[^{35}S]-GTPγS binding and immunoprecipitation of Gα-subunits.* Determination of G-protein activation was by [^{35}S]-GTPγS binding and immunoprecipitation of specific Gα-subunits as previously described (Akam et al., 2001) using membranes (25μg) incubated with 1μM GDP and 1nM [^{35}S]-GTPγS (1250Ci mmol^{-1}; GE Healthcare UK Ltd, Bucks, U.K.) in assay buffer (composition (mM) HEPES, 10; NaCl, 100; $MgCl_2$ 10; pH 7.4). Where appropriate, tubes contained 10μM GTPγS to determine non-specific binding and/or agonist at the required concentration. Reactions were allowed to proceed (2min, 37^{o}C) and then terminated by addition of ice-cold assay buffer and membranes pelleted by centrifugation. Pellets were solubilised, pre-cleared and incubated overnight at 4^{o}C with 5μl $G\alpha_{q/11}$-specific antisera (1:100 dilution) (Bundey and Nahorski, 2001). Immune complexes were isolated with Protein A Sepharose beads (GE Healthcare UK Ltd, Bucks, U.K.), collected by centrifugation and extensively washed. Beads were re-suspended in scintillation fluid and [^{35}S] determined.

2.7 Data analysis

For all experiments data are reported as the mean±/+S.E.M. for *n* experiments. Statistical analysis was carried out using one-way ANOVA and where $P<0.05$ followed by Dunnett's range test. In all cases, * represents $P<0.05$; ** $P<0.01$ and *** $P<0.001$ by the range test.

3. Results

3.1 Methacholine and gonadotropin-releasing hormone activate similar signalling pathways. Challenge of αT3-1/M_3 cells with either methacholine (100μM) to activate the recombinant muscarinic M_3 receptors or gonadotropin-releasing hormone (1μM) to activate endogenously expressed gonadotropin-releasing hormone receptors, resulted in rapid and approximately equivalent increases of the $[Ca^{2+}]_i$ consisting of a rapid transient peak followed by a lower but sustained plateau phase (Fig. 1A). Transfection of cells with the Ins(1,4,5)P_3 biosensor, eGFP-$PH_{PLC\delta1}$, resulted in intense plasma membrane fluorescence consistent with binding to membrane PtdIns(4,5)P_2 (Fig. 1B). Upon addition of either methacholine (100μM) or gonadotropin-releasing hormone (1μM) there were rapid and approximately equivalent translocations of membrane fluorescence to the cytosol, resulting in a marked increase in cytosolic fluorescence (Fig. 1B,C). Typically this increased cytosolic fluorescence subsided over the following 1-2min, although there were instances in some cells where the cytosolic fluorescence was rather more sustained. G-protein-coupled receptor-mediated activation of $G\alpha_{q/11}$ was assessed in membrane preparations of αT3-1/M_3 cells by the immunoprecipitation of $G\alpha_{q/11}$ and determination of bound [^{35}S]-GTPγS following agonist challenge. Methacholine and gonadotropin-releasing hormone resulted in marked and equivalent increases in the binding of [^{35}S]-GTPγS to $G\alpha_{q/11}$ (Fig. 2).

3.2 Effects of RGS-myc constructs on agonist-mediated Ins(1,4,5)P_3 generation. We have previously demonstrated that RGS proteins containing a C-terminal *myc* epitope tag (RGS-*myc*; Tovey and Willars, 2004) inhibit phosphoinositide and Ca^{2+} signalling by muscarinic M_3 receptors in HEK 293 cells in a manner that is indistinguishable form the inhibition mediated by their untagged counterparts (Tovey and Willars, 2004). Here we co-transfected αT3-1/M_3

cells with an RGS-*myc* construct and eGFP-$PH_{PLC\delta1}$ to determine the impact of RGS protein expression on gonadotropin-releasing hormone receptor- and muscarinic M_3 receptor-mediated activation of PLC. Co-transfection of cells with eGFP-$PH_{PLC\delta1}$ and either RGS2-*myc* or RGS3-*myc* markedly reduced, and in some cells abolished the response to methacholine (Figs. 3 and 4A). These effects of the RGS proteins were particularly apparent at higher agonist concentrations, causing a partial collapse of the concentration-response curves but with no significant effect on agonist potency (Fig. 4B). Furthermore, in those cells expressing either RGS2-*myc* or RGS3-*myc* that did respond to a maximal concentration of methacholine (100μM), the kinetics of the responses were significantly slowed (rates (1/rise time to peak (s^{-1})): control, 0.094±0.008; RGS2-*myc*, 0.068±0.005; RGS3-*myc*, 0.051±0.004; mean±S.E.M., n=16-21, $P<0.05$ and $P<0.01$ respectively by Dunnett's test following oneway ANOVA). In contrast, RGS4-*myc* had no effect on either the magnitude of methacholine-mediated responses (Fig. 4A and B) or the kinetics of the Ins(1,4,5)P_3 response (1/rise time to peak (s^{-1}): 0.107+0.010, n=25). Transfection of cells with a control plasmid (LacZ-*myc*; see Tovey and Willars, 2004) resulted in methacholine-mediated signalling that was indistinguishable from that of controls (Fig. 4A). This additional control was used in all instances where RGS-*myc* constructs were used in functional assays. In each instance this construct had no effect and for simplicity the data are not shown for subsequent experiments.

Expression of either RGS2-*myc* or RGS3-*myc* significantly reduced Ins(1,4,5)P_3 responses to a maximal concentration of gonadotropin-releasing hormone (1μM) (Fig. 4C). In those cells expressing either RGS2-*myc* or RGS3-*myc* that responded to gonadotropin-releasing hormone (RGS2-*myc* 8 out of 14 cells; RGS3-*myc* 18 out of 21 cells), the kinetics of the responses were significantly slowed (rates (1/rise time to peak (s^{-1})): control, 0.097±0.008; RGS2-*myc*, 0.040±0.004; RGS3-*myc*, 0.058±0.009; mean±S.E.M., n=8-29, $P<0.01$ by Dunnett's test

following oneway ANOVA). In contrast RGS4-*myc* had no effect on the magnitude (Fig. 4C) or kinetics (rise-time) of the Ins(1,4,5)P_3 response (1/rise time to peak (s^{-1}): 0.107+0.010, n=13).

The subcellular distribution of RGS-*myc* proteins was assessed by immunocytochemistry using an anti *myc*-epitope antibody. Transfection of αT3-1/M_3 cells with RGS2-*myc* resulted in predominantly nuclear staining with some localisation at the plasma membrane (Fig. 5). RGS3-*myc* was localised predominantly in the cytosolic compartment, with evidence of nuclear exclusion whilst RGS4-*myc* showed an even distribution throughout the cell (Fig. 5). Challenge of either endogenously expressed gonadotropin-releasing hormone receptors with gonadotropin-releasing hormone (1μM, 5min) or recombinantly expressed muscarinic M_3 receptors with methacholine (100μM, 5min) did not affect the subcellular distribution of the RGS constructs as assessed by immunocytochemistry (data not shown).

To ensure co-expression of the Ins(1,4,5)P_3 biosensor and the recombinant RGS protein and to allow live cell imaging of the subcellular localisation of the RGS proteins, we generated RGS proteins with C-terminal CFP tags (RGS-CFP). Co-transfection of cells with an RGS-CFP construct and eYFP-$PH_{PLC\delta1}$ allowed dual imaging of the fluorescently-tagged proteins and determination of the impact of RGS proteins on agonist-mediated PLC activation. Expression of RGS-CFP proteins in HEK 293 cells expressing recombinant human muscarinic M_3 receptors (HEK/M_3; Tovey and Willars, 2004) resulted in a pattern of inhibition of Ins(1,4,5)P_3 generation similar to that of the *myc*-tagged proteins and untagged counterparts described in a previous study (Tovey and Willars, 2004). Thus, RGS2-CFP and RGS3-CFP markedly inhibited muscarinic receptor-mediated signalling, whereas RGS4-CFP had no effect (Fig. 6).

Co-transfection of αT3-1/M_3 cells with both an RGS-CFP protein and eYFP-$PH_{PLC\delta1}$ resulted in the majority of transfected cells co-expressing both proteins (see Fig. 7B(i) and C(i) for example). The patterns of subcellular distribution of the RGS proteins were identical to those of their *myc*-tagged counterparts in unstimulated cells (see Figs. 5 and 7C for example of RGS3-CFP and data not shown for RGS2/4-CFP). Furthermore, activation of either gonadotropin-releasing hormone receptors (gonadotropin-releasing hormone, 1μM) or muscarinic M_3 receptors (methacholine, 100μM) did not influence the distribution of these constructs when imaged in real-time over a 3-4min period (see Fig. 7C for example of RGS3-CFP and data not shown for RGS2/4-CFP). The pattern of signal inhibition was identical to that seen with the *myc*-tagged constructs. Thus, expression of either RGS2-CFP or RGS3-CFP resulted in a marked inhibition of PLC activation mediated by either methacholine or gonadotropin-releasing hormone (Figs. 7A and B and Fig. 8) as judged from the reduced maximal increases in cytosolic fluorescence. In contrast, RGS4-CFP had little impact on signalling by either type of receptor (Fig. 8).

Data from the experiments with either the CFP- or *myc*-tagged RGS proteins and the Ins(1,4,5)P_3 biosensors clearly indicate a high degree of co-expression when αT3-1/M_3 cells are co-transfected. Thus, we also used this strategy to determine the impact of RGS-*myc* proteins on receptor-mediated diacylglycerol generation using the diacylglycerol biosensor, eGFP-PKCγ$C1_2$. In cells transfected with eGFP-PKCγ$C1_2$ alone, fluorescence in unstimulated cells was located in the cytosol and the nucleus. However, immediately following challenge with either gonadotropin-releasing hormone (1μM) or methacholine (100μM), there was a reduction in cytosolic fluorescence and an increase in fluorescence associated with the plasma membrane (Fig. 9A and B and Fig. 10) consistent with recruitment of the biosensor following

the generation and plasma membrane location of diacylglycerol. The expression of RGS2-*myc* or RGS3-*myc* resulted in a marked reduction in diacylglycerol formation in response to either methacholine (Figs. 9 and 10A) or gonadotropin-releasing hormone (Fig. 10B) as judged from a reduced loss of cytosolic fluorescence. In contrast, expression of RGS4-*myc* had no effect on receptor-mediated diacylglycerol generation (Fig. 10).

To assess the ability of the recombinantly expressed RGS proteins to inhibit signalling by effector antagonism we employed a constitutively active mutant of $G\alpha_q$ (CA-$G\alpha_q$; Q209L) that is resistant to RGS protein GTPase activating protein activity (Heximer et al., 2001). Co-transfection of αT3-1/M_3 cells with both CA-$G\alpha_q$ and the Ins(1,4,5)P_3 biosensor, eYFP-$PH_{PLC\delta1}$, resulted in predominantly cytosolically located fluorescence, which was in contrast to the plasma membrane localisation of fluorescence when cells were transfected with eYFP-$PH_{PLC\delta1}$ alone (Fig. 11). These data indicate a markedly enhanced level of Ins(1,4,5)P_3 in cells as a consequence of the expression of CA-$G\alpha_q$. The co-expression of RGS2-CFP, RGS3-CFP or RGS4-CFP along with both CA-$G\alpha_q$ and eYFP-$PH_{PLC\delta1}$ did not alter the predominantly cytosolic localisation of eYFP-$PH_{PLC\delta1}$ fluorescence (Figs. 11 and 12). Furthermore, expression of the CA-$G\alpha_q$ did not influence the subcellular distribution of the RGS-CFP constructs (data not shown).

4. Discussion

Using biosensors to monitor single-cell PLC activity, we demonstrate that RGS2 and 3 but not RGS4 markedly reduce $G\alpha_{q/11}$-dependent signalling by either gonadotropin-releasing hormone receptors or muscarinic M_3 receptors in an identical cell background. Real-time imaging showed that signal inhibition occurs during the initial, most likely physiologically relevant phase of receptor activation. Although inhibition of gonadotropin-releasing hormone receptor-mediated signalling by RGS2 was not seen previously, the pattern of inhibition amongst the RGS proteins is generally consistent with previous studies on both gonadotropin-releasing hormone receptors and muscarinic M_3 receptors when expressed independently in a number of different cell types (Neil et al., 1997; Castro-Fernandez and Conn, 2002; Castro-Fernandez et al., 2002; Tovey and Willars, 2004).

The lack of a C-terminal tail on the gonadotropin-releasing hormone receptor and its inability to undergo agonist-dependent phosphorylation, β-arrestin recruitment and rapid desensitisation result in sustained PLC activation following agonist challenge (Willars et al., 1998). Despite this, the magnitude of the initial $Ins(1,4,5)P_3$- and Ca^{2+} responses on agonist addition are similar (Willars et al., 1998), consistent with the equivalent activation of $G\alpha_{q/11}$ shown here. Despite major differences between receptors and the difference in phosphorylation status following agonist activation, RGS2, 3 and 4 influence signalling by these two receptors in a similar manner. This is in contrast to examples where the receptor appears to play a role in RGS protein-dependent regulation of signalling (Xu et al., 1999; Saitoh et al., 2002; Ghavami et al., 2004; Cabrera-Vera et al., 2004; Wang et al., 2002). Such selectivity may be a consequence of either direct interactions between receptors and RGS proteins or indirect interactions via scaffolding proteins (Snow et al., 1998; Bernstein et al., 2004; Wang et al., 2005; Jeanneteau et al., 2004; Kovoor et al., 2005; Garzon et al., 2005b;

for review see Willars, 2006). Of particular interest, RGS2 binds strongly to the third intracellular loops of M_1 and M_5 muscarinic receptors but relatively weakly to the same region of the M_3 muscarinic receptor (Bernstein et al., 2004). Importantly, despite such differential interactions, neither RGS2 nor RGS4 show specificity against signalling by these $G\alpha_{q/11}$-coupled muscarinic receptors recombinantly expressed in HEK 293 cells (Bodenstein et al., 2007). Although binding sites for RGS proteins within G-protein-coupled receptors have not been defined precisely, interactions between the $G\alpha_{q/11}$-coupled muscarinic receptors and some B/R4 RGS family members (eg. RGS2) involves at least the third intracellular loop (Bernstein et al., 2004). Given the major structural differences and the almost total lack of homology between the third intracellular loops of the gonadotropin-releasing hormone receptor and the $G\alpha_{q/11}$-coupled muscarinic receptors, it is perhaps unlikely that these receptors would selectively recruit identical RGS proteins, particularly as there is selectivity between the more homologous M_1, M_3 and M_5 muscarinic receptors.

Although the current study provides no evidence for receptor-selective effects of RGS2, 3 and 4, there were apparent differences in the extent of signal inhibition amongst the RGS proteins, with RGS4 being particularly poor. Although the RGS4 used was of rat origin, this is unlikely to account for its lack of effect as this has been reported elsewhere including studies using the human variant against muscarinic M_3 receptors and other $G\alpha_{q/11}$-coupled receptors (eg. Bodenstein et al., 2007). Direct comparison of the relative effects of the different RGS proteins is difficult as, where inhibition occurs, this will be critically dependent upon the level of RGS protein expression. Indeed, it is possible that differences in the expression levels of the RGS proteins contribute to differences in the extent of signal inhibition seen in the present study. Thus, RGS4 expresses relatively poorly compared to RGS2 and 3, most likely as a consequence its relatively short half-life due to proteasomal degradation (Bodenstein et al.,

2007). However, even when expressed at a level at which RGS2 and 3 have significant inhibitory effects on muscarinic M_3 receptor-mediated signalling, RGS4 still has little or no effect (Tovey and Willars, 2004). Another possibility is that selectivity arises as a consequence of different subcellular distributions of the different RGS proteins. Thus, both *myc*- and CFP-tagged versions of the RGS proteins showed similar subcellular distributions with RGS2 showing predominantly nuclear and plasma membrane localisation, RGS3 localising in the cytosol and RGS4 distributing evenly throughout the cell. With the possible exception of the plasma membrane localisation of RGS2, perhaps due to a membrane targeting domain (Bernstein et al., 2000), the distributions provide little insight into their relative abilities to inhibit signalling by either receptor type. Furthermore, activation of either gonadotropin-releasing hormone receptors or muscarinic M_3 receptors did not alter the subcellular distribution of RGS proteins observed either by immunocytochemistry or in real time by confocal imaging of CFP-tagged proteins. Although the expression of G-proteins and G-protein-coupled receptors has been reported to influence the subcellular distribution of, for example RGS2, 3 and 4 (Roy et al., 2003; Dulin et al., 1999), our data suggest that these RGS proteins must be either in the vicinity of activated receptors at sufficiently high concentrations or, if recruitment occurs, this represents a small proportion of the pool of recombinant RGS proteins. It is possible that the different RGS proteins have different abilities to regulate signalling by $G\alpha_{q/11}$ as a consequence of such selective recruitment but equally, differences in expression levels and/or different affinities for $G\alpha_{q/11}$ could be responsible. Indeed it is possible that the over-expression of RGS proteins in the present study masks receptor selectivity and the challenge is to determine if the effects of endogenously expressed RGS are truly regulated by the nature of the G-protein-coupled receptor.

In addition to their function as GTPase activating proteins, RGS proteins may also influence signalling by either effector antagonism or by inhibiting guanine nucleotide dissociation. Indeed effector antagonism has been reported as the major mechanism by which RGS2 and 3 inhibit signalling by muscarinic M_3 receptors overexpressed in COS-7 cells (Anger et al., 2004). In contrast, we were unable to find evidence for such an effect of these RGS proteins on muscarinic M_3 receptors overexpressed in HEK 293 cells (Tovey and Willars, 2004). Here, we initially sought to determine possible effector antagonism by determining the effects of RGS protein expression on AlF_4^--induced Ca^{2+} signalling. AlF_4^- directly activates G-proteins, producing an active conformation that is resistant to GTPase activity but susceptible to effector antagonism. Although we were able to evoke oscillatory Ca^{2+} signalling with AlF_4^- (50mM NaF, 50μM $AlCl_3$) in HEK 293 cells, which is consistent with our previous study (Tovey and Willars, 2004), Ca^{2+} signalling was totally refractory to AlF_4^- in αT3-1/M_3 cells (25-100mM NaF, 25-100μM $AlCl_3$) (data not shown). As an alternative strategy we used CA-$G\alpha_q$ which is resistant to GTPase activating proteins but still susceptible to effector antagonism (Heximer et al., 2001). Here we show that neither RGS2, 3 nor 4 could reverse the effects of CA-$G\alpha_q$ on the distribution of eYFP-$PH_{PLC\delta1}$ indicating that the overexpressed RGS proteins were not effector antagonists of CA-$G\alpha_q$. Despite this, we cannot exclude the possibility that overexpressed or indeed endogenously expressed RGS proteins inhibit endogenous $G\alpha_{q/11}$ either fully or partly independently of enhancing GTPase activity.

Perhaps one of the clearest examples of receptor-selective effects of endogenously expressed RGS proteins is the observation that knock-down of either RGS3 or RGS5 in A-10 smooth muscle cells selectively reduces activation of extracellular signal-regulated kinases by muscarinic M_3 receptors and angiotensin AT1a receptors respectively (Wang et al., 2002). These receptors are thought to both couple via $G\alpha_{q/11}$, therefore implicating a role for the

receptor in the selective effects of the RGS proteins. Thus, although there is clear evidence that receptors can select RGS proteins, whether this is widespread and the circumstances under which this occurs requires careful analysis. Here, in agreement with another recent study showing a lack of receptor selectivity by some B/R4 RGS proteins (Bodenstein et al., 2007), we show that despite major structural differences, muscarinic M_3 receptors and gonadotropin-releasing hormone receptors couple to an equivalent level through $G\alpha_{q/11}$ and are equally susceptible to inhibition by members of the B/R4 RGS sub-family.

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Figure Legends

Fig. 1. Muscarinic- and gonadotropin-releasing hormone receptor-mediated Ca^{2+}-signalling and PLC-activation in αT3-1/M_3 cells. A) αT3-1/M_3 cells were loaded with fluo-3 and imaged by confocal microscopy with fluorescence recorded as an index of $[Ca^{2+}]_i$. Challenge of cells with either methacholine (100μM; upper, dotted trace) to activate the recombinant muscarinic M_3 receptors or gonadotropin-releasing hormone (1μM; lower, solid trace) to activate endogenously expressed gonadotropin-releasing hormone receptors, resulted in rapid and approximately equivalent increases of $[Ca^{2+}]_i$ consisting of a rapid transient peak followed by a lower but sustained plateau phase. Data are the average of approximately 30 cells and are representative of at least 3 independent experiments. **B)** αT3-1/M_3 cells were transfected with the Ins(1,4,5)P_3 biosensor, eGFP-$PH_{PLC\delta1}$, and imaged by confocal microscopy 48h later. Under basal (non-stimulated) conditions eGFP-$PH_{PLC\delta1}$, was located predominantly at the plasma membrane consistent with binding to membrane PtdIns(4,5)P_2. Upon addition of either methacholine (100μM) or gonadotropin-releasing hormone (1μM) there were rapid translocations of membrane fluorescence to the cytosol, resulting in a marked increase in cytosolic fluorescence. Images show cells under basal (unstimulated) conditions (i) and as an example, at the point of the maximal change in cytosolic fluorescence following addition of methacholine (100 μM) (ii). Size bar in the bottom left hand corner of bi and ii approximates to 10μM. **C)** Traces showing examples of agonist-mediated changes in cytosolic fluorescence following challenge of cells expressing eGFP-$PH_{PLC\delta1}$ with either methacholine (100μM; dotted lines) or gonadotropin-releasing hormone (1μM; solid lines). Data are presented as the fold increase in cytosolic fluorescence over basal levels and are representative of at least n=3.

Fig. 2. Coupling of muscarinic and gonadotropin-releasing hormone receptors to $G\alpha_{q/11}$ G-protein subunits.

Membrane preparations were incubated in the presence of GDP, [^{35}S]GTPγS and where applicable either methacholine (100μM) or gonadotropin-releasing hormone (1μM) (Stimulated) for 2min. Non-specific binding (NSB) was determined using 10μM GTPγS. Immunoprecipitation was carried out using antibodies specific for $G\alpha_{q/11}$ and associated ^{35}S was determined. Agonist-dependent stimulations (Stimulated – Basal values) were not significantly different for methacholine (2299±63 c.p.m.) and gonadotropin-releasing hormone (2167±86). Data are mean+/±S.E.M., n=3.

Fig. 3. Single-cell imaging of Ins(1,4,5)P_3 production in response to stimulation of recombinant muscarinic M_3 receptors. A) (i) Typical single-cell confocal images of αT3-1/M_3 cells transiently transfected with the Ins(1,4,5)P_3 biosensor, eGFP-$PH_{PLC\delta1}$. Under resting conditions (0s), the biosensor is localized to the plasma membrane, but upon agonist stimulation (100μM methacholine), eGFP-$PH_{PLC\delta1}$ translocates to the cytosol (20s) corresponding to the production of Ins(1,4,5)P_3. **A) (ii)** In αT3-1/M_3 cells transiently cotransfected with eGFP-$PH_{PLC\delta1}$ and RGS3-*myc*, the translocation of the eGFP-tagged biosensor is reduced, corresponding to an inhibition of Ins(1,4,5)P_3 production. Size bars in the bottom left hand corner images approximate to 10μM. **B)** Sample traces of the change in cytoplasmic eGFP fluorescence upon muscarinic M_3 receptor stimulation in αT3-1/M_3 cells. Traces represent αT3-1/M_3 cells transiently transfected with eGFP-$PH_{PLC\delta1}$ alone (upper trace) or cells transiently cotransfected with eGFP-$PH_{PLC\delta1}$ and RGS3-*myc* (lower trace). The arrow represents the time point for the addition of methacholine (100μM).

Fig. 4. Effects of RGS-*myc* constructs on Ins(1,4,5)P_3 generation mediated by either methacholine or gonadotropin-releasing hormone. A) Summary of data from the type of experiments described in Figure 3. Data represent the mean+S.E.M. of the peak increase in cytoplasmic eGFP fluorescence immediately following the addition of 100μM methacholine for 20 to 35 cells from at least 6 different coverslips. Statistical comparisons are by one-way ANOVA with Dunnett's range test; *, $P < 0.05$; ***, $P < 0.001$. **B)** Concentration-response curves (0.01-100μM methacholine) for single-cell Ins(1,4,5)P_3 production in control αT3-1/M_3 cells and αT3-1/M_3 cells transiently transfected with either RGS2-*myc*, RGS3-*myc*, RGS4-*myc* or a LacZ-*myc* control vector. Data represent the mean±S.E.M. of the maximal change in cytoplasmic eGFP fluorescence immediately following agonist addition for 10-30 cells from at least 3 different coverslips. The pEC50 values were: Control, 5.69±0.04; RGS2-*myc*, 5.97±0.07; RGS3-*myc*, 5.80±0.04; RGS4-*myc*, 5.95±0.08; LacZ-*myc*, 5.53±0.17; data are mean±S.E.M., n=3. **C)** Experiments were performed as described in Figure 3 with the exception that cells were challenged with 1μM gonadotropin-releasing hormone. Data represent the mean+S.E.M. of the peak increase in cytoplasmic eGFP fluorescence for 15 to 30 cells from at least 6 different coverslips. Statistical comparisons were by one-way ANOVA with Dunnett's range test; *, $P < 0.05$; ***, $P < 0.001$.

Fig. 5. Subcellular localization of RGS-*myc* proteins. αT3-1/M_3 cells were transiently transfected with either RGS2-*myc*, RGS3-*myc* or RGS4-*myc*. After 48h, the *myc*-tag was immunolocalised using an anti-*myc* antibody, which was subsequently labelled with a FITC-conjugated secondary antibody. Confocal images are typical of at least three different transient transfections and immunolabelling experiments. Size bars in the bottom left hand corner images approximate to 10μM.

Fig. 6. Effects of RGS-CFP constructs on methacholine-mediated Ins(1,4,5)P_3 generation in HEK 293 cells expressing recombinant muscarinic M_3 receptors. An HEK 293 cell line with stable expression of recombinant, human muscarinic M_3 receptors was transiently transfected with eYFP-$PH_{PLC\delta1}$ alone or in combination with either RGS2-CFP, RGS3-CFP or RGS4-CFP. After 48h, cells were imaged by confocal microscopy and challenged with 100μM methacholine. The maximal change in cytosolic fluorescence was determined and expressed as a fold increase over basal levels with basal subtraction ((F/F_0)-1). Data are mean+S.E.M. from at least 20 cells in 3 independent experiments. Statistical comparisons were by one-way ANOVA with Dunnett's range test; ***, $P<0.001$.

Fig. 7. Effects of RGS-CFP constructs on methacholine-mediated Ins(1,4,5)P_3 generation. αT3-1/M_3 cells were transiently transfected with eYFP-$PH_{PLC\delta1}$ alone or in combination with RGS3-CFP. After 48h, CFP and YFP fluorescence was imaged by confocal microscopy and the cells challenged with 100μM methacholine. **A)** Images showing the distribution of YFP fluorescence in cells transfected with eYFP-$PH_{PLC\delta1}$ alone under basal (unstimulated) conditions **(i)** or at the peak of increased cytosolic fluorescence following the addition of methacholine **(ii)**. Cytosolic YFP fluorescence was determined and expressed as F/F_0 to show the increase associated with agonist stimulation **(iii)**. **B)** Image showing distribution of YFP fluorescence in cells transfected with both eYFP-$PH_{PLC\delta1}$ and RGS3-CFP following the addition of methacholine. **(i)**. Cytosolic YFP fluorescence was determined and expressed as F/F_0 to show the lack of effect of agonist stimulation **(ii). C)** Image showing the cytoplasmic distribution of CFP fluorescence immediately following with methacholine in the same cells as b **(i)**. Cytosolic CFP fluorescence was determined and expressed as F/F_0 to show the lack of effect of agonist stimulation **(ii)**. All data are representative of $\geq$10 cells in 3

independent experiments. Size bars in the bottom left hand corner of images approximate to 10μM.

Fig. 8. Effects of RGS-CFP constructs on Ins(1,4,5)P_3 generation mediated by either methacholine or gonadotropin-releasing hormone. αT3-1/M_3 cells were transiently transfected with eYFP-$PH_{PLC\delta1}$ alone or in combination with either RGS2-CFP, RGS3-CFP or RGS4-CFP. After 48h, cells having YFP fluorescence and where appropriate also CFP fluorescence were imaged by confocal microscopy and the cells challenged with either 100μM methacholine **(i)** or 1μM gonadotropin-releasing hormone **(ii)**. The maximal change in cytosolic YFP fluorescence was determined and expressed as a fold increase over basal levels with basal subtraction ((F/F_0)-1). Data are mean+S.E.M., n≥10 cells in 3 independent experiments. Statistical comparisons were by one-way ANOVA with Dunnett's range test; ***, P<0.001.

Fig. 9. Single-cell imaging of diacylglycerol production in response to stimulation of recombinant muscarinic M_3 receptors. A) (i) Typical single-cell confocal images of αT3-1/M_3 cells transiently transfected with the diacylglycerol biosensor eGFP-PKCγ$C1_2$. Under resting conditions (0s) the eGFP-tagged biosensor is localised homogeneously across the cell cytoplasm and nucleus, but upon agonist stimulation (100μM methacholine) the eGFP translocates to the plasma membrane (20s) corresponding to the production of diacylglycerol. **A) (ii)** In αT3-1/M_3 cells transiently co-transfected with both eGFP-PKCγ$C1_2$ and RGS3-*myc* the translocation of the eGFP-tagged biosensor is reduced, corresponding to an inhibition of diacylglycerol production. Size bars in the bottom left hand corner of images approximate to 10μM. **B)** Sample traces of the change in cytoplasmic eGFP fluorescence with time upon muscarinic M_3 receptor stimulation in αT3-1/M_3 cells. Traces represent αT3-1/M_3 cells

transiently transfected with eGFP-PKCγC1$_2$ alone (lower trace) and cells transiently co-transfected with both eGFP-PKCγC1$_2$ and RGS3-*myc* (upper trace). The arrow represents the time point for the addition of methacholine (100μM). Data are representative of 10-20 cells from at least 6 different coverslips.

Fig. 10. Effects of RGS-CFP constructs on diacylglycerol generation mediated by either methacholine or gonadotropin-releasing hormone. αT3-1/M$_3$ cells were transiently transfected with eGFP-PKCγC1$_2$ alone or in combination with either RGS2-*myc*, RGS3-*myc* or RGS4-*myc*. After 48h, cells were imaged by confocal microscopy and challenged with either 100μM methacholine **(i)** or 1μM gonadotropin-releasing hormone **(ii)**. The maximal change in cytosolic GFP fluorescence was determined and expressed as F_0/F so that the greatest reduction in cytosolic fluorescence is represented by the greatest increase in the ratio. Data are mean+S.E.M., n = 10-20 cells from at least 6 different coverslips. Statistical comparisons were by one-way ANOVA with Dunnett's range test; ***, P<0.001.

Fig. 11. Lack of effect of RGS3-CFP on changes in the cellular distribution of eYFP-PH$_{PLC\delta1}$ induced by CA-Gα_q. αT3-1/M$_3$ cells were transiently transfected with eYFP-PH$_{PLC\delta1}$ alone **(A)** or in combination with either CA-Gα_q **(B)** or CA-Gα_q and RGS3-CFP **(C)**. After 48h, cells having YFP fluorescence and where appropriate also CFP fluorescence were imaged by confocal microscopy **(Ai, Bi and Ci)**. Pixel intensities across the lines drawn over individual cells were determined to show the profile of fluorescence **(Aii, Bii and Cii)**. Data show predominantly membrane localisation of eYFP-PH$_{PLC\delta1}$ when expressed alone **(Aii)**, an even distribution across the cell when expressed with CA-Gα_q **(Bii)** and no effect of RGS3-CFP on this distribution associated with the expression of CA-Gα_q **(Cii)**. Data are

representative of ≥10 cells over 3 separate experiments. Size bars in the bottom left hand corner of images approximate to 10μM.

Fig. 12. Effects of RGS-CFP proteins on the cellular distribution of eYFP-$PH_{PLC\delta1}$ when expressed in combination with CA-Gα_q. αT3-1/M_3 cells were transiently transfected with eYFP-$PH_{PLC\delta1}$ alone or in combination with either CA-Gα_q or CA-Gα_q and an RGS-CFP. For each cell, the average eYFP fluorescence intensity along the line of interest was taken at the two points were it crossed the membrane, this was then divided by the average cytosolic fluorescence along the line of interest. A high value therefore indicates predominantly membrane localization whereas a low level indicates predominantly a cytosolic localization. These data demonstrate that expression of CA-Gα_q with eYFP-$PH_{PLC\delta1}$ caused a marked, agonist-independent, cytosolic localisation of eYFP-$PH_{PLC\delta1}$ that was unaffected by co-expression of RGS proteins. Data are the mean±S.E.M. for ≥10 cells over 3 separate experiments. For ***, P<0.001 versus control cells (oneway ANOVA with Dunnett's range test).

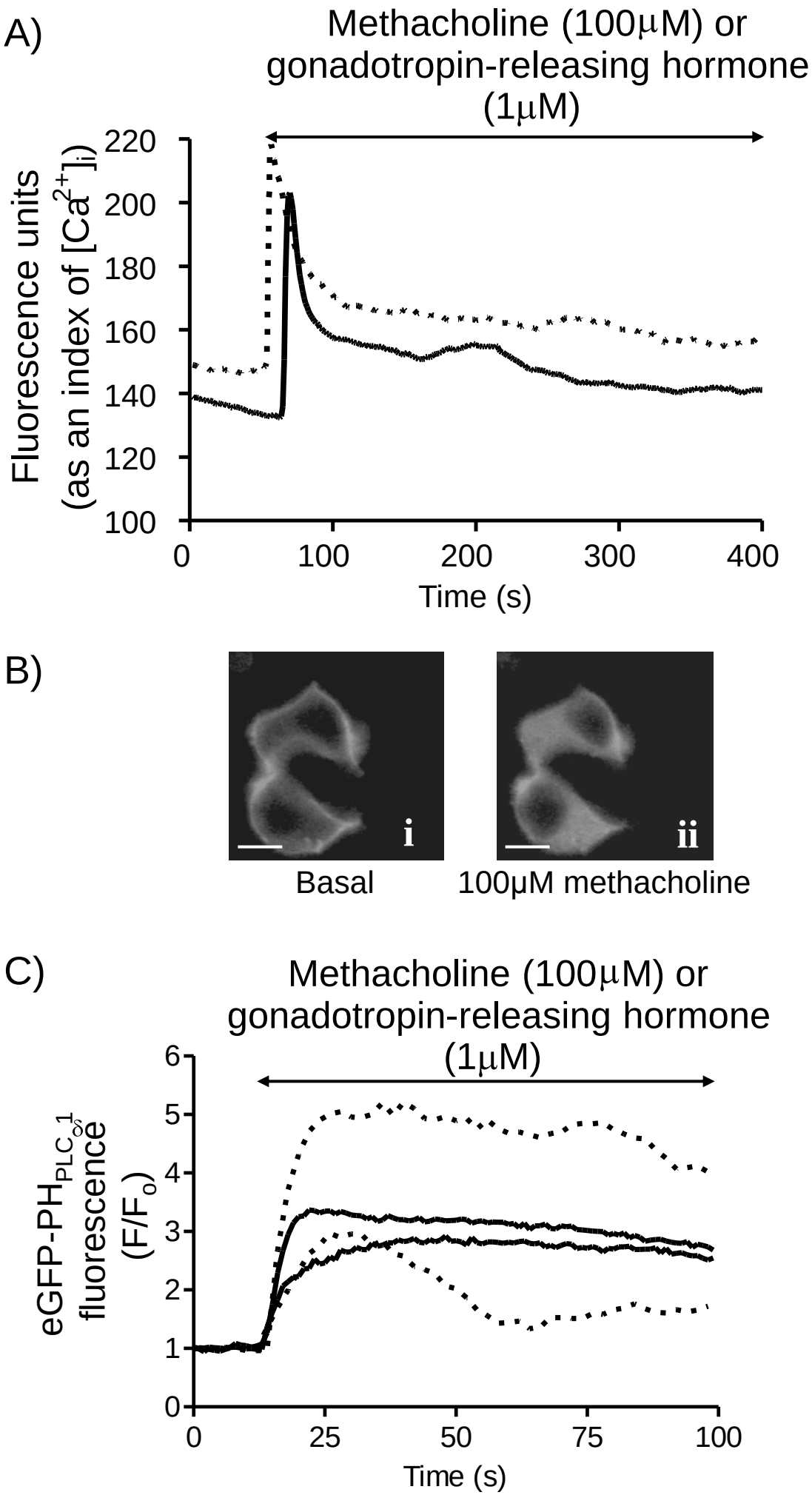

A)
Methacholine (100μM) or
gonadotropin-releasing hormone
(1μM)
Fluorescence units
(as an index of [Ca2+]i)
220
200
180
160
140
120
100
0
100
200
300
400
Time (s)
B)
i
ii
Basal
100μM methacholine
C)
Methacholine (100μM) or
gonadotropin-releasing hormone
(1μM)
eGFP-PHPLCδ1
fluorescence
(F/Fo)
6
5
4
3
2
1
0
0
25
50
75
100
Time (s)

Fig. 1

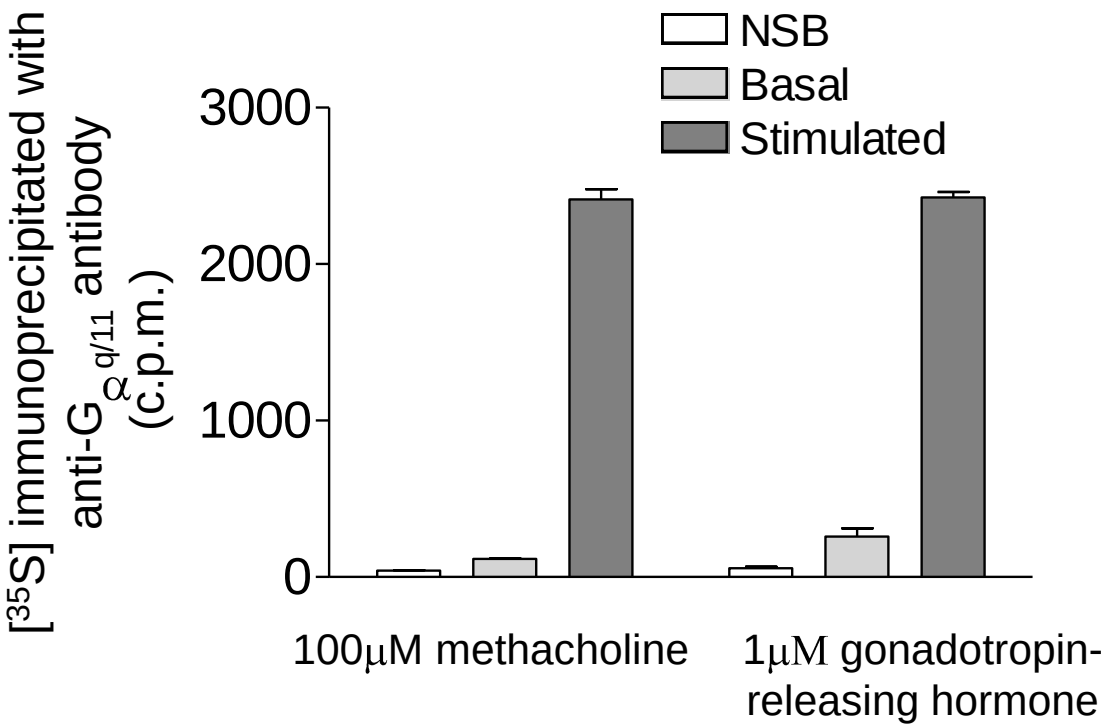

NSB
Basal
Stimulated
[35S] immunoprecipitated with anti-Gαq/11 antibody (c.p.m.)
3000
2000
1000
0
100μM methacholine
1μM gonadotropin-releasing hormone

Fig. 2

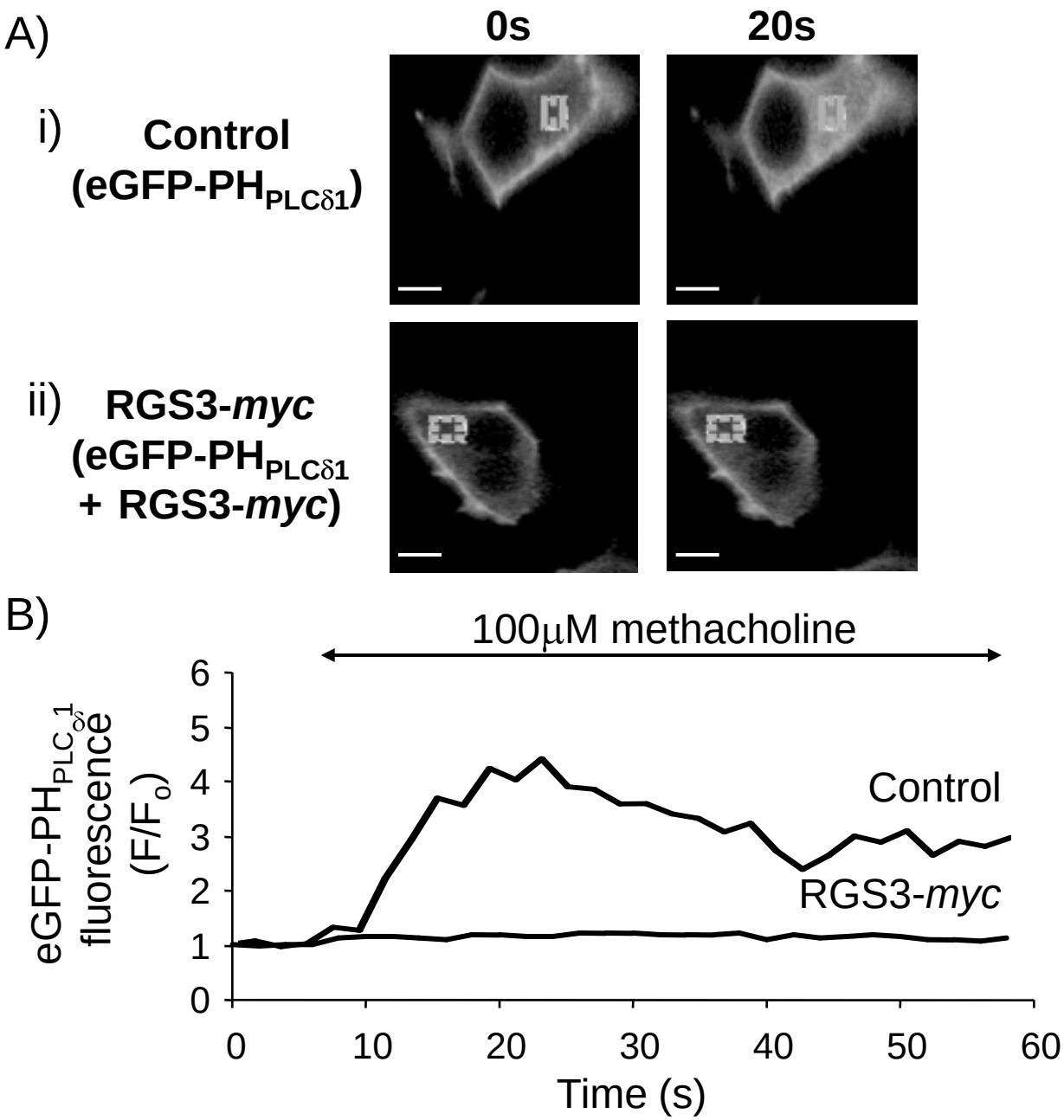

A)
0s
20s
i) Control (eGFP-PH$_{PLC\delta1}$)
ii) RGS3-*myc* (eGFP-PH$_{PLC\delta1}$ + RGS3-*myc*)
B)
100μM methacholine
eGFP-PH$_{PLC\delta1}$ fluorescence (F/F$_o$)
Control
RGS3-*myc*
Time (s)
0
1
2
3
4
5
6
0
10
20
30
40
50
60

Fig. 3

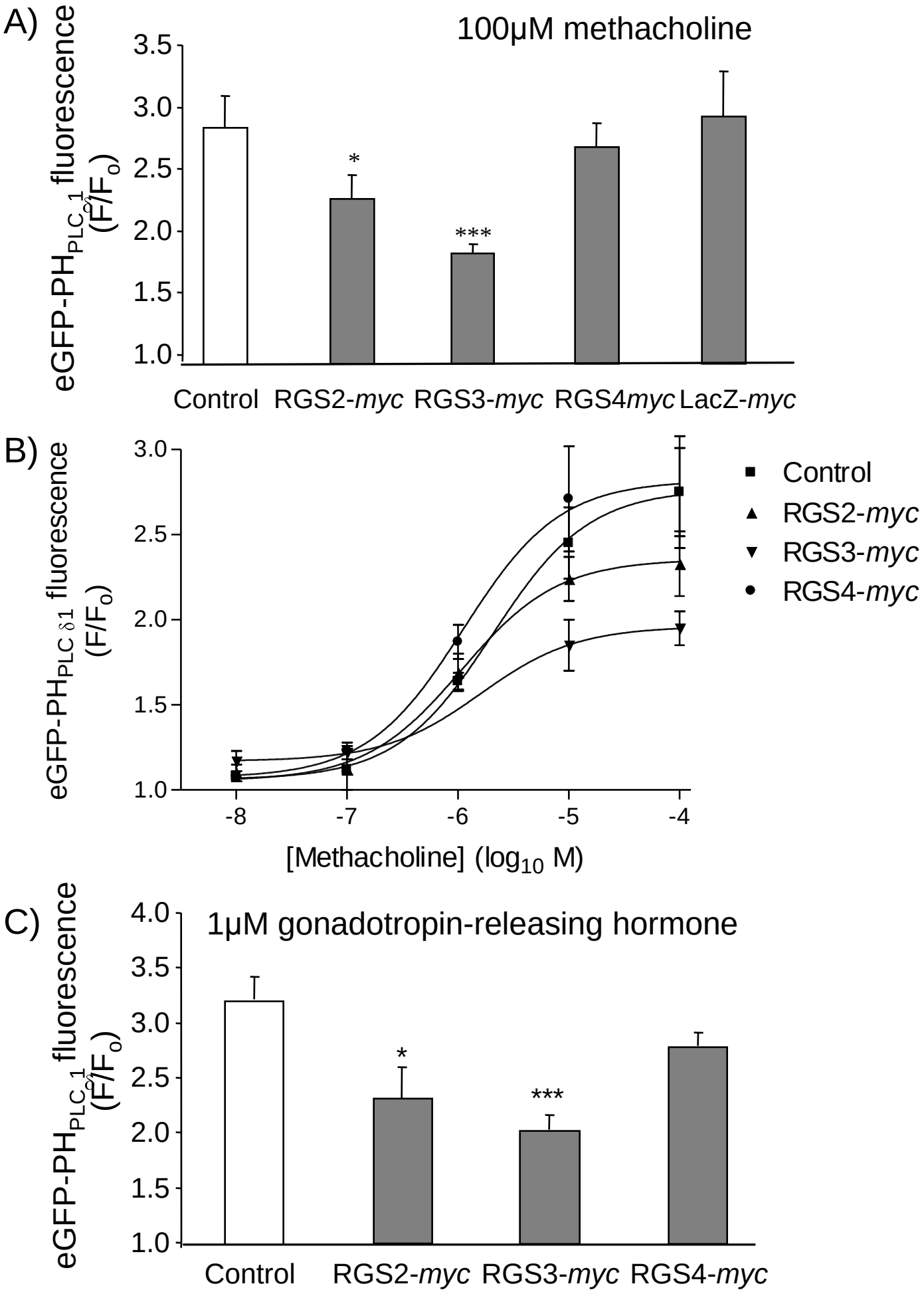
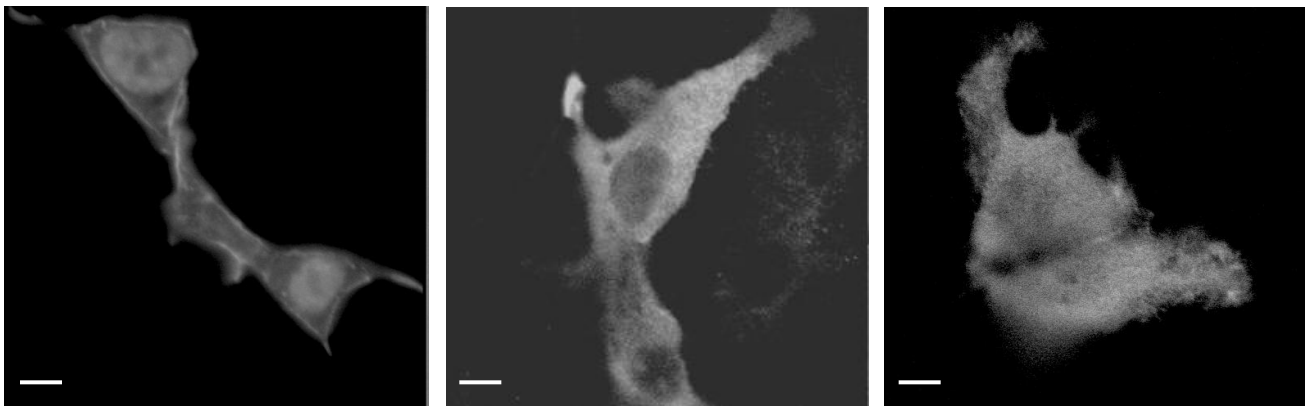

A)
100μM methacholine
eGFP-PH$_{PLC\delta1}$ fluorescence (F/F$_o$)
3.5
3.0
2.5
2.0
1.5
1.0
*
***
Control
RGS2-*myc*
RGS3-*myc*
RGS4*myc*
LacZ-*myc*
B)
eGFP-PH$_{PLC\delta1}$ fluorescence (F/F$_o$)
3.0
2.5
2.0
1.5
1.0
-8
-7
-6
-5
-4
[Methacholine] (log$_{10}$ M)
Control
RGS2-*myc*
RGS3-*myc*
RGS4-*myc*
C)
1μM gonadotropin-releasing hormone
eGFP-PH$_{PLC\delta1}$ fluorescence (F/F$_o$)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
*
***
Control
RGS2-*myc*
RGS3-*myc*
RGS4-*myc*

Fig. 4



RGS2-*myc* RGS3-*myc* RGS4-*myc*

Fig. 5

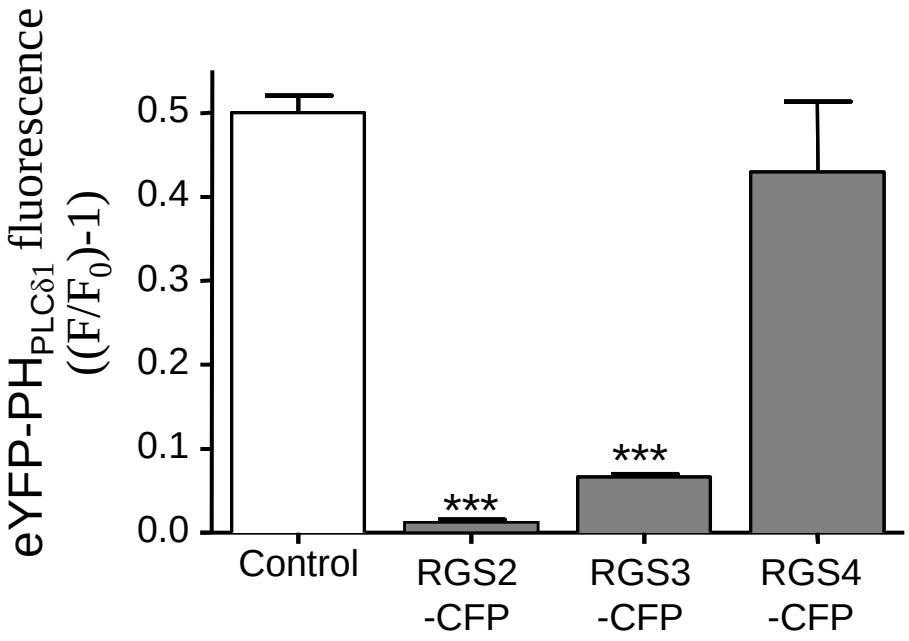

eYFP-PH$_{PLC\delta1}$ fluorescence
((F/F$_0$)-1)
0.5
0.4
0.3
0.2
0.1
0.0
***
***
Control
RGS2
-CFP
RGS3
-CFP
RGS4
-CFP

Fig. 6

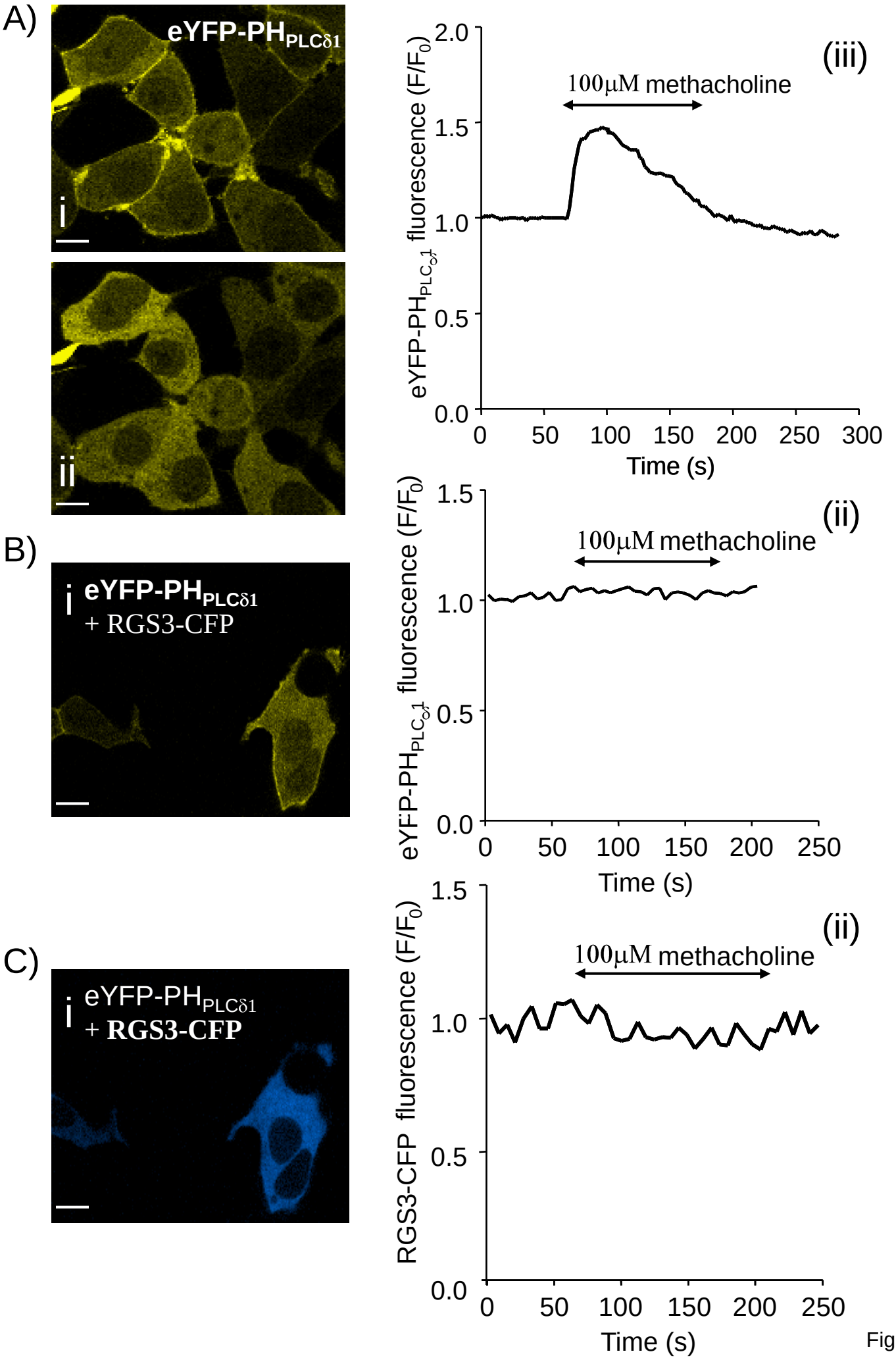
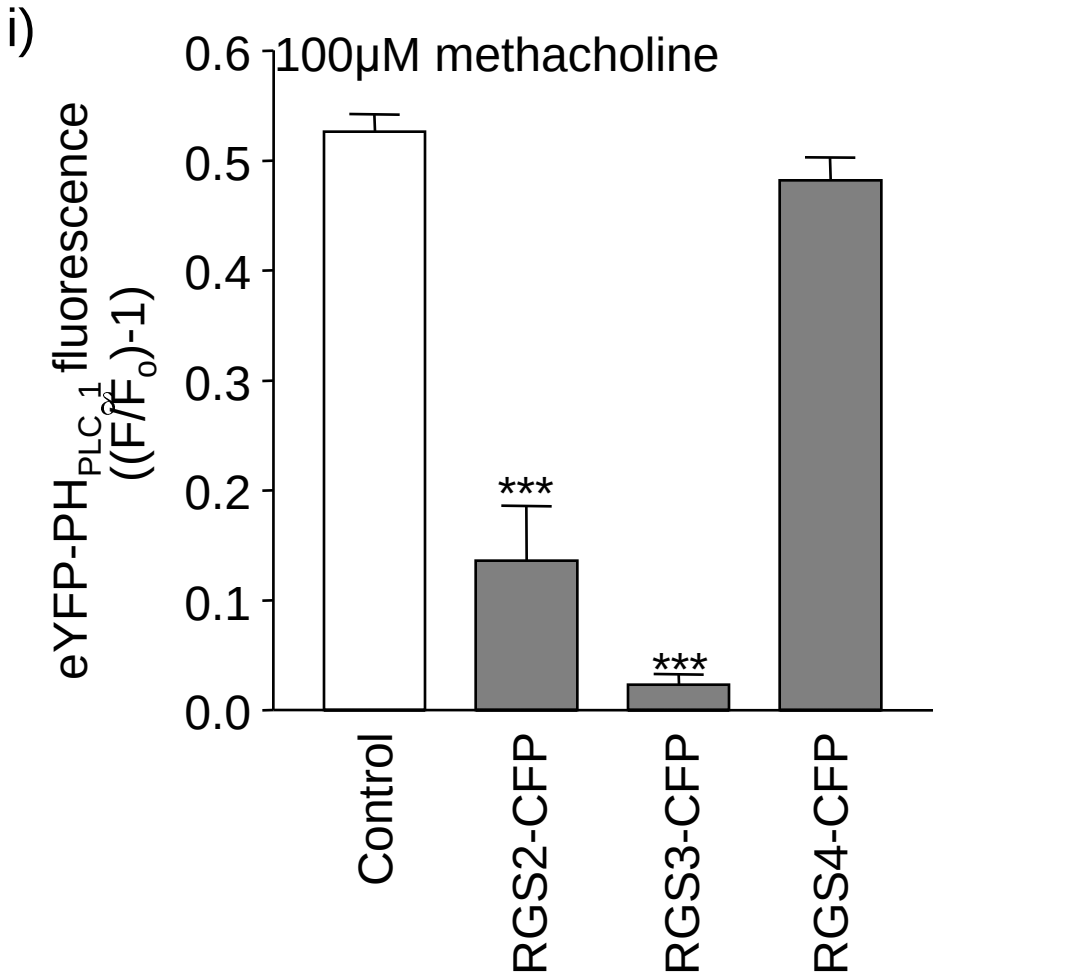

A)
eYFP-PHPLCδ1
i
ii
(iii)
100μM methacholine
eYFP-PHPLCδ1 fluorescence (F/F0)
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
Time (s)
B)
i eYFP-PHPLCδ1
+ RGS3-CFP
(ii)
100μM methacholine
eYFP-PHPLCδ1 fluorescence (F/F0)
1.5
1.0
0.5
0.0
0
50
100
150
200
250
Time (s)
C)
i eYFP-PHPLCδ1
+ RGS3-CFP
(ii)
100μM methacholine
RGS3-CFP fluorescence (F/F0)
1.5
1.0
0.5
0.0
0
50
100
150
200
250
Time (s)

Fig. 7


i)
100μM methacholine
eYFP-PH$_{PLC\delta1}$ fluorescence ((F/F$_o$)-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
***
Control
RGS2-CFP
RGS3-CFP
RGS4-CFP

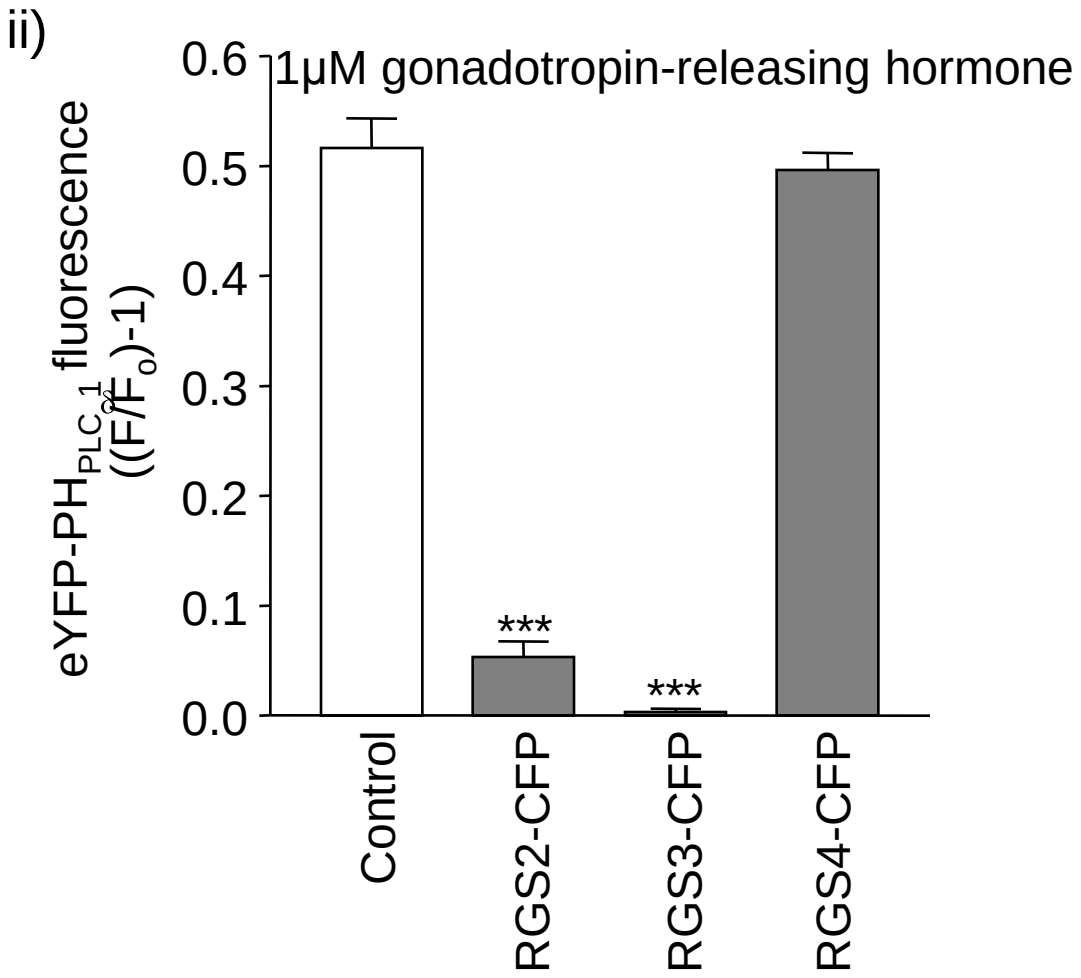
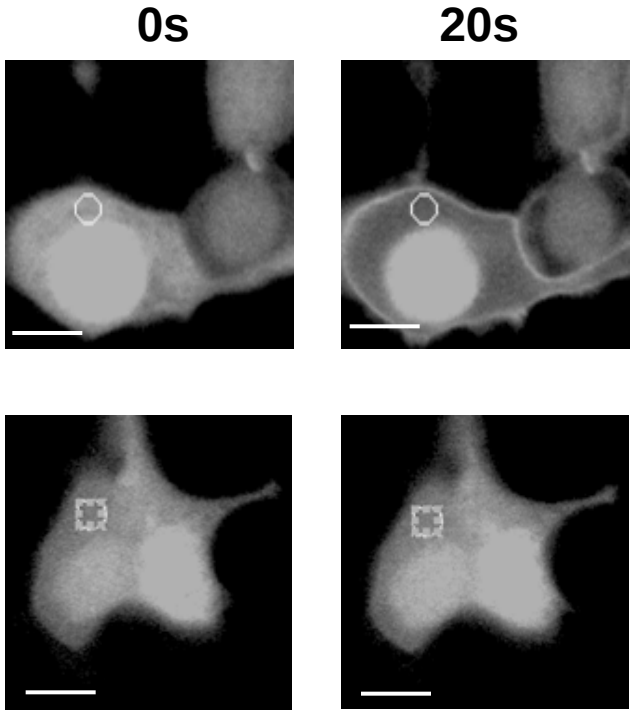

ii)
1μM gonadotropin-releasing hormone
eYFP-PH$_{PLC\delta1}$ fluorescence ((F/F$_o$)-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
***
Control
RGS2-CFP
RGS3-CFP
RGS4-CFP

Fig. 8

A)


0s
20s
i) Control
(eGFP-PKCγC1$_2$)
ii) RGS3-*myc*
(eGFP-PKCγC1$_2$
+ RGS3-*myc*)

B)

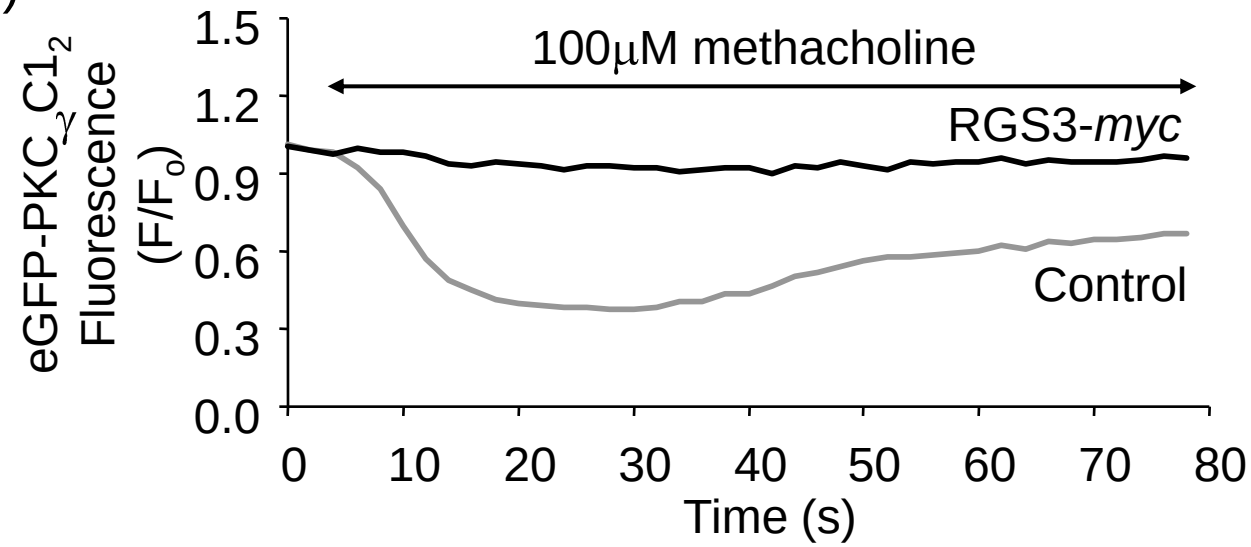

100μM methacholine
RGS3-*myc*
Control
eGFP-PKCγC1$_2$ Fluorescence (F/F$_o$)
1.5
1.2
0.9
0.6
0.3
0.0
0
10
20
30
40
50
60
70
80
Time (s)

Fig. 9

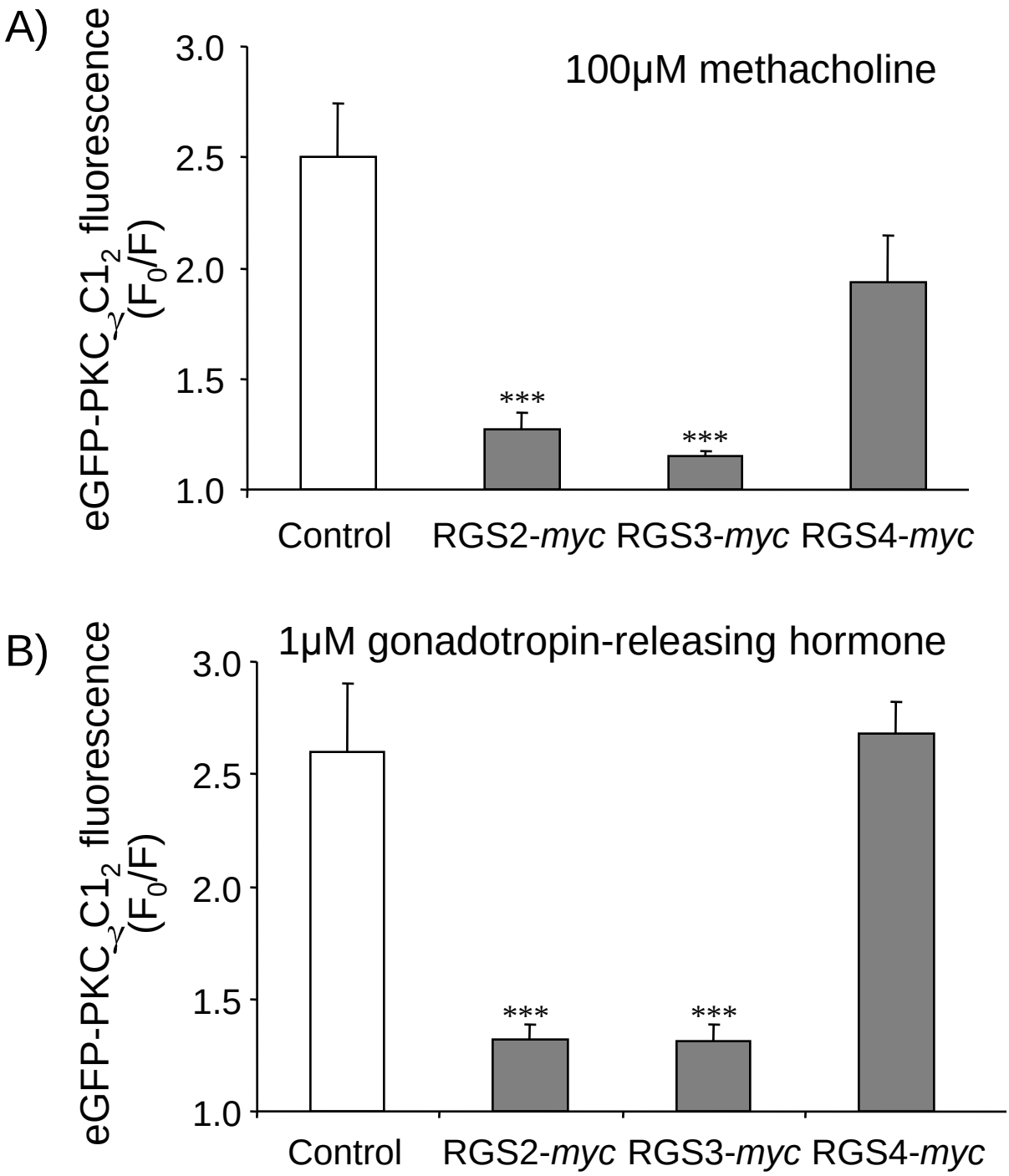
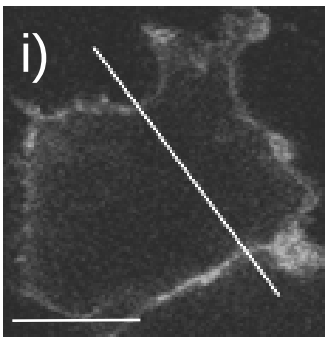
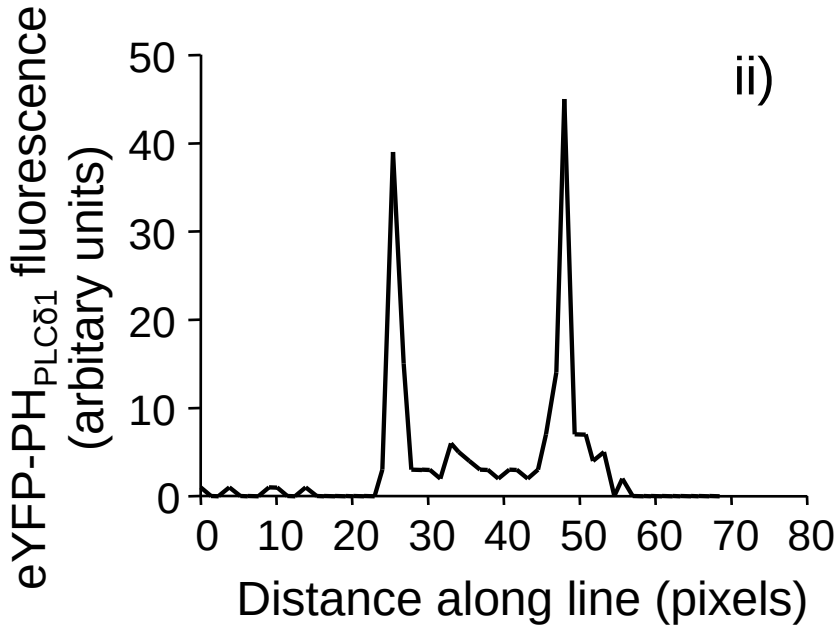

A)
100μM methacholine
eGFP-PKCγC1$_2$ fluorescence (F$_0$/F)
3.0
2.5
2.0
1.5
1.0
***
***
Control
RGS2-*myc*
RGS3-*myc*
RGS4-*myc*
B)
1μM gonadotropin-releasing hormone
eGFP-PKCγC1$_2$ fluorescence (F$_0$/F)
3.0
2.5
2.0
1.5
1.0
***
***
Control
RGS2-*myc*
RGS3-*myc*
RGS4-*myc*

Fig. 10

A)

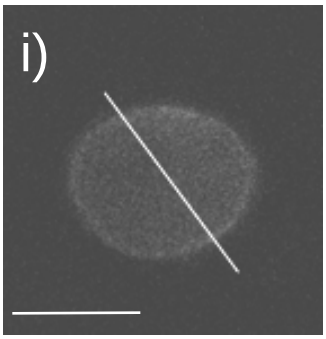
eYFP-PH$_{PLC\delta1}$ alone

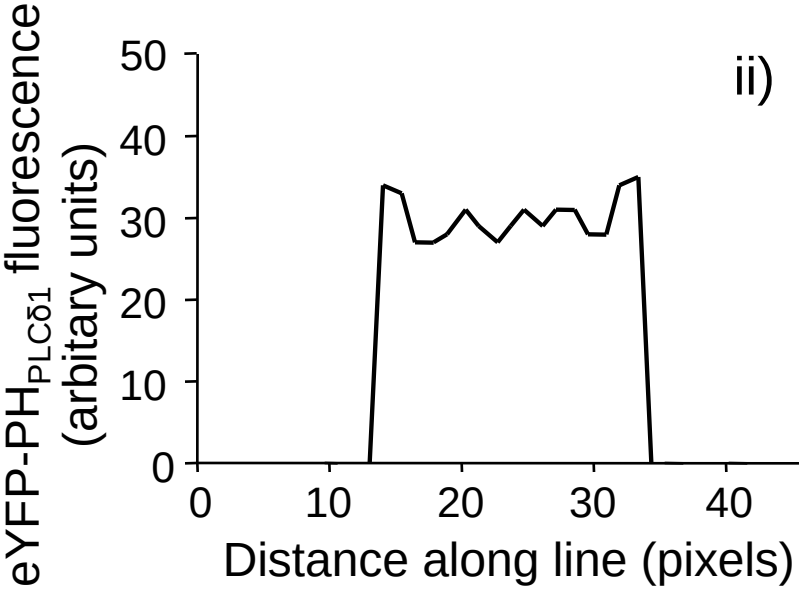




B)

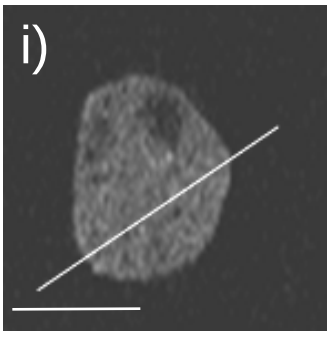
eYFP-PH$_{PLC\delta1}$ + CA-Gα_q





C)

eYFP-PH$_{PLC\delta1}$ + CA-Gαq + RGS3-CFP



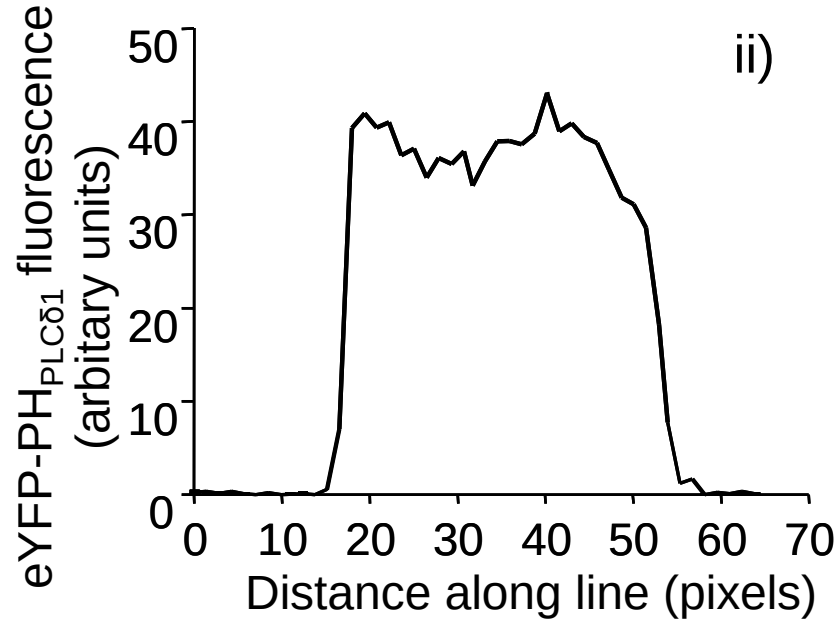


Fig. 11

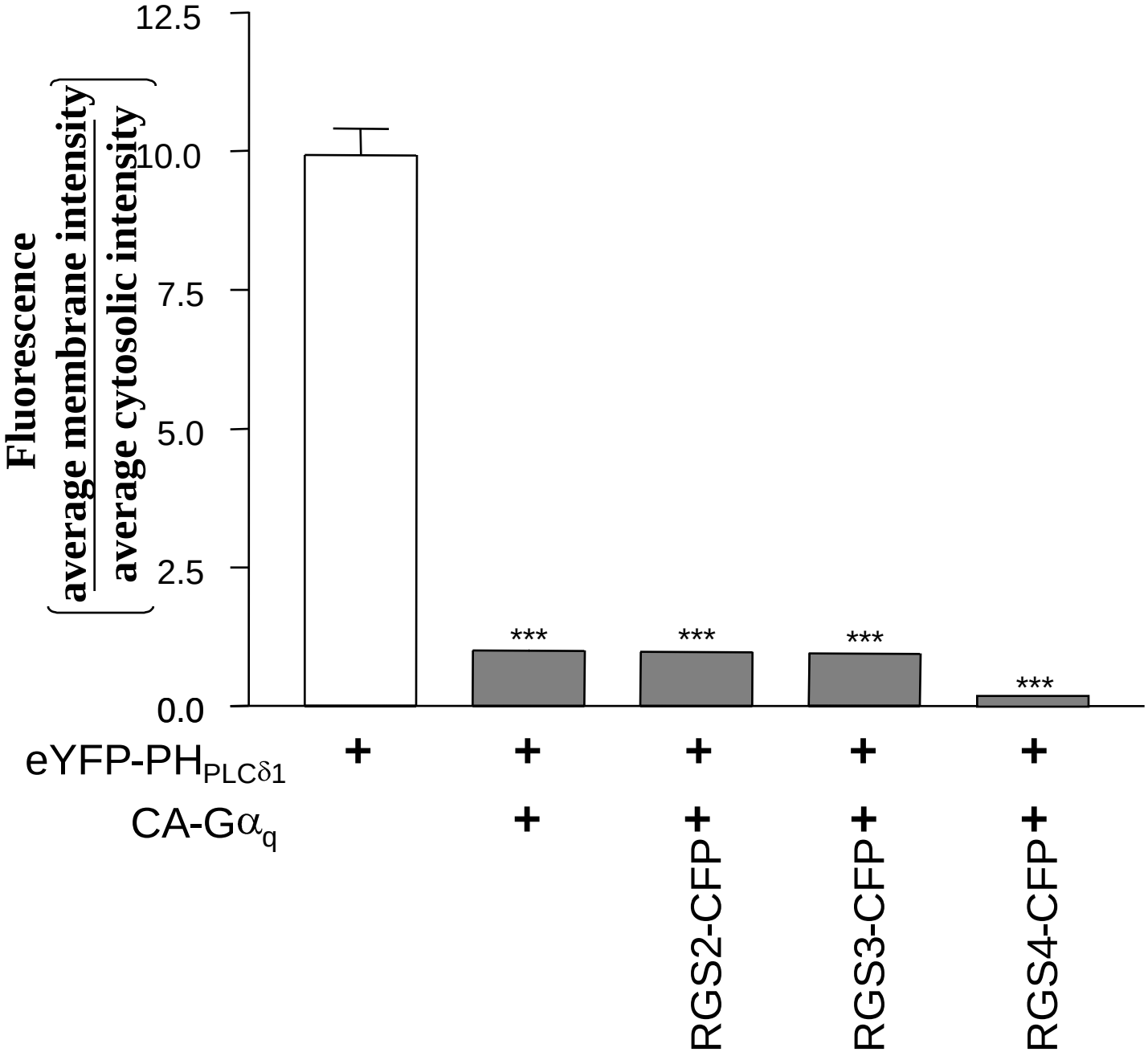

Fluorescence
average membrane intensity
average cytosolic intensity
12.5
10.0
7.5
5.0
2.5
0.0
***
***
***
***
eYFP-PHPLCδ1
CA-Gαq
+
+
+
+
+
+
+
+
+
RGS2-CFP
RGS3-CFP
RGS4-CFP

Fig. 12