The effect of GPCR crosstalk on intracellular Ca^{2+} responses and downstream signalling

Thesis submitted for the degree of Doctor of Philosophy at the

University of Leicester

By

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September 2010

Abstract

This thesis studies crosstalk between $G\alpha_{q/11}$-coupled muscarinic M_3 receptors and $G\alpha_s$-coupled β_2-adrenoceptors leading to a synergistic increase in intracellular Ca^{2+}. The aims were to generally understand the integration of signalling and specifically the potential interactions between these receptors in physiological and pathological settings, for example in asthma and chronic obstructive pulmonary disease (COPD). This study demonstrated that only in the presence of a muscarinic agonist did stimulation of β_2-adrenoceptors elicit a Ca^{2+} response. Both full and partial agonists of the muscarinic M_3 receptor were used to examine differences in the pattern of Ca^{2+} signalling both mediated by the muscarinic M_3 receptor and the β_2-adrenoceptor. Crosstalk was dependent on the concentration of the muscarinic agonist, being more robust and consistent in the presence of a partial rather than a full muscarinic agonist. Under crosstalk conditions, single cell imaging highlighted pronounced alterations in the pattern of Ca^{2+} signalling, such as oscillatory frequency. This Ca^{2+} signal was independent of extracellular Ca^{2+} but was dependent on thapsigargin-sensitive stores. Investigation of the potential mechanism indicated that exchange proteins directly activated by cAMP (Epac) and protein kinase A (PKA) were not involved. An alternative mechanism was suggested, with localised increases in cAMP directly sensitising $Ins(1,4,5)P_3$ receptors. Downstream signalling events regulated by Ca^{2+}, including extracellular signal-regulated kinases (ERK) and nuclear factor-kappaB (NF-kB) were examined to determine if their regulation altered in the presence of crosstalk. Although crosstalk experiments were unsuccessful in rat tracheal smooth muscle cells, due to loss of muscarinic M_3 receptor expression, it is still possible that this crosstalk could alter the regulation of cell function and be an important factor in treating diseases such as asthma and COPD.

Publications

Paper

- Kurian N., **Hall C.J.,** Wilkinson G.B., Sullivan M., Tobin A.B and Willars G.B (2009). Full and partial agonists of the muscarinic M_3 receptor reveal single and oscillatory Ca^{2+} responses by β_2-adrenoceptors. *Journal of Pharmacology and Experimental Therapeutics*. 330(2):1-11

Abstracts

- **Hall C.J.,** Brown A., Wilkinson G.F and Willars G.B. Increased intracellular Ca^{2+} signalling mediated via crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors is associated with a synergistic effect on ERK activation. *Young Physiologists' Symposium* 23-24th September 2009, Leicester, U.K.

- **Hall C.J.,** Brown A., Wilkinson G.F and Willars G.B. Increased intracellular Ca^{2+} signalling mediated via crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors is associated with a synergistic effect on ERK activation. *British Pharmacological Society* - 3rd Focused Meeting Cell Signalling British Pharmacological Society 20-21st April 2009, Leicester, U.K.

- **Hall C.J.,** Brown A., Wilkinson G.F. and Willars G.B. Co-stimulation of muscarinic M_3 acetylcholine receptors and β_2-adrenoceptors causes a synergistic effect on the release of intracellular Ca^{2+} and ERK activation. *British Pharmacological Society pA2 6(4) abstract 090*, Winter meeting 16-18th December 2008, Brighton, U.K.

Acknowledgements

Firstly, a big thank you to my supervisor Gary Willars for helping throughout my PhD both with laboratory advice and endless thesis reading. Many thanks also to AstraZeneca for their financial support and use of equipment, and to my thesis committee members John Challiss and Christine Pullar.

As for my fellow members of CPP, I can't thank Sophie, Claire, Karen and Sam enough for their unlimited support throughout my PhD. Karen your cakes got me through so many rough times and you knew everything about my project which still amazes me today! Sophie you have kept a constant smile on my face and I'm impressed I made it through my PhD without being injured from your karate moves! Rachel thank you for listening to my moans about science and Jo for appreciating every step as we slogged along together! Yan your help in the lab was invaluable, I couldn't have coped without your endless molecular knowledge and thank you for taking over Karen's 'lab mum' role when she left. I would also like to acknowledge Khaled and Neil for being brave enough to share a lab with me and giving advice when needed!

The greatest appreciation goes to my parents, Granny, sister and Jason. Thank you for your love and support, without which I would never have got this far. Jason thank you for spending so many days as a 'thesis widower', for your help and support during my regular moments of stress and the belief that I could do it. A massive thank you to Gill Buswell - What a friend you have been both helping with Murphy and being an ear to talk to, even if you didn't always understand what I was on about!

Lastly, this thesis is in loving memory of my Grandpa who knew I would be able to do it and has been with me the whole way. I'm just sorry you're not here to see it finished.

Contents

Abbreviations

AC	Adenylate cyclase
ACh	Acetylcholine
ATP	Adenosine tri-phosphate
Ca^{2+}	Calcium
cAMP	Adenosine 3′,5′-cyclic monophosphate
CCE	Capacitative Ca^{2+} entry
CK1α	Casein kinase α
CTX	Cholera toxin
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTT	1,4-dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EDTA	Ethyl-n-diamine tetra acetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Foetal bovine serum
Fluo-4AM	Fluo-4-acetoxymethyl ester
GAP	GTPase-activating proteins

GEF	Guanine nucleotide exchange factor
GFP	Green fluorescence protein
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GTP	Guanosine 5′-triphosphate
HA	Haemaglutinin
HBSM	Human bronchial smooth muscle cell line
HEK 293	Human embryonic kidney cells 293
$Ins(1,4,5)P_3$	Inositol 1,4,5-trisphosphate
KHB	Krebs'-HEPES buffer
mACh	Muscarinic acetylcholine
MAPK	Mitogen-activated protein kinase
MCh	Methacholine
mRNA	Messenger RNA
NA	Noradrenaline
Oxo	Oxotemorine
PBS	Phosphate-buffered saline
PDE	Phosphodiesterase
$PtdIns(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A

PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PTX	Pertussis toxin
RGS	Regulators of G-protein signalling
TCA	Trichloroacetic acid
XeC	Xestospongin C

Chapter 1: Introduction

G-protein coupled receptors (GPCRs) are responsible for the transduction of a vast array of stimuli into intracellular signals, allowing the cell to respond appropriately. There are different types of GPCRs co-expressed on the surface of the cell and these may be stimulated concurrently by the same or different ligands, activating more than one type of G-protein at any one time. The resultant cellular responses reflect an integration of these signals (crosstalk) and therefore understanding such signal integration is critical for understanding the physiology, pathology and therapeutics of both normal and diseased tissues. Although crosstalk often results in desensitisation of responses this thesis will focus on crosstalk between GPCRs that results in enhanced signalling, specifically calcium

1.1 G-protein coupled receptors

Multicellular organisms have the ability to send messages between cells via transmitters, receptors and effector molecules. The process involves signalling from one cell to another cell leading to an intracellular signal and an appropriate response from the second cell. One of the major ways in which messages are detected from outside cells is via cell surface receptors. These receptors can convert extracellular messages into signalling cascades to produce the required outcome. These responses can be rapid, such as synaptic transmission or much slower, even occurring over hours in the case of gene transcription. One of the largest and most diverse families of membrane receptors are the GPCRs. These receptors are members of a superfamily that are thought to share a common structure, which consists of seven transmembrane hydrophobic α-helixes connected by hydrophilic loops with an extracellular amino terminus (N-terminus) and a cytoplasmic carboxyl-terminus (C-

terminus) (Kristiansen *et al.,* 2004). These receptors are termed GPCRs because when a ligand binds to the receptor a conformational change occurs leading to the activation of a G-protein and stimulation of a pathway of effector proteins. Exactly which effector molecules are activated depends on the type of heterotrimeric G-protein coupled to the receptor. GPCRs are also known as seven transmembrane domain receptors due to their structure and the emergence of G-protein independent signalling by the receptors (Tabata *et al.,* 2007, Gsandtner *et al.,* 2005, Heuss and Gerber, 2000).

1.1.1 GPCR classification

Members of the GPCR superfamily have diverse primary structures and are therefore sub-divided into classes or families based on a variety of criteria including their native ligand, phylogenetic development, amino acid sequence or the structure of their N-terminus. The first attempt at classifying the superfamily occurred in 1993 with the receptors being separated into 'clans' based on their hydrophobic domain sequences (Attwood and Findlay, 1994). In 1994 the well known classification system containing families A to F, which organised receptors mainly according to their sequence homology and native ligand, was developed (Kolakowski, 1994). However, after the human genome was drafted, the GRAFS system was devised which contains 802 known or predicted human GPCRs. It therefore omits Family D and E, which do not contain human receptors. It also further subdivided the Family A receptors into 6 sub-classifications and Family B into secretin and adhesion receptors (Fredriksson *et al.,* 2005). The currently accepted classification of receptors has been developed by the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification and is based on the GRAFS system

(http://www.iuphar-db.org/iuphar-rd). The classes of GPCR include; Family A (rhodopsin-like receptors), B (secretin-like receptors), C (metabotropic neurotransmitter type receptors), D (pheromone receptors), E (cAMP receptors) and F (Frizzled/Smoothened receptors) (Davies *et al.,* 2008, Foord *et al.,* 2005, Kristiansen 2004, Pin *et al.,* 2007). Families D, E and F include GPCRs from both vertebrates and invertebrates.

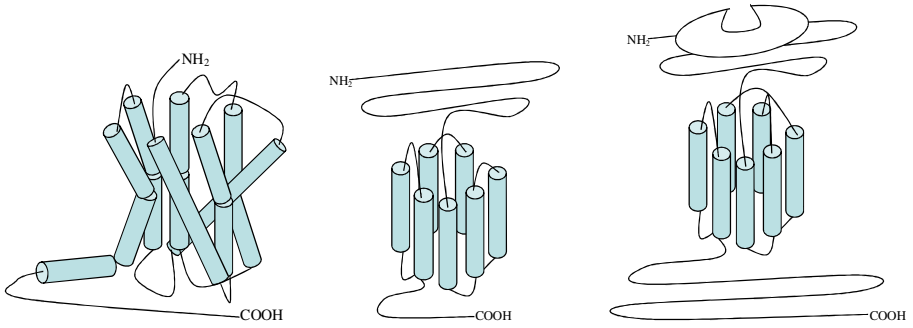
The largest family of GPCRs is Family A, also know as the rhodopsin-like family and will be the focus of this thesis (Fredriksson *et al.,* 2005, Lattin *et al.*, 2007, Kristiansen 2004). These consist of many receptors including rhodopsin, adenosine, neuropeptide and olfactory with over 80% of the group members being found in humans. These receptors are sub-classified into six groups due to their phylogenetic sequence: the biogenic amine receptors, opsin receptors, bradykinin receptors, chemokine receptors, olfactory receptors and melatonin receptors (Fredriksson *et al.,* 2005). Family A receptors have the typical seven transmembrane (7TM) structure with a short N-terminus (Figure 1.1). The receptors share specific sequence motifs within these 7TM regions, although they are still diverse in structure. Family A GPCRs are characterised by 20 highly conserved amino acids within their structure and contain a highly conserved triplet of amino acids, Asp-Arg-Tyr, known as the DRY motif. This motif lies between the third transmembrane helix and the second intracellular loop and studies have suggested that this sequence may have an important role in signalling. For example, mutation of the aspartate 115 residue in the DRY motif of the histamine H_2 receptor results in a great increase in constitutive activity and increased agonist affinity and signalling (Alewijnse *et al.,* 2000). The middle arginine is thought to be the main residue involved in signal transduction because mutations here prevent signalling (Alewijnse *et al.,* 2000, Flanagan, 2005, Sung *et al.,* 1991). The DRY motif has an

important role in both structural stability of the receptor and also in constraining the receptor in an inactive form. Its roles have been recently reviewed (Flanagan, 2005, Rovati *et al.,* 2007).

Agonist binding to Family A receptors occurs deep inside the receptor, between TM helices III to VII, as demonstrated by spectroscopic analysis of the fluorescent antagonist of the β_2-adrenoceptor, carazolol (Tota *et al.,* 1990). Site-directed mutagenesis studies have shown that residues in the second extracellular loop (ECLII) can be important in the binding of some small molecule ligands. A disulfide bridge forms between a cysteine in the ECLII and a cysteine near the N-terminus of the TM helix III allowing the ECLII to be close to the binding site and therefore interact with ligands (Kim *et al.,* 1996).

The serpentine structure of GPCRs was based originally on two-dimensional structure of bacteriorhodopsin (Unwin and Henderson, 1975). However this was not an accurate representation of receptor composition and the development of the three-dimensional tertiary structure of bacteriorhodopsin, which resembles that of the rhodopsin receptor in mammals, presented a better model. However, the functions of these two receptors are different and they have only slight homology in their amino acid sequence. Both receptors belong to the 7TM receptor family but only rhodopsin is a GPCR. The three-dimensional crystal structure of the bovine rhodopsin receptor gave an insight into the mechanisms of receptor activation and G-protein interactions (Palczewski *et al*., 2000).

However, rhodopsin differs from other GPCRs because it does not have an extracellular 'ligand'. Instead light causes the retinal molecule, within the receptor, to undergo a conformational change on absorbance of a photon (Palczewski *et al*., 2000). This receptor was therefore not the best model for the structure and function of other GPCRs and in recent years structures of ligand-activated (inverse agonist) GPCRs have been obtained by crystallography, namely the human β_2-adrenoceptor, the avian β_1-adrenoceptor and the human A_{2A} adenosine receptor (Rosenbaum *et al.,* 2007, Rosenbaum *et al.*, 2009). These crystal structures confirmed that the overall structure of bovine rhodopsin was reflected in these other receptors, although the proline kink in TMI of the rhodopsin receptor was not present in the human β_2-adrenoceptor.



Family A GPCR Family B GPCR Family C GPCR

Figure 1.1 Schematic diagram showing the structure of GPCRs from the three Families. All the receptor Families contain 7TM domains, an N-terminus involved in ligand binding and a C-terminus involved in activation of the G-protein and phosphorylation of the receptor. The Family B and C receptors have much longer N-termini than Family A with family C containing a VFT in which the ligand binds. The C-terminus of Family C also contains many more amino acids than Family A and B. (Adapted from Strosberg and Nahmias, 2007).

There were also differences between the inactive states of these two receptors with the rhodopsin receptor being held in the inactive form by an 'ionic lock' between the R135 in

the DRY motif and E247 in TMVI. These interactions are not seen in the inactive, antagonist bound human β_2-adrenoceptor (Lagerström and Schiöth, 2008).

Family B contains both liganded and orphan receptors (http://www.iuphar-db.org/PRODDATABASE/FamilyMenuForward?familyId=17). Secretin-like Family B receptors are sub-divided into secretin and adhesion receptors, of which there are 15 and 33 members respectively. Family B GPCRs are distinguishable from other families by their long extracellular N-terminal hormone binding domain that is between 35 and 350 amino acids (see Figure 1.1). Ligand binding to Family B receptors follows a 'two-domain model' of binding with the C-terminus of the ligand interacting with the extracellular domain of the receptor and the N-terminus of the ligand interacting with the extracellular loops and transmembrane helices of the receptor (Parthier *et al.,* 2009). The endogenous ligands for these receptors are peptide hormones such as glucagon (Parthier *et al.,* 2009). The receptors share 21-67% sequence identity and most of the variation occurs in the N-terminus (Lagerström and Schiöth, 2008). Within this tail there are six conserved cysteine residues thought to form three disulphide bonds (Unson *et al.,* 2002). Site directed mutagenesis and cross-linking experiments have shown that these cysteines, along with conserved cysteine residues in the first and second extracellular loops, stabilise the receptor in both its inactive form and when activated, upon ligand binding (Lagerström and Schiöth, 2008, Kristiansen, 2004). Until now no full Family B crystal structure has been developed.

The seven transmembrane helices of Family C receptors have no sequence similarity with other GPCRs except for a few conserved residues also present in Family A receptors (Kristiansen 2004, Pin *et al.,* 2003). Members of this Family include metabotropic

glutamate receptors, γ-aminobutyric acid$_B$ ($GABA_B$) receptors, the Ca^{2+}-sensing receptor, putative pheromone receptors, sweet and amino acid taste receptors and orphan Family C receptors. Most GPCRs within Family C, but not orphan receptors, have a large extracellular domain, the venus fly trap (VFT), connected to the heptahelical domain by a cysteine rich region, except the $GABA_B$ receptor subunits, $GABA_{B1}$ and $GABA_{B2}$. (Pin *et al.,* 2004). These subunits form the fully functioning $GABA_B$ receptor with the $GABA_{B1}$ subunit containing the VFT responsible for ligand binding and $GABA_{B2}$ activating the G-protein (see Figure 1.1) (Pin *et al.,* 2003, Pin *et al.,* 2004, Kristiansen 2004). When an agonist binds to the VFT a conserved, intra-domain disulfide bridge forms between the VFT and the 'heptahelical G-protein activating domain' causing the VFT to close (Rondard *et al.,* 2006). In metabotropic-glutamate type 2 receptors (mGluR2) the Cys-518 in the cysteine rich region cross-links with the Cys-234 in the VFT. The VFT domain adopts two major conformations, an inactive open conformation and a closed activate conformation stabilised by the agonist (Rondard *et al.,* 2006).

Receptors are generally thought to be monomers but both Family C receptors and those of other families can form dimers (Pin *et al.,* 2004, Rondard *et al.,* 2006). It is necessary for some members of Family C to form homo- or hetero-dimers for stabilisation of the VFT and heptahelical domain allowing receptor activation (Rondard *et al.,* 2006). Family C dimers must undergo a conformation change in both heptahelical domains and to allow activation (Rondard *et al.,* 2006). When expressed alone $GABA_{B1}$ is retained intracellularly as an immature glycoprotein whereas $GABA_{B2}$ is transported to the cell surface but unable to bind GABA or signal. When the two receptors are co-expressed they reach the cell surface and are fully functional (Couve *et al.,* 1998, Pin *et al.,* 2007, White *et al.,* 1998).

Dimerisation does not just occur between members of Family C. In β_2-adrenoceptors, addition of a synthetic peptide corresponding to TMVI interfered with receptor dimerisation and limited agonist activation of adenylate cyclase (Herbert *et al.,* 1996). The β_2-adrenoceptor dimerises during protein synthesis and maturation prior to cell surface expression and during internalisation (Sartaria *et al.,* 2007). CXCR1 and 2 receptors have also been shown to form homo- and hetero-dimers (Wilson *et al.,* 2005). Dimerisation of receptors has been shown to occur due to constitutive activity, such as the $GABA_{B1}$ and $_{B2}$ dimers, or induced on agonist activation for example the gonadotropin-releasing receptor (see Milligan, 2010 for full review, Milligan *et al.,* 2005).

Members of GPCR Families D and E are found in fungi and *Dictyostelium* whereas Family F frizzled/smoothened receptors are found in mammals. There are 10 frizzled and 1 smoothened receptor with have sequence similarities with Family B receptors. However, the N-terminus of the frizzled receptors is even more cysteine rich than Family B receptors (Foord *et al.,* 2002, Kristiansen 2004).

1.2 GPCR activation

The mechanism by which GPCRs activate their pathways could be thought of as a simple off (no ligand bound)/on (ligand bound) switch, caused by conformational changes of the receptor. This model was first suggested by del Castillo and Katz (Del Castillo and Katz, 1957). However, this suggested that all receptor ligands had the same effect. The complexity of GPCR signalling first started to emerge after the β-adrenoceptor was found to exhibit two affinity states for ligands. This led to the development of a new model, the

ternary complex model (Figure 1.2). The model was extended and predicted that in the presence of GDP, an agonist caused a stable, high-affinity complex to form between the receptor (R), agonist (A) and G-protein (G). When a G-protein is not associated with the receptor, the complex is dissociated and the receptor is in a low affinity state (A-R). Using radioligand binding, the correlation suggested that full agonists were able to shift the equilibrium more in favour of an active receptor, than partial agonists (reviewed in Park *et al.,* 2008).

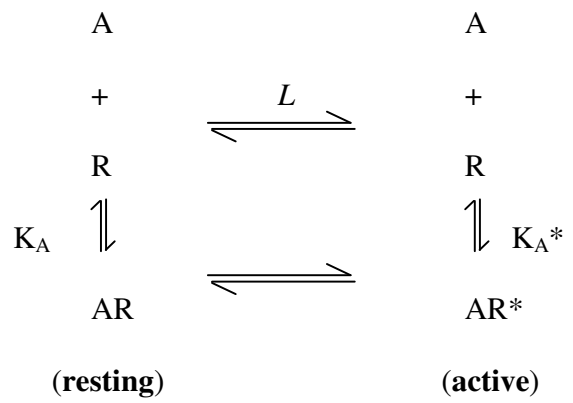


Figure 1.2 Ternary complex model of receptor activation. The receptor exists in two states resting (R) and active (R*). In the absence of agonist (A) the state of the receptor is in equilibrium (constant *L*). The affinity the agonist has for the two states is governed by the dissociation equilibrium constants K_A and K_A*. (Adapted from Leff, 1995, Park *et al.,* 2008).

Studying constitutively active GPCRs (receptors able to signal without the need to bind a ligand) has led to a greater understanding of receptor activation and resulted in the extended ternary complex model. This hypothesises that receptors exist in equilibrium between two states, inactive (R) and active (R*) (Gether and Kobilka, 1998, Shenoy and Lefkowitz, 2003). When the receptor is not bound to a ligand it is held mainly in the R state by

intramolecular interactions within the transmembrane helices. Once an agonist binds, the interactions in R are disrupted and the receptor relaxes into the R* conformation, allowing contact with a G-protein. Full agonists bind preferentially to R* and therefore stabilise this form of the receptor shifting the equilibrium. Partial agonists favour binding to R* with antagonists having no preference on which receptor state they bind too whereas inverse agonists reduce the activity of constitutively active receptors or act as antagonists in non-constitutively active GPCRs and therefore have a preference for R (Gether and Kobilka, 1998, Shenoy and Lefkowitz, 2003). This extended ternary complex model is however unable to explain the multiple active states of GPCRs, which can be stabilised by different ligands. Several studies have indicated that GPCRs may exist in multiple conformation states with intermediate receptor conformations between the active and inactive forms (Hill, 2006, Vauquelin *et al.,* 2005). Real-time fluorescence spectroscopy has demonstrated that receptors undergo different conformations between inactive and active, after ligand binding. Thus, cysteine residues in the external loops of purified β_2-adrenoceptors were labelled with conformationally sensitive fluorophores. Different changes in fluorescence intensity were observed when the receptors were challenged with different agonists (Giraldo, 2004, Ghanouni *et al.,* 2001).

Receptor activation is not only dependent on the conformation of the receptor but also its binding to G-proteins. Some GPCRs are promiscuous and bind to more than one type of G protein. The 'three-state' or 'multiple state' model suggests that GPCRs may have different conformations allowing coupling to different G-proteins when ligands are bound. This occurs because different ligands expose distinct regions of the intracellular domains which couple with the G-proteins allowing a single receptor type to activate more than one

pathway. For example, multiple G-protein-coupled states of the α_2-adrenoceptor have been demonstrated using guanine nucleotide analogs (Seifert *et al.,* 1999). The multiple state model can explain why agonists can control the strength, amplitude and pattern of signal by regulating the type of activation of the receptor and therefore the pathway (Seifert and Dove, 2009). Evidence has suggested that different areas on the intracellular loops of GPCRs activate different G-proteins (Gether and Kobilka, 1998). This allows the receptor to be set in a particular tertiary conformation and could expose different G-protein binding sites. This model also allows for the concept that signalling-selective agonism can occur with different agonists exhibiting different affinities for a receptor and also stabilise different active conformations (Seifert and Dove, 2009). These conformations determine the affinity of the receptor for the downstream effector molecules that mediate its signalling. This model shows that although two agonists may bind to a receptor, the potencies, efficacies and nature of signalling of those may be different, thereby varying the biological affect they have.

1.3 GPCR signalling

The traditional way in which a GPCR is thought to signal is via a G-protein-dependent pathway. However, the importance of G-protein-independent signalling is now being recognised (Kristiansen, 2004). G-protein-dependent signalling is the focus of this thesis and in order for a GPCR to transducer a signal in this manner, a heterotrimeric G-protein must be activated. The heterotrimeric G-proteins consist of three subunits: α, β and γ with molecular masses of ~39-45, 35-39 and 6-8 kDa, respectively (Kristiansen, 2004). So far at least 28 distinct G-protein α-subunits have been identified and these include 16 splice

variants and different genes. The β and γ subunits are closely bound by disulfide bonds and therefore considered as one unit. G-protein activation and deactivation occurs in a cycle (Figure 1.3). When the G-protein is in its basal heterotrimeric state the three subunits α, β and γ are associated. In the absence of agonist binding, the Gα subunit is bound to guanosine 5'-diphosphate, GDP. The activated receptor functions as a guanine nucleotide exchange factor (GEF) promoting the exchange of GDP for GTP. The binding of GTP causes a conformational change in the G-protein and the separation of the Gα subunit from the obligate dimer of the βγ subunit. In order to terminate signalling, the G-protein, which has intrinsic GTPase activity, hydrolyses GTP back to GDP allowing re-association of the subunits and returning the G-protein to its basal GDP-bound state. GTPase-activating proteins (GAPs) can increase the speed at which the GTPase reaction occurs. A diverse family of proteins known as regulators of G-protein signalling (RGS) proteins act as GAPs for the α-subunit. These RGS proteins regulate G-protein activation and therefore the effector proteins, controlling the kinetics and amplitude of signalling (Jones *et al*., 2004). In some cases the effector molecules themselves have GAP activity. For example PLCβ has GAP activity within its C-terminal tail and is able to enhance the GTPase activity of the G-protein, $G\alpha_{q/11}$, that activates it (Chidiac and Ross, 1999, Ilkaeva *et al.,* 2002, Paulssen *et al.,* 1996).

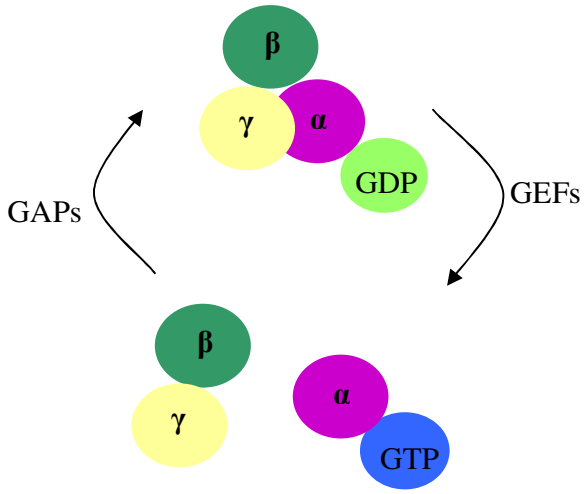


Figure 1.3. Heterotrimer G-protein activation/inactivation cycle. G-proteins remain in a GDP-bound basal state until activated by a ligand bound receptor which acts as a guanine nucleotide exchange factor (GEF). The GEF catalyses the release of GDP from the α-subunit and enhances the binding of GTP. The GTP-bound α-subunit separates from the βγ-dimer and they each regulate their own effectors. The G-protein signalling is terminated with the hydrolysis of GTP back to GDP by GTPase-activating proteins (GAPs). This allows the α-subunit and βγ-dimer to re-associate into a heterotrimer.

There are 16 known genes that encode for Gα–subunits. These subunits are divided into 4 families based on sequence similarities: $G\alpha_s$; $G\alpha_i$; $G\alpha_{q/11}$ and $G\alpha_{12}$; (Cabrera-Vera *et al.,* 2003, Downes and Gautam, 1999). Members of the $G\alpha_s$ family activate adenylate cyclase (AC) (see Table 1.1 and Figure 1.4) whereas activation of $G\alpha_i$ inhibits the actions of AC 5 and 6 (see Table 1.1 and Figure 1.4). Stimulation of $G\alpha_{q/11}$-coupled receptors leads to an increase in phospholipase C (PLC) activity and hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) into inositol trisphosphate (Ins(1,4,5)P_3) and 1,2-diaglycerol (DAG). Presently thirteen mammal PLC isoenzymes have been identified and have been divided into 6 groups: PLC-β, -γ, -δ, -ζ, -ε, -η (Suh *et al.,* 2008).

Subunit	Family	Subtype	Effector
α	$G\alpha_s$	$G\alpha_s$	↑ AC ↑ L-type Ca^{2+} channel ↑ voltage-gated Na^+ channel ↑ cAMP activated Cl^- channel
		$G\alpha_{olf}$	↑ AC
	$G\alpha_i$	$G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$	↓ Cl^- channel ↑ K^+ channel ↓ Ca^{2+} channel Rap 1 ↓ Na^+ channel ↓ AC
		$G\alpha_{oA}$, $G\alpha_{oB}$	↑ K^+ channel ↓ AC ↓ Cl^- channel
		$G\alpha_{t1}$, $G\alpha_{t2}$	↓ Ca^{2+} channel ↑ cGMP-PDE
		$G\alpha_z$	↑ cGMP-PDE
	$G\alpha_q$	$G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15 \text{ or } 16}$	↑ PLC_β
	G_{12}	$G\alpha_{12}$ $G\alpha_{13}$	↑ Na^+/H^+ exchanger ↑ PLD ↑ p115RhoGEF ↑ iNOS
βγ	Combinations of β_{1-5} and γ_{1-12} dimers		↑ AC II, IV, VII ↓ AC I ↑ K^+ channels ↑ PI_3 kinase ↑ PKD ↑ PLC_β ↑ PKC ↑ GPCR kinases

Table 1.1. Classification of Gα-subunit and βγ-dimer subtypes and their effectors.

↑Indicates stimulation and ↓indicates inhibition of effector. iNos, inducible nitric oxide synthase; PLD, phospholipase D; (Adapted from Gudermann *et al.,* 1997, Hermans, 2003, Kristiansen, 2004).

Within this family are the PLC-β isoenzymes containing PLC-β1 to 4 (Suh *et al.,* 2008). These isoforms can be activated by both Gα and βγ subunits, with the exception of PLC-β4 which is not activated by βγ. The other PLC-β isoforms are activated by Gβγ-dimers in the rank order PLC-β1 ≥ PLC-β3 > PLC-β2 (Jhon *et al.,* 1993, Rhee, 2001, Smrcka and Sternweis, 1993). $G\alpha_{12/13}$-coupled proteins are implicated in the regulation of Rho, a monomeric G-protein and guanine nucleotide exchange factor (RhoGEF) (Table 1.1).

There are five genes encoding for the β-subunit. $\beta_{1\text{-}4}$ are ubiquitously expressed and share approximately 80-90% homology whereas β_5 is expressed in the central nervous system and only shares 50% similarity with the other subunits. β_5 is able to interact with RGS7 via a binding site on RGS7, which has great homology with the γ-subunit (Levay *et al.,* 1999). Each β-subunit contains approximately 340 amino acids and has a molecular weight of ~35,000Da (Cabrera-Vera *et al.,* 2003). The γ-subunits are the most diverse in amino acid sequence out of the three G-protein parts. There are twelve γ-subunits and they form dimers with the β-subunit using their N-terminal tail to make several interactions, via disulfide bonds, with the β subunit (Cabrera-Vera *et al.,* 2003, Downes and Gautam, 1999).

Both the α-subunit and the βγ-dimer are able to activate effectors in their own right (Bouvier 2001, Gether *et al.,* 1998, Zhang *et al.,* 1996). The βγ-dimer has been shown to regulate a range of enzymes, channels and effector molecules (see Table 1.1) (Cabrera-Vera *et al.,* 2003, Wickman *et al.,* 1994). When co-transfections of $\beta_5\gamma_2$ or $\beta_1\gamma_2$ were carried out in COS-7 cells, both combinations of dimers were able to activate PLC-β2 but only $\beta_1\gamma_2$ was able to activate extracellular signal-regulated kinase ERK via activation of PLC-β2 (Zhang *et al.,* 1996). This demonstrates another method for selective receptor transduction

allowing an extensive range of signalling from GPCRs. The mechanisms by which the receptor mediates G-protein independent signalling is unknown, although evidence suggests this can be via the JAK/STAT pathway and GRK/β-arrestin (Ahn *et al.,* 2004, Zhai *et al.,* 2005). G-protein independent signalling via the GRK/β-arrestin pathway has also been demonstrated for β_2-adrenoceptors (Shenoy *et al.,* 2006).

Activation and therefore regulation of the receptor is not only dependent on the degree and type of conformational change but also on intermolecular contacts allowing expression and activation of the GPCR at the cell surface. Dimerisation is one important method in which expression and activation of receptors can be regulated, as demonstrated by the $GABA_B$ receptor (Milligan, 2004, Milligan, 2009). Protein scaffolds are another method by which receptors can ensure they are expressed at the cell surface, allowing the receptor to bind the ligand, causing activation of the effector and generation of second messenger molecules. Some scaffold proteins act as platforms to bring the components of a pathway together whereas others can also affect the activation of the effectors. Several scaffold proteins have been identified, one example being the A-kinase anchoring proteins (AKAPs) family of proteins which target PKA and other signalling proteins to specific subcellular locations (Andreeva *et al.,* 2007, Colledge and Scott, 1999, Malbon *et al.,* 2004). Scaffold proteins also have an important role in signal termination of some receptors. AKAP250 is important in the desensitisation/resensitisation cycle of the β_2-adrenoceptor. Suppression of AKAP250 using antisense RNA prevented trafficking and resensitisation of the receptor showing these scaffold proteins to have a diverse function in many different pathways (Malbon *et al.,* 2004).

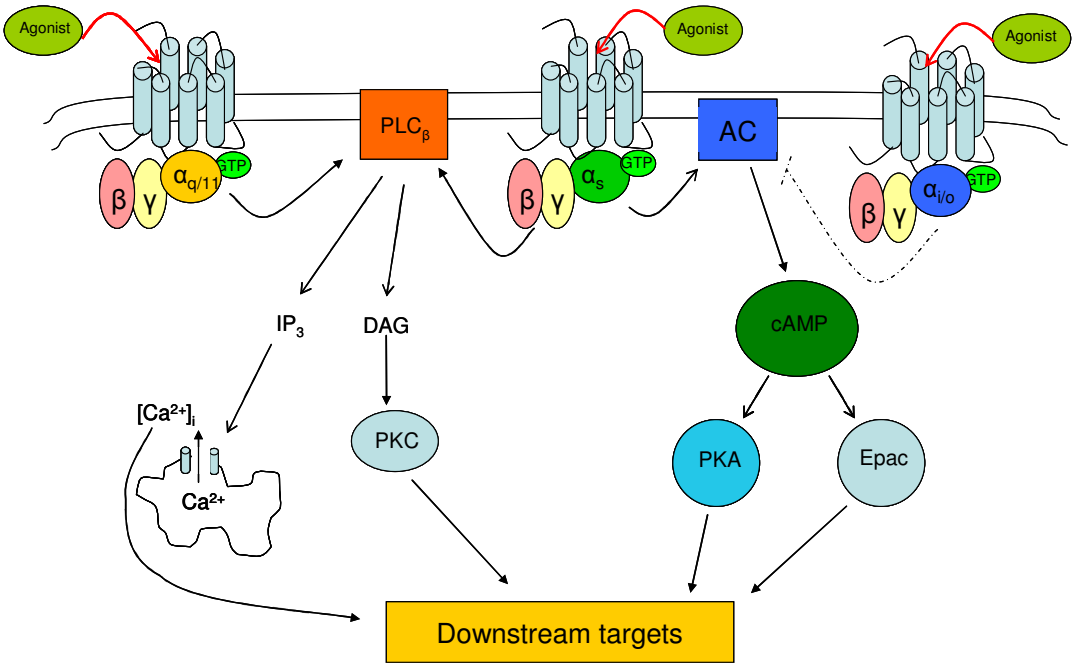


Figure 1.4. Signal transduction pathways for the G-protein subunits. Stimulation of a G$\alpha_{q/11}$-coupled receptor leads to activation of phopholipase C$_\beta$ (PLC$_\beta$) via the α-subunit, causing hydrolysis of PtdIns(4,5)P_2 to Ins(1,4,5)P_3 and DAG. These molecules trigger release of intracellular Ca^{2+}, via Ins(1,4,5)P_3 receptors and protein kinase C (PKC) activation respectively. Stimulation of a Gα_s-coupled receptor leads to activation of adenylate cyclase which catalyses the conversion of cAMP. This protein activates protein kinase A (PKA), Guanine nucleotide exchange factor activated by cAMP (Epac) and also cyclic-nucleotide gated channels. Stimulation of a Gα_i-coupled receptor inhibits adenylate cyclase activity preventing the production of cAMP. Certain βγ-dimer combinations are able to activate or inhibit proteins and channels including PLC$_\beta$, AC and other effector molecules.

1.4 Regulation of signalling

Signalling pathways do not have a fixed on and off switch. The sensitivity and responsiveness can be regulated to allow fine control of cellular activity allowing adaptation to the changing environment and outcome needed. For example, receptors can become less able to mediate signalling (desensitised). There are two forms of desensitisation, homologous and heterologous (Gainetdinov *et al.,* 2004, Kelly *et al.,* 2008). Homologous desensitisation is the loss of receptor sensitivity following ligand binding to those receptors. In this paradigm, the receptor is phosphorylated by G-protein coupled receptor kinases (GRKs) (see section 1.4.2) (Gainetdinov *et al.,* 2004, Kelly *et al.,* 2008, Tobin *et al.,* 2008). GRKs phosphorylate agonist activated receptors, uncoupling the G-protein and allowing the recruitment of one of four β-arrestins, a key step in GPCR internalisation and hence desensitisation (Lefkowitz and Shenoy, 2005, Luttrell and Lefkowitz, 2002). The GRKs distinguish activated and inactivated GPCRs because they are catalytically activated by the stimulated receptor, leading to homologous desensitisation (section 1.3.2) (Gainetdinov *et al.,* 2004, Kelly *et al.,* 2008). Heterologous desensitisation is a feedback mechanism controlled by second-messenger-dependent protein kinases (e.g. PKA and PKC) and can occur even in the absence of agonist binding to the receptor (see section 1.4.1). These kinases can phosphorylate both the receptor and effectors proteins including AC and PLC to regulate their function.

1.4.1. Desensitization by second messenger-dependent protein kinases

The second messenger-dependent proteins kinases, PKA and PKC, are activated downstream of $G\alpha_s$ and $G\alpha_q$-coupled receptors respectively. Phosphorylation of receptors

by PKA and PKC was originally thought to be the main mediator of receptor phosphorylation and desensitisation (Kelly *et al.,* 2008). Although GRKs and β-arrestins have more recently been shown to play a role in GPCR homologous desensitisation, heterologous desensitisation following phosphorylation by second messenger-dependent protein kinases is the primary mechanism by which some receptors regulate signalling. Activated PKA and PKC phosphorylate serine and/or threonine residues in the intracellular loops and C-terminus of many GPCRs. For example, PKA targets Ser262 in the third intracellular loop and Ser346 in the C-terminal tail of the β_2-adrenoceptor (Clark *et al.,* 1989, Hausdorff *et al.,* 1989). In rat C6 glioma cells, phosphorylation at serine and threonine sites (Thr298, Ser320, and Ser335) of the A2A adenosine receptor is increased by phorbol ester activation of PKC or endothelin-1-mediated activation of PKC (Palmer and Stiles, 1999). This prevents the GPCRs interacting with a G-protein and they are therefore unable to signal (Figure 1.5). How PKA and PKC uncouple the GPCR from the G-protein is not know but the phosphorylation is likely to sterically inhibit the interaction between the G-protein and receptor (Kelly *et al.,* 2008). Mutation of the PKA and PKC consensus sequences within a number of GPCRs has been shown to reduce phosphorylation and desensitisation of the receptors. For example, in HEK 293 cells transfected with β_1-adrenoceptors, mutation of the single PKA/PKC consensus site (S301A) led to a decrease in the ability of PKC to phosphorylate the receptor (Guimond *et al.,* 2005). A similar loss of PKC regulation was demonstrated when the β_2-adrenoceptor expressed in HEK 293 cells was mutated at the PKC consensus sites S261A; S262A; S345A and S346A, preventing phosphorylation by PKCα (Guimond *et al.,* 2005). Similar results have been demonstrated after mutation of PKC consensus sequences in other GPCRs (Benya *et al.,* 1995, Fujimoto *et al.,* 1999, Shi *et al.,* 2007).

In order for receptors to be recycled back to the cell surface, rather than degraded, the internalised receptor must undergo dephosphorylation (Section 1.4.3). The desensitisation mechanisms described, therefore allow for both heterologous and homologous phosphorylation of receptors and indeed many receptors are susceptible to both forms. For example, the β_2-adrenoceptor is known to be phosphorylated by PKA on the third intracellular loop (Ser262) and by GRKs on the C-terminal tail (Ser355 and Ser365) (Seibold *et al.,* 2000, Tran *et al.,* 2004). Signal amplification also allows the stimulation of a small number of receptors to produce a great increase in PKA and PKC activation therefore increasing the number of receptors phosphorylated.

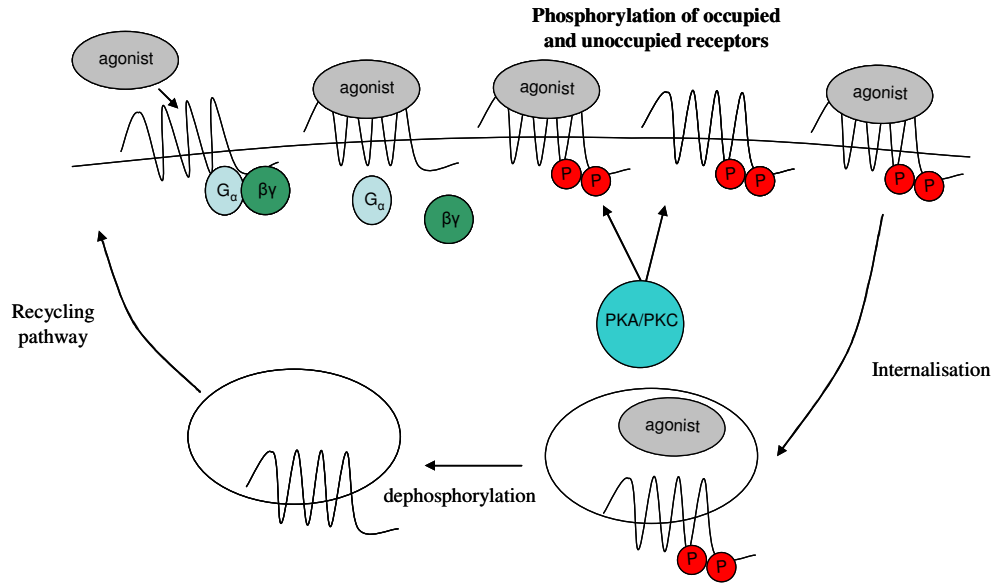


Figure 1.5. Heterologous desensitisation. The agonist-occupied or unoccupied receptor is phosphorylated by a protein kinase causing a conformational change in the receptor allowing a β-arrestin to bind. This prevents further interaction between the receptor and G-protein and the receptor is recycled to the cell surface. (Adapted from Kelly *et al.,* 2008)

These different processes may also be dependent on the position or the receptors within tissue and therefore the concentrations of agonist they are likely to encounter. For example GRK-mediated β_2-adrenoceptor phosphorylation is important for desensitisation at noradrenergic nerve endings where noradrenaline concentration is high (Arriza *et al.,* 1992). In contrast, PKA phosphorylation occurs in tissues such as peripheral blood vessels where less β_2-adrenoceptors are occupied. The two desensitisation processes may interact as both PKA and PKC have been shown to activate GRK2 (Ally *et al.,* 2003, Chuang *et al.,* 1995, Krasel *et al.,* 2001, Kelly *et al.,* 2008). GRK-mediated desensitisation involves phosphorylation by a GRK which increases the affinity for and binding of a β-arrestin protein. The β-arrestin prevents further coupling of a G-protein and targets the receptor for clathrin-coated internalization (see figure 1.6) (Maudsley *et al.,* 2005, Kelly *et al.,* 2008). There are seven known GRKs and they all only phosphorylate agonist-occupied receptors. GRK1 and 7 are retinal kinases involved in the regulation of rhodopsin photoreceptors and GRK2-6 are more widely expressed (Kristiansen, 2004). GRKs all share characteristics but are distinct enzymes. GRK 2, 3, 5 and 6 are ubiquitously expressed in mammalian tissue whereas 1, 4 and 7 are found in specific organs (Ribas *et al.,* 2007). GRKs all have a common structure with a conserved catalytic domain of approximately 270 amino acids and an N-terminus of around 185 amino acids, thought to be important for receptor recognition and membrane anchoring (Garcia-Higuera *et al.,* 1994, Kohout and Lefkowitz, 2003, Reiter and Lefkowitz, 2006). Unlike the other enzymes in the family, GRK2 and 3 have a pleckstrin homology domain in the C-terminal tail which contains a βγ-binding site. GRK 4-6 have a PtdIns(4,5)P_2 binding motif in the N-terminal which increases the kinase activity (Eichmann *et al.,* 2003, Pitcher *et al.,* 1998).

The requirement for the receptor to be occupied in order for GRK-mediated phosphorylation and β-arrestin binding was demonstrated by fluorescence resonance energy transfer (FRET). This method was used to detect interactions between β_2-adrenoceptors and arrestins with the β-arrestin being CFP-tagged and the β_2-adrenoceptor was YFP-tagged. In unstimulated HEK 293 cells, the receptor resided in the plasma membrane and β-arrestin was located in the cytosol (Barak *et al.,* 1997). During agonist exposure, β-arrestin translocated to the plasma membrane and formed a complex with the receptor. The spatial proximity of the two proteins brought CFP and YFP close together, which after excitation of the CFP, led to FRET and emission from YFP. The experiments demonstrated that on removal of agonists, β-arrestins detached from the β_2-adrenoceptor, even if the receptor was still phosphorylated by GRKs (Krasel *et al.,* 2005). Arrestins are also important in trafficking receptors from the cell surface. The arrestins bind to the clathrin adaptor protein AP2 and also to clathrin to facilitate the removal of the desensitised receptor into clathrin-coated pits to be internalised (Gainetdinov *et al.,* 2004). The receptors can then either be recycled (via a recycling endosome) to the cell surface (Figure 1.6 pathway 1) or targeted by lysosomes for degradation (via late endosomes) (Figure 1.6 pathway 2). The outcome for the receptor depends on the extent of agonist stimulation. Normally the GPCR would undergo degradation after hours of agonist treatment although this differs for different receptors. Downregulation and degradation of the receptors occurs more slowly than desensitization (Pfleger *et al.,* 2007).

GRKs are able to carry out phosphorylation- and arrestin-independent desensitization. This has been shown for GRK2 and 3 at $G\alpha_{q/11}$-coupled receptors (Ferguson, 2001). On activation of the receptor, the G-protein dissociates and GRK2 binds to the Gβγ-dimer. This

prevents re-association of the Gβγ-dimer and $G\alpha_{q/11}$ preventing further activation of the G-protein by the GPCR. For some receptors such as the metabotropic glutamate receptors (mGluRs), this represents the most important mechanism of desensitization (Dhami *et al.,* 2004, Ferguson, 2001). Phosphorylation not only terminates GPCR signalling but can also alter the G-protein and therefore the pathway activated by the receptor. For example, PKA phosphorylation of the β_2-adrenoceptor prevents the receptor coupling to $G\alpha_s$ but promotes binding to $G\alpha_i$. This switching of G-protein allows the β_2-adrenoceptor to cause ERK1/2 activation (Daaka *et al.,* 1997, Zamah *et al.,* 2002). However, this phenomenon has only been demonstrated for a small number of GPCRs.

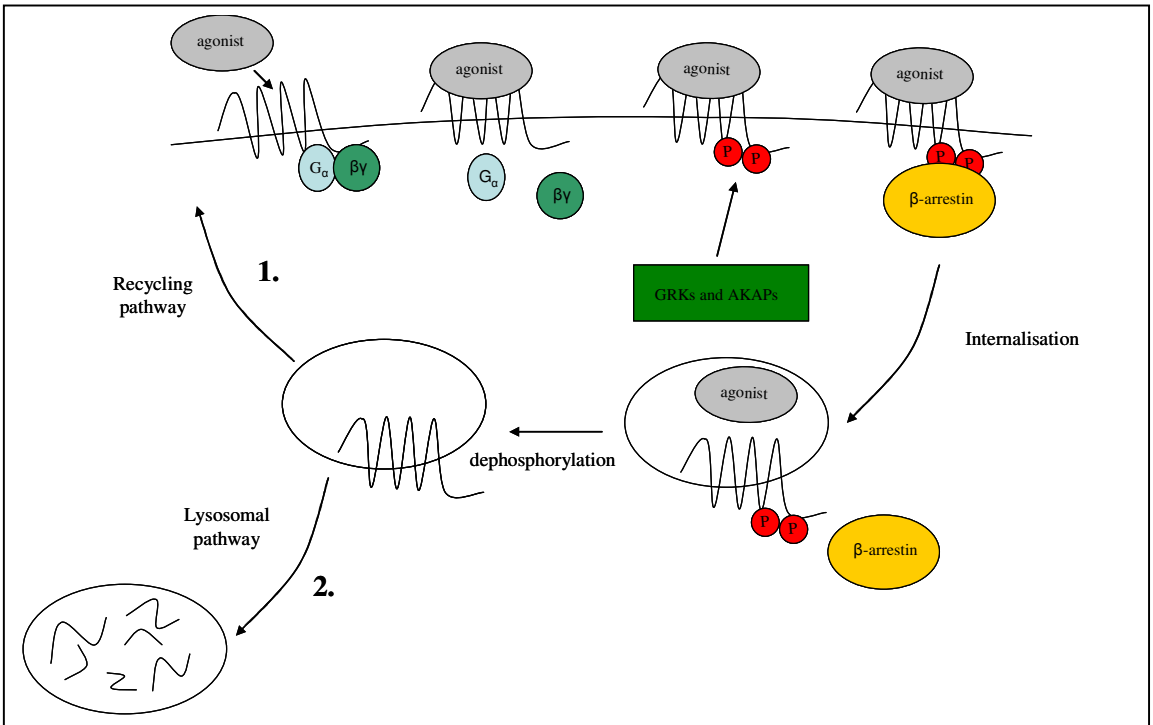


Figure 1.6. Homologous desensitisation. The agonist-occupied receptor is phosphorylated by members of the GRK family. A β-arrestin binds and prevents further interaction between the receptor and G-protein. The receptor is dephosphorylated and either recycled to the cell surface (pathway 1) or degraded (pathway 2). (Adapted from Billington and Penn, 2003, Kelly *et al.,* 2008).

1.4.2. Other proteins involved in receptor phosphorylation

In addition to GRKs, other kinases are able to phosphorylate agonist occupied GPCRs. For example, casein kinase 1α (CK1α) can phosphorylate the M_1 and M_3 muscarinic receptors on serine residues in the third intracellular loop of the GPCRs (Budd *et al.,* 2000, Luo *et al.,* 2008, Tobin *et al.,* 1997, Waugh *et al*., 1999). Although CK1α offers an alternative route for phosphorylation of receptors it is not required for receptor desensitisation. CK1α has also been demonstrated as important in a novel pathway for activation of ERK by muscarinic M_3 receptors (Budd *et al.,* 2001, Tobin *et al.,* 2002). A phosphorylation deficient muscarinic M_3 receptor (removal of Lys370-Ser425 in the third intracellular loop) significantly reduced activation of ERK1/2. This demonstrates the importance of phosphorylation of this receptor in activation of the pathway.

The AKAP scaffolding molecules represent dynamic platforms allowing the interaction of activated receptors with a variety of kinases and phosphatases to regulate signalling (see Malborn *et al.,* 2004 for full review). AKAP79 and AKAP250 were the first AKAPs shown to interact with membrane bound GPCRs bringing various kinases to the receptor. However, these AKAPs direct downstream signalling in different directions. AKAP250 is essential for recycling of the receptor whereas AKAP75 switches the signalling from the AC pathway to one involving mitogen-activated protein kinases (MAPK) (Wang *et al.,* 2006). The β_2-adrenoceptor interacts with AKAP79, PKA and PKC allowing phosphorylation and regulation of signalling (Fraser *et al.,* 2000). In the β_2-adrenoceptor, the recognition motifs for AKAP79 and AKAP250 (Arg329 to Leu413 domain) are found in the ICLIII and C-terminal tail (Fan *et al.,* 2001). AKAP79 also facilitates phosphorylation by PKA of GRK2, limiting GRK2 activity. This leads to an increase of

Gβγ-dimer binding to GRK2, enhancing translocation of the kinase to the membrane and increasing receptor phosphorylation.

There are two major pathways by which receptors can be internalised: one via clarthrin-coated pits and the other by calveolae (Anderson, 1998, Grady *et al.,* 1997, Koenig and Edwardson, 1997, Okamoto *et al.,* 1998). The molecular mechanisms for sequestration of agonist-occupied GPCRs, to clathrin-coated pits, has been best studied for the β_2-adrenoceptor and involves phosphorylation by GRKs. Phosphorylated receptors bind β-arrestin, which then targets the receptors to clathrin-coated pits (see section 1.4.1). Caveolae are small invaginations of the plasma membrane containing high levels of cholesterol, glycosphingolipids and caveolin (a 20–24kDa integral membrane protein) (Anderson, 1998, Okmaoto *et al.,* 1998). Caveolae are specialised plasma membrane microdomains which contain the receptor, G-protein and proteins involved in formation and budding, such as dynamin and are routes for internalisation in some circumstances.

1.4.3. Resensitisation of receptors

Resensitisation of GPCRs involves endocytosis, the dephosphorylation of receptors by protein phosphatases (PPs) in endocytic vesicles, and receptor recycling to the plasma membrane. The stability of the GPCR-β-arrestin complexes seems to dictate the fate of the receptor, with GPCRs such as the α_{-1B} adrenoceptor and dopamine D_1 receptor binding β-arrestin-2 with a higher affinity than β-arrestin-1 (Oakley *et al.,* 1999, Oakley *et al.,* 2000, Perry and Lefkowitz, 2002). These receptors undergo rapid dissociation of the receptor/β-arrestin-2 complex in endosomes and rapid recycling to the plasma membrane. Other GPCRs including the AT_{1A} angiotensin II receptor and NK_1 neurokinin receptor have

similar affinities for the two β-arrestins which also dissociate more slowly, therefore recycling to the cell membrane at a slower rate (Oakley *et al.,* 1999, Oakley *et al.,* 2000, Perry and Lefkowitz, 2002). The rate of recycling of a receptor therefore depends on the rate of dissociation of the receptor/β-arrestin complex.

1.5. GPCR crosstalk

Cells express many different types of receptor and at any one time several of these receptors types can be activated by the same or different ligands. These stimulated receptors activate effector molecules and downstream pathways leading to specific outcomes for the cell. However, crosstalk between these pathways can lead to an increase or decrease in signalling or completely alter the pathway activated by interactions between the G-proteins or the effector molecules within the transduction pathway (Cordeaux and Hill, 2002). Crosstalk can have an effect on the specificity of the signal generated within the cell, altering the cellular outcome and potentially altering the physiology of the tissue and providing new therapeutic targets. For example, in a rat aorta smooth muscle cell line (A7r5), Arg^8-vasopressin stimulates release of Ca^{2+} from intracellular stores via the V_{1A} receptor and inhibits ACIII activity (Dyer *et al.,* 2005). This inhibition occurs because $Ins(1,4,5)P_3$-evoked Ca^{2+} activates Ca^{2+}-calmodulin dependent protein kinase II which phosphorylates ACIII, preventing any further signalling by the cAMP pathway (Dyer *et al.,* 2005). Heterologous desensitisation, as demonstrated in this example, is the most common outcome where crosstalk leads to a decrease in signalling.

Crosstalk can also enhance or reveal signalling. This thesis focuses on crosstalk between GPCRs which causes enhanced intracellular Ca^{2+} signalling. The regulation of intracellular Ca^{2+} is important because Ca^{2+} is highly versatile and regulates different cellular functions. For example, at synaptic junctions Ca^{2+} triggers exocytosis within milliseconds whereas to activate gene transcription, leading to outcomes such as increased proliferation, Ca^{2+} signalling must be sustained for minutes to hours. The Ca^{2+} concentration in the cell is controlled by a balance in the mechanisms between Ca^{2+} influx and removal. The removal can be both into intracellular stores via Ca^{2+}-ATPase pumps and out of the cell via Ca^{2+} exchange proteins e.g. the N^{+}/Ca^{2+} exchanger and the plasma-membrane Ca^{2+}-ATPase pumps. To increase the intracellular Ca^{2+} concentration in the cell, cytoplasmic Ca^{2+} can be released through $Ins(1,4,5)P_3$ and ryanodine receptors from internal stores (Berridge *et al.,* 2003). This initial release of Ca^{2+} can then lead to Ca^{2+}-induced Ca^{2+}-release (CICR), a mechanism by which cytoplasmic Ca^{2+} activates the release of further Ca^{2+} from intracellular stores via $Ins(1,4,5)P_3$ and ryanodine receptors. Further CICR occurs after plasma membrane depolarisation has activated voltage-gated Ca^{2+} channels, leading to influx of extracellular Ca^{2+} (Berridge *et al.,* 2003). Depletion of the intracellular Ca^{2+} stores causes capacitative Ca^{2+} entry, into the cell, via a mechanism involving the protein stromal interaction molecule (STIM). Other channels can be involved in Ca^{2+} entry into the cell and include receptor operated channels such as N-methyl-D-aspartate (NMDA) and nicotinic acetylcholine (nACh) receptors. Differences in the amplitude, frequency and pattern of Ca^{2+} signalling can alter the outcome of the cell and these aspects of Ca^{2+} signalling can be regulated by crosstalk (Di Capite *et al.,* 2009, Iino, 2007, Berridge, 1997, Berridge *et al.,* 2003). For example, in mouse parotid acinar cells activation of AC by forskolin, to elevate cAMP levels, synergistically increases the carbachol-mediated intracellular Ca^{2+} response

and converts oscillatory Ca^{2+} signals into a sustained response (Bruce *et al.,* 2002). The mechanism involves PKA phosphorylation of Ins(1,4,5)P_3 type 2 receptors and results in enhanced fluid secretion and exocytosis. In HEK 293 cells the recombinantly expressed $G\alpha_i$-coupled chemokine receptor, CXCR2, can only elevate intracellular Ca^{2+} in the presence of an activated $G\alpha_{q/11}$-coupled $P2Y_2$ nucleotide receptor, which is endogenously expressed in these cells (Werry *et al.,* 2002). This increase in Ca^{2+} signalling is caused by enhanced PLC activity with the $G\beta\gamma$ subunits, from the CXCR2, increasing the $P2Y_2$ nucleotide receptor-mediated activation of PLC by $G\alpha_{q/11}$.

There are many mechanisms by which crosstalk between GPCRs, coupled to different G-proteins, can alter intracellular Ca^{2+} signalling. These mechanisms are discussed in the following sections (see Werry *et al.,* 2003 for review).

1.5.1. Crosstalk between $G\alpha_s$ and $G\alpha_i$-coupled receptors

Activation of $G\alpha_s$- and $G\alpha_i$-coupled receptors activate and inhibit the AC pathway respectively, regulating cAMP levels and therefore downstream molecules within the pathway. To date nine isoforms of mammalian AC have been discovered all of which are activated by $G\alpha_s$ (see section 1.3). In HEK 293 cells stably expressing the $G\alpha_i$–coupled muscarinic M_2 receptor, a synergistic increase in cAMP was seen. This enhanced signalling occurred when the cells were pre-incubated with carbachol, to activate muscarinic M_2 receptors and then treated with either forskolin to elevate cAMP or prostaglandin to activate $G\alpha_s$-coupled prostaglandin-E_1 receptors (Thomas and Hoffman, 1996). This enhanced signalling was brought about by the interaction of $G\beta\gamma$-dimer, from the

muscarinic M_2 receptor, with ACVI. In HEK 293 cells stably expressing ACII and $G\alpha_i$–coupled dopamine D_{2L} or D_4 receptors, when either of these receptors were activated, in the presence of activated $G\alpha_s$-coupled β_2-adrenoceptors, ACII activity was potentiated (Watts and Neve, 1997). The potentiation was caused by $G\beta\gamma$-dimers, released from the dopamine receptors, enhancing AC activity. However, $G\beta\gamma$ can only affect ACII in the presence of an activated $G\alpha_s$-coupled receptor (Taussig *et al.,* 1993). There is also evidence to suggest that AC can be regulated by other proteins including kinases, calmodulin and Ca^{2+} (Cooper *et al.*, 1995, Cooper, 2003, Soderling *et al.,* 1999). With AC having the potential to be regulated by these other proteins it is possible for crosstalk to occur between receptors activating AC and receptors able to release signalling molecules.

cAMP causes cellular effects through downstream proteins including PKA and Epac (Ponsioen *et al.,* 2004). The specificity of this signalling is thought to occur via spatial regulation and the use of protein scaffolds. This allows cAMP to activate PKA or Epac, only within a localised area, with the help of AKAPs (see sections 1.3 and 1.4.2) (Wang *et al.,* 2006). Second messenger-dependent protein kinases such as PKA and PKC can also regulate AC activity. In HEK 293 cells transfected with ACII, activation of PKC by either $G\alpha_{q/11}$-coupled receptors or phorbol esters facilitates the activity of this AC stimulation by $G\alpha_i$-coupled receptors (Tsu and Wong, 1996). PKC has also been shown to potentiate ACVI activity after sensitisation by $G\alpha_i$-coupled D_{2L} dopamine receptors (Beazely and Watts, 2005).

1.5.2 Crosstalk between $G\alpha_{q/11}$ and $G\alpha_i$-coupled receptors

There are many examples of crosstalk involving $G\alpha_{q/11}$- and $G\alpha_i$-coupled receptors particularly with G-protein modulation of PLC activity (see Werry *et al.,* 2003 for full review). For example, in CHO cells stably transfected with the $G\alpha_i$-coupled adenosine A_1 receptor, a synergistic increase in [^{3}H]-inositol phosphate formation (as an index of PLC activity) was observed on activation of both this receptor and the endogenously expressed $G\alpha_{q/11}$-coupled CCK_A receptor (Dickenson and Hill, 1996). As this mechanism involves enhanced PLC activity it is likely that increased Ca^{2+} signalling also occurs. Such synergism may occur as $G\alpha_{q/11}$ and the $G\beta\gamma$-dimer (from the $G\alpha_i$-coupled receptor) interact with PLC at different sites (Lee *et al.,* 1993, Suh *et al.,* 2008). Indeed, PLCβ3 has been shown to account for synergistic signalling between $G\alpha_{q/11}$- and $G\alpha_i$-coupled receptors (Philip *et al.,* 2010). The synergy involves a two-state allosteric mechanism (between active and inactive states) with the synergistic state enhancing the active state of PLCβ3 when both receptor ligands are bound. The synergism requires a low level of basal activity in the absence of ligand and only becomes significant when the activity level rises about 0.1% of the maximal response. This synergy is unique to PLCβ3 and does not occur with PLC$\beta_{1,2}$ and PLCβ4 (Philip *et al.,* 2010). This mechanism is potentially highlighted in RAW264.7 cells, where synergy between the $G\alpha_i$-coupled C5a receptor and $G\alpha_{q/11}$-coupled $P2Y_6$ receptor is mediated by enhanced PLCβ3 activity and does not involve either PLCβ_2 or PLCβ_4 (Roach *et al.,* 2008).

The pleckstrin homology domain and EF hand motif of PLC has a high affinity for the $\beta\gamma$-dimer (Kuang *et al.,* 1996, Wang *et al.,* 1999). In HEK 293 cells, PLC activity is enhanced

by the Gβγ-dimer released from the recombinant $G\alpha_i$-coupled CXCR2 receptor, after activation of PLC by the endogenous $G\alpha_{q/11}$-coupled $P2Y_2$ receptor (Werry *et al.,* 2002). In COS-7 cells, $G\alpha_{q/11}$-mediated PLC-β activation was augmented on stimulation of the $G\alpha_i$-coupled δ and k-opioid receptors (Chan *et al.,* 2000). This synergistic increase in PLC activity was reduced by pertussis toxin (a compound which catalyzes ADP-ribosylation of $G\alpha_i$ and prevents G-protein interaction with the GPCR) again highlighting the involvement of the Gβγ-dimer from the $G\alpha_i$–coupled opioid receptors. The involvement of the Gβγ-dimer in crosstalk has also been shown in COS cells over-expressing Gβγ-dimers. Thus, agonist-stimulated cells transfected with the Gβγ-dimer showed a synergistic increase in the activity of PLC-β when comparing to either agonist- or Gβγ-mediated PLC activation (Zhu and Birnbaumar, 1996, Quitterer *et al.,* 1999). This mechanism of enhanced PLC activity has been demonstrated in a variety of cells. For example, in rabbit ciliary body epithelium cells, crosstalk between $G\alpha_{q/11}$-coupled muscarinic receptors and $G\alpha_i$-coupled α_2-adrenoceptors synergistically increased total [^{3}H]- InsPx accumulation (Cilluffo *et al.,* 2000). An increase in intracellular Ca^{2+} was also observed as a consequence of the synergistic increase in PLC activity. Both the $G\alpha_i$-coupled $GABA_B$ and $G\alpha_{q/11}$-coupled $mGluR1_A$ receptors are expressed in mouse purkinje cells and co-activation of the two receptors result in a synergistic increase in PLC activity as a consequence of $G\alpha_{q/11}$ and Gβγ activation of PLC (Rives *et al.,* 2009).

Alternative mechanisms of crosstalk between $G\alpha_{q/11}$- and $G\alpha_i$-coupled receptors have been suggested that do not directly increase activation of PLC. For example, inositol phosphate generation mediated by crosstalk between $G\alpha_{q/11}$-coupled P2Y or bradykinin B_2 receptors and either the α_{2C}-adrenoceptor or A_1 adenosine receptor was driven by βγ-dimers of the

latter $G\alpha_i$-coupled receptors but did not involve increased PLC activity (Quitterer *et al.,* 1999). The Gβγ-dimer was suspected to promote Gα activation, therefore accelerating $G\alpha_{q/11}$-coupled receptor stimulation of PLC. The mechanism for this is unclear but the free Gβγ-dimers from $G\alpha_i$-coupled receptors could bind to excess $G\alpha_{q/11}$(GDP) subunits, producing heterotrimeric G-proteins able to re-associate with receptors (Gerwins *et al.,* 1992, Tomura *et al.,* 1997). Gβγ-dimers have also been shown to directly sensitise $Ins(1,4,5)P_3$ receptors increasing Ca^{2+} signalling or mediating Ca^{2+} oscillations rather than a sustained signal (Neylon *et al.,* 1998, Xu *et al.,* 1996, Zeng *et al.,* 1996). The exact mechanism is still to be elucidated. However a direct association between Gβγ-dimers and $Ins(1,4,5)P_3$ receptors has been suggested. Indeed, in aortic smooth muscle cells from adult Wistar Kyoto rats, $G\alpha_i$ and $Ins(1,4,5)P_3$ receptors were co-purified from microsomal membrane fractions (Neylon *et al.,* 1998). Pertussis toxin treatment of the cells attenuated the rate of $Ins(1,4,5)P_3$ receptor stimulated Ca^{2+} release, again suggesting a role for Gβγ-dimers.

1.5.3 Crosstalk between $G\alpha_{q/11}$ and $G\alpha_s$-coupled receptors

Of most relevance to this thesis is crosstalk between $G\alpha_{q/11}$ and $G\alpha_s$-coupled receptors and there are a variety of examples of such crosstalk within the literature. For example, P2Y-mediated Ca^{2+} responses are potentiated by $G\alpha_s$-coupled A_{2B} receptors in type-1 cultured rat astrocytes (Jimenez *et al.,* 1999). The mechanism appears to involve Gβγ-dimers released from $G\alpha_s$ as neither forskolin nor dibutyryl-cAMP elicit the crosstalk.

Mechanisms of crosstalk between $G\alpha_{q/11}$ and $G\alpha_s$-coupled receptors have also been described that are independent of $G\beta\gamma$ enhancement of PLC activity. For example, PKA has been shown to enhance $Ins(1,4,5)P_3$ receptor-mediated release of intracellular Ca^{2+}. Thus, in mouse parotid acinar cells, forskolin potentiates carbachol-evoked increases in Ca^{2+} (Bruce *et al.,* 2002). Forskolin was also able to convert oscillatory Ca^{2+} signals into sustained signals and allow sub-threshold concentrations of carbachol to signal through Ca^{2+}. This potentiation was independent of Ca^{2+} entry or increased $Ins(1,4,5)P_3$ levels, therefore enhanced PLC activity was not involved. $Ins(1,4,5)P_3$ type 2 receptors were shown to be directly phosphorylated by PKA therefore enhancing the release of intracellular Ca^{2+}. $Ins(1,4,5)P_3$ receptors have been shown to be phosphorylated by PKA at serine 1755 and 1589 (Ferris *et al.,* 1991, Furuichi *et al.,* 1989). Phosphorylation of $Ins(1,4,5)P_3$ receptors has also been shown as the mechanism of increased intracellular Ca^{2+} release in other cell types (Burgess *et al.,* 1991, Hajnoczky *et al.,* 1993).

PKC also has a role in crosstalk between some $G\alpha_{q/11}$ and $G\alpha_s$-coupled receptors, although the outcome of such crosstalk is not enhanced intracellular Ca^{2+} signalling. For example, PKC is able to activate certain isoforms of AC (ACII, IV and VII) (Hanoune and Defer, 2001). In RAW 264.7 mouse macrophage cells PKCε and/or μ potentiates AC activity following treatment with phorbol ester to activate PKC (Lin and Chen, 1998). Several groups have also demonstrated that phorbol ester treatment can increase AC activity (Cumbay and Watts, 2005, Frings, 1993, Jacobowitz *et al.,* 1993, Jacobowitz *et al.,* 1994, Lustig *et al.,* 1993). In Sf9 insect cells, PKCα increases both the basal activity of ACII and its responsiveness to G-protein subunits (Zimmerman and Taussig, 1996). The activation of PKC by a GPCR, rather than phorbol esters, has also been shown to enhance AC activity.

Thus, addition of UTP to J774 mouse macrophage cells caused PKC-mediated phosphorylation and enhanced activation of ACII after stimulation of the $G\alpha_s$-coupled prostaglandin E_1 receptor (Lin and Chen, 1997). These examples demonstrate that the nature of the PKC isoform involved in crosstalk with AC can differ.

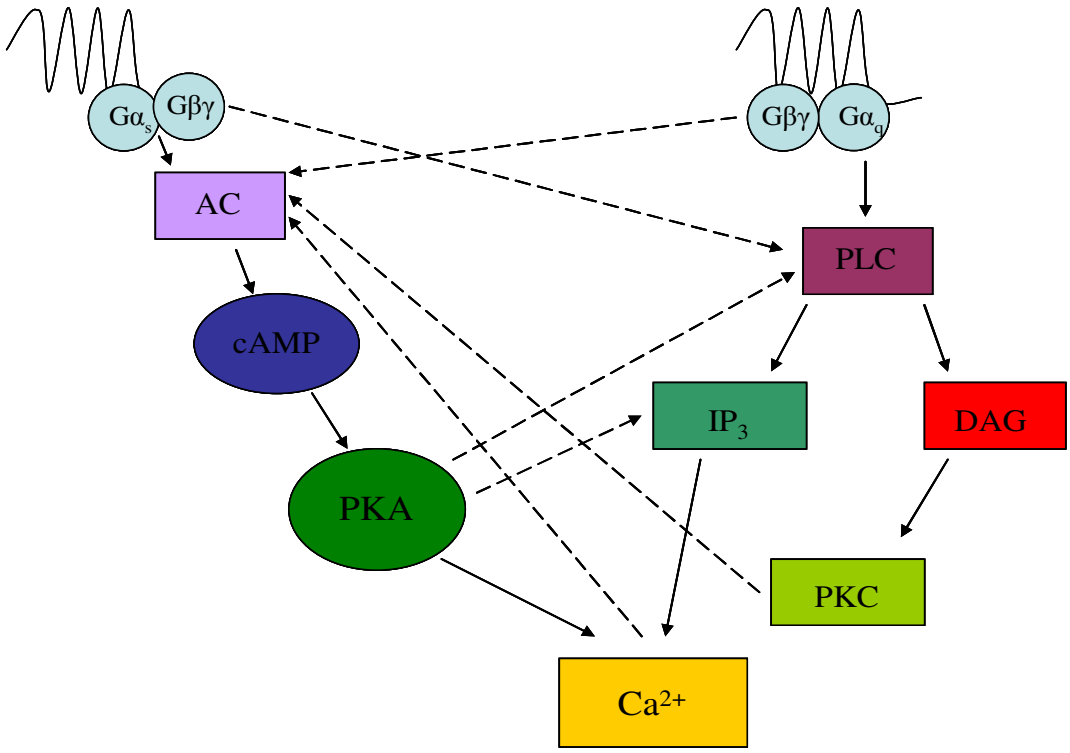


Figure 1.6. Potential mechanisms of crosstalk between $G\alpha_{q/11}$- and $G\alpha_s$-coupled receptors. Solid arrows demonstrate the pathways activated by each receptor and the dashed arrows indicate the crosstalk mechanisms at each stage of the pathways (see text for details).

1.5.4 Pharmacological relevance and therapeutic potential of crosstalk

Crosstalk demonstrates a method of fine tuning cellular functions and is an important factor when understanding diseases and their therapeutic control. Enhanced gene expression has

been demonstrated as an outcome of synergistic signalling between GPCRs. For example, in rat osteoblast cells, activation of the $G\alpha_s$-coupled parathyroid hormone (PTH) receptor potentiates intracellular Ca^{2+} signalling from nucleotide stimulation of the $G\alpha_{q/11}$-coupled $P2Y_1$ receptor (Buckley *et al.,* 2001). Furthermore, co-stimulation of these receptors increases the phosphorylation of the cAMP response element-binding protein (CREB) and enhanced c-Fos gene expression in a synergistic manner. This crosstalk may have important implications for PTH-induced signalling in bone, highlighting a physiological or therapeutic relevance to such crosstalk. Importantly this example illustrates that crosstalk can alter gene expression. By activating different receptors or combinations of receptors it may therefore be possible to alter gene expression.

GPCR crosstalk depends on co-expression of the receptors in the tissue or cell. Examples of crosstalk have been shown in recombinant systems between receptors known to be co-expressed in physiologically/therapeutically relevant cell types including muscarinic M_3 receptors and β_2-adrenoceptors. These receptors are co-expressed on airway smooth muscle and control contraction and relaxation of the tissue respectively (Billington and Penn, 2002). It is therefore important for diseases including asthma and chronic obstructive pulmonary disease (COPD) that the signalling patterns between these two receptor types are understood.

1.6 Muscarinic receptors

Muscarinic receptors are members of the Family A GPCR superfamily. Their endogenous ligand is acetylcholine which is released predominantly from pre- and postganglionic

parasympathetic fibres and preganglionic sympathetic fibres. Acetylcholine mediates actions through muscarinic receptors and also nicotinic receptors, which are ligand gated ion channels (Caulfield and Birdsall, 1998, Wess, 2004). Muscarinic receptors are expressed on both the pre- and post-synaptic membranes found throughout the central nervous system (Caulfield and Birdsall, 1998). They mediate physiological functions such as muscle contraction, appetite, cardiovascular actions and memory. Alterations in muscarinic receptor expression and signalling are involved in a number of disease states including Alzheimer's disease (Caulfield and Birdsall, 1998, Eglen *et al.,* 1999, Wess *et al.,* 1993).

The muscarinic family contains five distinct subtypes, M_1, M_2, M_3, M_4 and M_5, (Caulfield and Birdsall, 1998, Luo *et al.,* 2008). All five of these receptors have been cloned with M_1 and M_2 subtypes being replicated by comparing the cDNA library from porcine cerebral and cardiac tissues (Kubo *et al.,* 1986). The M_3, M_4 and M_5 receptors were cloned by screening genomic and cDNA libraries from brain tissue (Bonner *et al.,* 1987, Bonner *et al.,* 1988, Peralta *et al.,* 1987). All of the muscarinic subtypes contain conserved prolines in their TM domains IV, V, VI and VII, which control the conformation of the receptor and changes occurring on ligand binding. They also have conserved cysteine residues in extracellular loops I and II which form disulfide bonds, stabilising the receptor and allowing activation and cell surface expression (Hulme *et al.,* 1990). A DRY motif between the third TM domain and second intracellular loop has also been demonstrated as important for the activation of the signalling pathway (Hulme *et al.,* 1990, Kurtenbach *et al.,* 1990, Zhu *et al.,* 1994). Therefore, finding agonists and antagonists which have high degrees of selectivity for the different subtypes is difficult. There are differences in the number of

amino acids that the receptors contain with M_1 having the smallest number (460) and the muscarinic M_3 receptor having the largest number (590). They all contain 149 conserved amino acids mainly in the transmembrane domains.

Muscarinic receptors are often co-expressed in tissues (see Table 1.2). However, the predominant subtype does not always elicit the most obvious physiological effects on the tissue. For example, in smooth muscle cells, although the muscarinic M_2 receptor is the predominant subtype (ratio 4:1 with M_3), contractions occurs following agonist addition predominantly through muscarinic M_3 receptors (Eglen *et al.,* 1994, Ehlert *et al.,* 1999).

The muscarinic family show 26.3% amino acid homology with most variability within the intracellular loops (Challiss and Tobin, IUPHAR (http://www.iuphar-db.org/DATABASE/FamilyIntroductionForward?familyId=2), 2009). The third intracellular loop shows most variability, with 2.7% identity between receptors compared to 66% in the most conserved TM domains. The ligand-binding sites of all the muscarinic receptor subtypes are very similar, involving TM domains III, V, VI and VII. On stimulation of muscarinic receptors, G-proteins are activated with M_2 and M_4 preferentially coupling to $G\alpha_{i/o}$ and M_1, M_3 and M_5 subtypes coupling to $G\alpha_{q/11}$ (Wess, 2004).

Muscarinic receptor subtype	Tissue where subtype expressed
M_1	forebrain, striatum, hippocampus, cerebral cortex
M_2	cardiac, smooth muscle, glandular function, medial septum
M_3	brain, smooth muscle, exocrine glands, cardiac
M_4	cortex, striatum, hippocampus, neuronal
M_5	brain stem nuclei, eye, hippocampus

Table 1.2. Tissue distribution of muscarinic subtypes from mRNA and immunoprecipitation studies of the receptor proteins (Hulme *et al.,* 1990, Wess, 2004)

The muscarinic receptors can undergo post-translational modifications with each receptor containing between two and five N-glycosylation sites (Asn-X-Ser/Thr) in the N-terminal sequence (Habecker *et al.,* 1993). However, site-directed mutagenesis studies of the M_2 receptor demonstrated that glycosylation was not required for either receptor expression or function (van Koppen and Nathanson, 1990). Treatment of the M_3 receptor with glycosidases also had little effect on the binding of the muscarinic antagonist [^{3}H]-3-quinuclidinyl benzilate (QNB) (Herron and Schimerlik, 1983). Cysteines in the C-terminus of the M_2 receptor have been shown to be palmitoylated (Hayashi and Haga, 1997). These receptors showed enhanced function but the palmitoylation was not required for interaction with G-proteins.

1.6.1 Muscarinic M_3 receptor

Muscarinic M_3 receptors are widely distributed in the central nervous system, exocrine glands and on smooth muscle (Felder *et al.,* 2000, Wess *et al.,* 2003, Wess, 2004). Muscarinic M_3 receptors have been shown to mediate smooth muscle contraction in tissues and organs including that of the airway, gastrointestinal tract and bladder (Eglen *et al.,*

1994, Wess *et al.,* 2004). The expression of this receptor has also been indicated in the heart, atria and in cardiac neurons controlling contraction of these tissues (Hassall *et al.,* 1993, Wang *et al.,* 2001, Wess, 2004). Molecular cloning studies in humans and rats distinguished five distinct genes for the five muscarinic sub-types (Section 1.6). In humans the muscarinic M_3 gene is found on chromosome 1 whereas in mice is found on chromosome 3 (Forsythe *et al.,* 2002, Matsui *et al.,* 2000). To determine the physiological roles of muscarinic M_3 receptors, knockout mice have been generated (Matsui *et al.,* 2000, Yamada *et al.,* 2001). Although knockout mice were the same weight as wild-type litter mates at birth, after 2-3 weeks they were significantly leaner and had a reduced appetite (Yamada *et al.,* 2001, Wess *et al.,* 2003). Smooth muscle function was also impaired in the knockout mice with animals having enlarged pupils, consistent with the lack of M_3 parasympathetic tone of the muscle (Matsui *et al.,* 2000). However, atropine still caused pupil dilation showing a function for other muscarinic subtypes in this tissue. Male knockout mice had expanded bladders, consistent with the lack of M_3-mediated muscle contraction, but for reasons unknown female mice did not suffer such distension (Matsui *et al.,* 2000). Carbachol caused a 40% reduction in the contraction of tracheal smooth muscle in the knockout mice compared to wild-type, again demonstrating a role for other muscarinic subtypes in the contraction (Stengel *et al.,* 2002). The M_3 receptor has also been implicated in cardiac function (Wang *et al.,* 2001). However, the knockout mice showed no difference in atrial rate compared to their wild-type litter mates. This lack of effect is a likely consequence of the M_2 subtype being the main subtype in the heart (Wang *et al.,* 2001).

1.6.2. Characterisation of muscarinic binding site

Agonist binding causes the receptor to undergo a conformational change (to R*) leading to the activation of a G-protein. For muscarinic receptors the binding occurs in a narrow cleft between the 7 TM domains approximately 10-15Å from the cell surface (Section 1.6.1) (Wess *et al.,* 1995). This core contains several conserved serine, threonine and tyrosine residues that interact with the ligand and act as hydrogen donors or acceptors (Wess *et al.,* 1991). Substituting the threonine and tyrosine residues (Y148F, T231A, T234A, Y506F, Y529F and Y533F) caused a 10-40 fold decrease in agonist affinity in wild-type receptors, with T231 and T234 being the most critical for agonist binding (Wess *et al.,* 1991). All of these residues are located at similar locations within the TM domains highlighting the location of ligand interaction. Another study using a mutant rat M_3 receptor showed that out of the 13 cysteine residues conserved across the muscarinic receptor subtypes, three are critically involved in ligand binding (Cys140, Cys220, Cys532) (Zeng *et al.,* 1999). The substitution of three non-conserved cysteines (Cys289, Cys310 and Cys419) in the third intracellular loop with alanine or lysine did not affect ligand binding or receptor function indicating that these residues are not critical for G-protein coupling.

Other more recent studies have shown agonist-induced conformational changes in the muscarinic M_3 receptor using *in situ* disulfide cross-linking experiments, after substitution of cysteine residues (Han *et al*., 2005). Agonist binding caused a disulfide bond to form between Ser151 and Cys532 suggesting the TM domains III and VII move towards one another on receptor activation. Mutational analysis of residues situated between TMVI and the third intracellular loop demonstrated the ligand-dependent switch action of TMVI (Ford *et al.,* 2002). Mutating the asparagine-threonine to tyrosine-proline between TMVI and the

third intracellular loop led to constitutive activity of the M_3 receptor and increased the efficacy of carbachol and acetylcholine by 15-20 fold (Ford *et al.,* 2002). This study suggests that interactions between TMVI and the third intracellular loop stabilise the R* conformation of the receptor.

N113 in TMII of the muscarinic M_3 receptor is important for coupling to the G-protein (Li *et al.,* 2005).When the point mutation D113N is present, the receptor cannot signal. However, when this mutation is expressed alone with either of the point mutations R165W, R165M or Y250D, in COS-7 cells, the function of the receptor is restored (Li *et al.,* 2005). It is therefore likely that Asp113, Arg165 and Tyr250 all interact to convert the inactive receptor into its active conformation.

1.6.3. Signalling via the muscarinic M_3 receptor

Muscarinic M_3 receptors preferentially couple to the pertussis toxin-insensitive $G\alpha_{q/11}$-coupled G-protein (Caulfield, 1993, Billington and Penn, 2002). This G-protein coupling can be ligand-specific. In CHO cells recombinantly expressing one of the muscarinic subtypes ($M_{1\text{-}5}$), distinct G-protein activation profiles were observed which were dependent on the receptor subtype (Akam *et al.,* 2001). Maximal [^{35}S]-GTPγS binding to stimulated $G\alpha_{q/11}$-coupled muscarinic M_1 or M_3 receptors occurred within 2min. The increases in $G\alpha_{q/11}$-coupled [^{35}S]-GTPγS binding after muscarinic M_1 or M_3 receptor stimulation differed substantially, with muscarinic M_1 receptors causing a 2- to 3-fold greater increase in [^{35}S]-GTPγS binding and requiring 5-fold lower concentrations of methacholine to stimulate an EC_{50} response (Akam *et al.,* 2001). The receptors can also be promiscuous,

particularly in recombinant systems. For example, cells expressing muscarinic M_3 receptors at physiological levels have shown coupling to pertussis toxin-sensitive $G\alpha_{i/o}$ using subtype-specific immunoprecipitation of Gα-subunits labelled with [α-^{32}P] GTP azidoanilide (Laugwitz *et al.,* 1994). Further, in CHO cells transfected with the human muscarinic M_3 receptor, carbachol stimulation increased the accumulation of cAMP (Burford *et al.,* 1995). The precise mechanisms of the signalling were unknown but involved AC activity and not the inhibition of phosphodiesterases, with a suggestion that M_3 receptors were able to directly couple to $G\alpha_s$-proteins. In HEK 293 cells, PLC activation and Ca^{2+} signalling via muscarinic M_3 receptors has also been shown to be mediated by both $G\alpha_{q/11}$- and $G\alpha_s$-coupled G-proteins (Evellin *et al.,* 2002). Thus, treatment with the AC inhibitor 2',5'-dideoxyadenosine (dd-Ado) significantly reduced carbachol-mediated PLC activity and Ca^{2+} signalling. PLC activation was shown to be partly cAMP-dependent with the $G\alpha_{q/11}$-coupled pathway activating PLC-β1 and the $G\alpha_s$-coupled pathway increasing PLC-ε activity (Evellin *et al.,* 2002). The stimulation of PLC-ε was thought to be via Epac1 and the GTPase, Rap2B.

Muscarinic receptors can also activate phospholipase D (PLD), via a tyrosine-kinase-dependent pathway, catalysing the hydrolysis of phosphatidylcholine to choline and phosphatidic acid (PA) (Mamoon *et al.,* 2004, Sandmann *et al.,* 1991). PA can be further converted by phospholipase A2 or phosphatidic acid phosphohydrolase to DAG or lysophosphatidic acid, respectively (Mamoon *et al.,* 2004). This pathway is therefore important for forming second messengers distinct from phosphoinositides. In HEK 293 cells stably expressing the muscarinic M_3 receptor, carbachol can potently activate PLD but the mechanism for this is poorly understood (Sandmann *et al.,* 1991). The tyrosine kinase

inhibitor genistein and the PKC inhibitor calphostin C inhibited PLD1 suggesting that PKC and tyrosine kinase activities may be involved (Mamoon *et al.,* 2004). The physiological functions of PLD are not well understood.

1.6.4. Regulation of muscarinic signalling

Following agonist stimulation of the muscarinic M_3 receptor rapid, agonist-dependent phosphorylation occurs via several kinases including GRKs, CK1α and PKC (Budd *et al.,* 2000, Willets *et al.,* 2002, Willets *et al.,* 2003, Tobin *et al.,* 1997, Tobin *et al*., 2008) (see also section 1.4). GRK phosphorylation and arrestin binding is a well established model of desensitisation of GPCRs (section 1.4.1). In SH-SY5Y neuroblastoma cells, GRK6 has been highlighted as particularly important for agonist-mediated phosphorylation of the muscarinic M_3 receptor (Willets *et al.,* 2002). Thus, the dominant negative ^{K215R}GRK6 inhibited methacholine-stimulated phosphorylation and uncoupled the receptor from its G-protein by 50% (Willets *et al.,* 2002). A dominant negative GRK5 mutant had no affect on muscarinic M_3 receptor phosphorylation demonstrating that GRK6 mediates at least some of the desensitisation in SH-SY5Y cells (Willets *et al.,* 2002). Further, neither GRK2, GRK3 nor CK1α had a role in agonist-specific muscarinic M_3 receptor phosphorylation (Willets *et al.,* 2003). However, in HEK 293 and COS-7 cells expressing a catalytically inactive form of CK1α, agonist-mediated phosphorylation of the muscarinic M_3 receptor was reduced by approximately 40% (Budd *et al.,* 2000). In this study, the functional role of receptor phosphorylation was also investigated using a mutant of the receptor (containing a deletion between Lys370-Ser425 of the third intracellular loop) that showed an 80% reduction in agonist-mediated phosphorylation. Perhaps surprisingly, the phosphorylation-

deficient receptor still underwent desensitisation (Budd *et al.,* 2000). Agonist-induced internalisation of the muscarinic M_3 receptor has been shown to be arrestin-independent (Lee *et al.,* 1998). Thus, the over-expression of arrestin had no affect on agonist-induced internalisation (Lee *et al.,* 1998). However, when dynamin-dependent endocytosis was blocked the muscarinic receptor could not internalise. Thus, the muscarinic M_3 receptor, at least in these cells, uses a dynamin-dependent pathway to internalise (Lee *et al.,* 1998). This finding has been reinforced by more recent studies with muscarinic M_3 receptors in SK-H-SH neuroblastoma cells in which the receptor co-immunoprecipitates with Gβγ, tubulin and clathrin, in carbachol-stimulated cells (Popova and Rasenick, 2004). These data suggest that Gβγ may allow the formation of a complex linking muscarinic M_3 receptors with tubulin and therefore reinforcing the idea of dynamin-dependent and arrestin-independent endocytosis of muscarinic M_3 receptors.

1.7 β-adrenoceptors

The catecholamines adrenaline and noradrenaline mediate their effects through adrenoceptors. They are released from the central nervous system and postganglion sympathetic fibres with adrenaline predominantly released from the adrenal medulla. These catecholamines affect tissues with sympathetic innervation and those with limited or no sympathetic innervation (e.g. lungs) (Civantos *et al.,* 2001). The first attempt to classify the adrenoceptors was made in 1948 in a study by Ahlquist (Bylund, 2007, Civantos *et al.,* 2001). This study was the first to use the closely related amines noradrenaline, methyl-noradrenaline, adrenaline, methyl-adrenaline and isoproterenol to examine functions in tissue from dogs, cats, rats and rabbits (reviewed in Civantos *et al.,* 2001). This group of

amines had the same rank order of potencies for controlling particular outcomes in different organisms and these data suggested that these functions were controlled by the same receptor, which was termed the 'α' adrenoceptor. However, these same amines had different rank orders of potencies when controlling different responses, highlighting that these responses were mediated through a different receptor, which was named the 'β' adrenoceptor. Following further studies, the α-adrenoceptors have been further divided into α_1-adrenoceptors (α_{1A}-, α_{1B} and α_{1D}-adrenoceptors), which are $G\alpha_{q/11}$-coupled and α_2-adrenoceptors ($\alpha_{2A/D}$-, α_{2B}- and α_{2C}-adrenoceptors), which are $G\alpha_{i/o}$-coupled (Civantos *et al.,* 2001). β-adrenoceptors have been divided into three different subtypes, the β_1-, β_2- and β_3-adrenoceptors, which are all coupled to $G\alpha_s$ (Strosberg, 1998). All of these adrenoceptors are Family A GPCRs.

β-adrenoceptors are involved in cardiovascular, respiratory, smooth muscle and neuronal functions. For example, β_2-adrenoceptors cause relaxation of airway smooth muscle through PKA-mediated phosphoryation of myosin light chain kinase (MLCK), therefore decreasing its affinity for calcium-calmodulin and myosin light chain (MLC) phosphorylation (Billington and Penn, 2002). The first non-visual mammalian GPCR to be cloned was the human β_2-adrenoceptor which is 413 amino acids long (Kobilka *et al.,* 1987). The β_2-adrenoceptor gene is intronless and has been shown to have a sequence homology of 87%, between the human and hamster receptor, with the carboxyl tails being most different (Kobilka *et al.,* 1987). Screening of a human placenta λgt11 library allowed the isolation of the β_1-adrenoceptor cDNA (Frielle *et al.,* 1987). This receptor is 477 amino acids in length (Strosberg, 1998). The β_3-adrenoceptor was identified by screening a human genomic library with the entire coding regions for the turkey β_1-adrenoceptor gene and the

human β_2-adrenoceptor gene (Emorine *et al.,* 1989). The human β_3-adrenoceptor gene is 402 amino acids and has a sequence homology of 50.7% and 45.5% with the human β_1-adrenoceptor and β_2-adrenoceptor respectively.

β-adrenoceptors are ubiquitously expressed in the human body (Table 1.3). Biochemical and quantitative studies have been carried out to localise the different receptor subtypes and determine their physiological roles.

β-adrenoceptor subtype	**Tissue where subtype expressed**
β_1	Heart, lung, cerebral cortex, anterior olfactory nucleus, pineal gland
β_2	Heart, lung, cerebral cortex, olfactory bulb, hippocampus, skeletal muscle, platelets, smooth muscle, bladder and liver
β_3	Brain, colon, adipose tissue and gall bladder

Table 1.3 Tissue distribution of β-adrenoceptors based on mRNA detection (Krief *et al.,* 1993, Nicholas *et al.,* 1993, Rodriguez *et al.,* 1995).

Although the β-adrenoceptors couple to $G\alpha_s$ they have been shown to promiscuously couple to $G\alpha_i$-proteins (Gauthier *et al.,* 1996). Both the β_2- and β_3-adrenoceptors have been shown to activate the ERK pathway by switching coupling from $G\alpha_s$- to $G\alpha_i$-proteins in several cell types including fibroblasts and adipocytes (Daaka *et al.,* 1997, Soeder *et al.,* 1999). After activation the receptor is rapidly phosphorylated by GRKs (see section 1.4).

β-adrenoceptors have been targeted therapeutically to treat several different diseases using both agonists and antagonists. Cardiomyocytes express both β_1- and β_2-adrenoceptors (β_1-adrenoceptors predominating) allowing both these receptor sub-types to be targeted in order to treat hypertension and angina following myocardial infarction and cardiac disrythmias (Wallukat, 2002). β_2-adrenoceptors have been targeted as treatments in asthma and COPD

leading to relaxation of airway smooth muscle (Giembycz and Newton, 2006). Agonist activation of the β_3-adrenoceptor has been shown to increase lipolysis and fat oxidation and presents a potentially useful target for the treatment of obesity (De Souza and Burkey, 2001).

1.7.1 The β_2-adrenoceptor

Smooth muscle contraction is often controlled via $G\alpha_q$-coupled receptors, such as muscarinic M_3 receptors, leading to the release of intracellular Ca^{2+}, which allows formation of Ca^{2+}-calmodulin complexes and the activation of MLCK Section 1.7). The kinase phosphorylates and therefore activates MLCs enabling actin to stimulate myosin ATPase, which is required for cross-bridge cycling and contraction. In contrast, β_2-adrenoceptors cause relaxation of smooth muscle via PKA-mediated phosphorylation of MLCK. This decreases the affinity of MLCK for Ca^{2+}-calmodulin, therefore reducing Ca^{2+}-calmodulin activity and MLC phosphorylation, allowing the smooth muscle to relax (Billington and Penn, 2003). The β_2-adrenoceptor (β_2-AR) has, therefore, great physiological relevance. Indeed receptor knockout studies have shown its role in cardiovascular, metabolic and reproductive functions. For example, β-adrenoceptors expressed on cardiac myocytes and cells of the sino-atrial node, control cardiac contraction (Devic *et al.,* 2001). Isoproterenol-mediated activation of the β_1-adrenoceptor (β_1-AR) in neonatal myocytes from β_2-AR-KO mice increased contraction via a PKA dependent pathway, whereas activation of the β_2-AR in cells from β_1-AR-KO mice decreased contraction in a PKA-independent manner (Devic *et al.,* 2001). Therefore, β_1-adrenoceptors and β_2-adrenoceptors couple to distinct pathways to control contraction in cardiac

myocytes. Mice with knock-out of all three β-adrenoceptor subtypes are obese and unable to maintain their body temperature highlighting the importance of β-adrenoceptors in resisting obesity and in temperature regulation (Jimenez *et al.,* 2002).

1.7.2 Ligand binding to the β_2-adrenoceptor

Deletion mutagenesis of the β_2-adrenoceptor showed that the ligand interacts with the hydrophobic core of the receptor and site-directed mutagenesis showed cysteine molecules on the extracellular loops to be crucial for ligand binding and receptor expression (Dohlman *et al.,* 1990, Strader *et al.,* 1994).

The study of ligand binding to the β_2-adrenoceptor has been possible with the development of the crystal structure for the receptor. In comparison to rhodopsin, the β_2-adrenoceptor has some key differences (Cherezov *et al.,* 2007). The β_2-adrenoceptor contains an unusual pair of disulfide bonds and a short helix in ECLII. This extra loop and the absence of structure in the N-terminal tail are important for ligand binding (Cherezov *et al.,* 2007, Hanson *et al.,* 2008, Rosenbaun *et al.,* 2007). The TM helices II, V, VI and VII all contain a proline-induced kink involved in structural rearrangement of the receptor, on agonist binding to allow activation of the G-protein (Yohannan *et al.,* 2004). Mutagenesis studies suggest that the binding pocket for agonists is deep within the β_2-adrenoceptor explaining why the extracellular loops between TM helices II and IV with VI and VII are short. There are two highly conserved cysteine residues in the second and third extracellular loops of the β_2-adrenoceptor (Cys106 and Cys184) which form disulphide bonds to stabilise the receptor structure (Cys106-Cys191 and Cys184-Cys190) (Cherezov *et al.,* 2007).

Substituting these residues with valine by site-directed mutagenesis destabilises the tertiary structure and alters binding of the ligand to the receptor.

The importance of serine residues in the β_2-adrenoceptor was highlighted using single and double mutants of Ser203, 204 and 207 in TMV (Del Carmine *et al.,* 2004). The affinities of six different catecholamine agonists with mutations in their ethanolamine tail were determined. The results demonstrated that as increasing numbers of interactions occurred between the receptor and ligand, the conformation of the receptor changed, showing the binding site not to be rigid (Del Carmine *et al.,* 2004). Biophysical studies with purified, fluorescently labelled β_2-adrenoceptors showed full and partial agonists stabilise the receptor in distinct conformational and functional states via different groups within the agonists (Ghanouni *et al.,* 2001, Swaminath *et al.,* 2004, Swaminath *et al.,* 2005). The three-dimensional crystal structure of the inactive state of the β_2-adrenoceptor bound to the inverse agonists carazolol and timolol has been recently solved (Cherezov *et al.,* 2007, Hanson *et al.,* 2008, Rasmussen *et al.,* 2007, Rosenbaum *et al.,* 2007). On binding of an agonist to the receptor, a conformational change occurs in the rotomer toggle switch (a conserved cluster on aromatic amino acids on TMVI, acting as a flexible hinge), breaking the polar interactions and allowing activation of the receptor (Shi *et al.,* 2002). Partial activation of the receptor can occur with partial agonists without activation of the toggle switch but for full agonism this change must occur. Ligand binding for β_2-adrenoceptors is mediated by polar and hydrophobic contacts between residues from TMII, V, VI and VII. Mutation of the highly conserved DRY motif in TMIII and VI increases constitutive activity of the receptor (Ballesteros *et al.,* 2001, Rasmussen *et al.,* 1999).

1.7.3 β_2-adrenoceptor signalling

β_2-adrenoceptors are one of the most studied GPCRs with the crystal structure of the human β_2-adrenoceptor recently being reported (Cherezov *et al.,* 2007). Deleting amino acids 267-273 in the third ICL prevents signalling of the receptor suggesting these residues are crucial for interaction with the G-protein (Hausdorff *et al.,* 1990).

β_2-adrenoceptors are co-expressed with muscarinic M_3 receptors in airway smooth muscle, controlling relaxation and contraction of the airway, respectively and this is of particular interest as the basis of this thesis is crosstalk between these two receptor types. The major effector of $G\alpha_s$ is activation of AC and in human airway smooth muscle cultures, RT-PCR has demonstrated the existence all AC subtypes except III and VIII, with isoform V being predominant (Billington *et al.,* 1999, Hanoune and Defer, 2001, Xu *et al.,* 2001). The isoforms share a large sequence homology in their catalytic sites and have the same predicted three dimensional structures with two hydrophobic domains and two cytoplasmic domains (Hanoune and Defer, 2001). The cytoplasmic domains, which make up the catalytic site, are subject to intracellular regulation by the $G\alpha_s$-subunit and also $G\alpha_i$ and $G_{\beta\gamma}$ subunits, Ca^{2+}, second messenger-dependent protein kinases (PKA, PKC and calmodulin), phosphatases (calcineurin) and Ca^{2+}-calmodulin (Hanoune and Defer, 2001). This demonstrates the regulation of AC by other molecules and effectors other than $G\alpha_s$. The AC isoform expressed in cells can be tissue- and species-specific. For example, in the mammalian heart, ACV and ACVI are predominantly expressed (Hu *et al.,* 2009). However, in the mammalian brain all nine isoforms of AC are present (Hanoune and Defer, 2001). Therefore AC isoforms can broadly be grouped by their regulation with Ca^{2+}-

calmodulin regulated AC isoforms including ACI, III and VIII, Ca^{2+}-inhibited isoforms being V and VI, Ca^{2+}-insensitive but PKC-stimulated isoforms including II, IV and VII and isoform IX being regulated by calcineurin (Cooper, 2003). Thus, Ca^{2+} can also regulate AC. In A7r5 smooth muscle cells, capacitative calcium entry (CCE) mediated by thapsigargin treatment, inhibits ACIII activity but Ca^{2+} entry via L-type channels or vasopressin mediated CCE did not have an affect on ACIII activity (Dyer *et al.,* 2005). Furthermore, when Ca^{2+} release occurred via Ins(1,4,5)P_3 receptors the AC inhibition lasted much longer than the Ca^{2+} signal whereas when the Ca^{2+} signal was evoked by CCE the AC inhibition only lasted as long as the Ca^{2+} response (Dyer *et al.,* 2005).

The β_2-adrenoceptor has also been shown to couple to $G\alpha_i$. In HEK 293 cells, stimulation of the endogenous β_2-adrenoceptor with isoprenaline resulted in a six-fold increase in MAPK phosphorylation (Daaka *et al.,* 1997). This mechanism involves the receptor being phosphorylated by PKA and switching coupling to $G\alpha_i$. The $G\beta\gamma$-dimer is released from the $G\alpha_i$ protein, which activates a pathway including proteins such as the tyrosine kinase c-Src, leading to MAPK activation (Daaka *et al.,* 1997). Receptor tyrosine kinases (RTKs) can also activate ERK in a G-protein-independent manner by autophosphorylation of tyrosine residues leading to recruitment of adaptor proteins and activation of ERK (Goldsmith and Dhanasekaran, 2007). The switching phenomenon has also been demonstrated in primary cultures such as tissue from human left ventricle biopsies of patients undergoing coronary bypasses (Pavoine *et al.,* 2003). Coupling of the β_2-adrenoceptor to $G\alpha_i$ activates the cytosolic phospholipase A2. In a failing or aging heart the activation of the AC pathway may decrease due to reduced expression of β_1-adrenoceptors and an impairment of coupling between the receptor and the AC pathway. Therefore, it could be possible for β_2-

adrenoceptor polymorphisms that exhibit an increase in coupling to the AC pathway to increase the chances of survival among patients with heart failure (Pavoine *et al.,* 2003). This demonstrates that genetic variability can be one factor determining the clinical outcome in diseases such as myocardial infarction.

1.7.4 Regulation of β_2-adrenoceptor signalling

β-arrestins do not only mediate desensitisation of the β_2-adrenoceptor but can act as scaffolding proteins leading to a negative feedback mechanism of signalling (Section 1.4). β-arrestins can target proteins such as phosphodiesterases (PDEs) leading to cAMP degradation and therefore prevent signalling by the receptor. In HEK 293 cells overexpressing β-arrestins and β_2-adrenoceptors, stimulation with isoprenaline targets PDE4 to the plasma membrane in a time-dependent manner (Perry *et al.,* 2002). The importance of PDEs in signalling is emphasised by the existence of approximately 19 genes encoding 40 isoenzymes separated into 10 different PDE families (Baillie and Houslay, 2005, Soderling and Beavo, 2000). PDE4 is a family of cAMP-specific phosphodiesterases encoded by four different genes (A, B, C, D) leading to more than 18 different isoforms (Baillie and Houslay, 2005). PDE8 and 9 have also been shown to be specific for cAMP degradation (Soderling and Beavo, 2000). In HEK 293 cells, a catalytically inactive mutant of PDE4 showed the receptor still had the ability to bind β-arrestin but could not recruit PDE4 to the plasma membrane after agonist addition (Perry *et al.,* 2002). Therefore, β-arrestins recruit PKA and target cAMP to sites of PKA activity at the plasma membrane allowing both receptor desensitisation and preventing further PKA signalling (Perry *et al.,* 2002). Recruitment of PDE4 by β-arrestin plays a key role in switching of the coupling of

the β_2-adrenoceptor from $G\alpha_s$ to $G\alpha_i$ (Baillie *et al.,* 2003). In both HEK 293 cells and cardiac myocytes the phosphorylation of the receptor by PKA has been shown to cause a switch of coupling from $G\alpha_s$ to $G\alpha_i$ enabling the β_2-adrenoceptor to activate ERK1/2 (Baillie *et al.,* 2003).

After β-arrestin binding to the carboxyl tail of the β_2-adrenoceptor, the receptor is internalised by a clathrin-dependent mechanism (Laporte *et al.,* 1999, Oakley *et al.,* 1999). Internalisation of the receptor occurs by a different mechanism to that of the G-proteins. Live-cell imaging of HEK 293 cells containing GFP-tagged β_2-adrenoceptors, $G\alpha_s$-subunits tagged with a cyan fluorescent protein (CFP) inserted into the internal loop and β_1- and γ_7-subunits tagged with a yellow fluorescent protein (YFP), was carried out to observe internalisation of receptors and G-proteins (Hynes *et al.,* 2004). This study demonstrated that after isoproterenol stimulation, GFP-β_2-adrenoceptor was internalised in different vesicles to $G\alpha_s$-CFP, β_1-YFP and γ_7-YFP, which all co-localised in the same vesicle. Another study investigated trafficking of $G\alpha_s$ using a $G\alpha_s$–GFP in C6 and NCF-7 cells (Allen *et al.,* 2005, Hynes *et al.,* 2004). On stimulation with isoprenaline, $G\alpha_s$–GFP moved from the plasma membrane and internalised into vesicles. These vesicles did not co-localise with markers for early or late endosomes suggesting a different mechanism was involved. The $G\alpha_s$-GFP also did not co-localise with the GFP-tagged β_2-adrenoceptor suggesting they use different pathways of endocytosis but did co-localise with vesicles treated with the lipid-raft marker, fluorescent cholera toxin B (to detect the caveolae invaginations) (Allen *et al.,* 2005). $G\alpha_s$-subunits, in membrane fractions, increased on agonist stimulation suggesting that $G\alpha_s$-subunits move into lipid rafts or caveolae after activation. Disrupting

these rafts by treatment with cyclodextrin prevented internalisation of $G\alpha_s$-subunits suggesting that these internalise via a mechanism using lipid rafts.

Receptors can either be recycled or degraded after internalisation (Section 1.4.1). Ubiquitination marks proteins for degradation by the 26S proteosome in a three step mechanism. Initially the ubiquitin enzyme is activated via the COOH-terminal glycine residue of the molecule. The activated ubiquitin is then transferred to a ubiquitin-carrying enzyme and catalysed by a protein ligase (E3) linking the COOH-terminal to the ε-amino group on the lysine residue of the substrate protein (Hershko and Ciechanover, 1998). In CHO cells, agonist stimulation of β_2-adrenoceptors led to the rapid ubiquitination of both the receptor and β-arrestins (Shenoy *et al.,* 2001). Proteosome inhibitors reduced receptor internalisation and degradation demonstrating a role for ubiquitination in these processes.

1.8 Thesis aims

The aim of this thesis is to explore crosstalk between muscarinic M_3 receptors and β_2-adrenoceptor, co-expressed endogenously in HEK 293 cells, which causes a synergistic increase in intracellular Ca^{2+} but also alters the pattern of Ca^{2+} signalling at the single cell level. Stimulating the muscarinic M_3 receptors with a partial agonist rather than a full modifies both the intracellular Ca^{2+} signalling response to the muscarinic agonist but also the crosstalk Ca^{2+} response. It is therefore important for further exploration of the patterns of Ca^{2+} signalling elicited by partial and full agonists, the patterns of the intracellular Ca^{2+} signalling and the mechanisms involved in the crosstalk. Events downstream of intracellular Ca^{2+} must be investigated to observe the potential consequences of this

crosstalk. Investigations in a more physiologically relevant system must also be considered to highlight potential reasons for the crosstalk.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Water

Water used for all cell culture and molecular biology techniques was 18MΩ ultra pure water from an ELGA System (ELGA Labwater, Marlow, U.K.).

2.1.2 Mammalian cell lines

Wild type, untransfected human embryonic kidney 293 (HEK 293) cells were used in most of the cell line experiments. HEK 293 stably expressing the GLP-1 receptor (HEK GLP-1R) were provided by AstraZeneca (Alderley Park, Cheshire, U.K.). The immortalised human bronchial smooth muscle cell line was kindly provided by Andrew J. Halayko and William T. Gerthoffer (University of Manitoba, Canada and University of Nevada School of Medicine, Nevada, U.S.A. respectively). The cell line was immortalised by stable expression of human telomerase reverse transcriptase (hTERT). The smooth muscle cells used to generate the cell line were from macroscopically healthy segments of second- to fourth-generation main bronchus obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma (Gosens *et al.,* 2006).

2.1.3 Standard laboratory chemicals, reagents and consumables

All standard laboratory reagents were obtained from Sigma-Aldrich (Poole, U.K.) or Fisher Scientific (Loughborough, U.K.) unless otherwise stated. Cell culture plastics were obtained from Nunc (Roskilde, Denmark). Borosilicate glass coverslips, 25mm diameter, were purchased from VWR International (Poole, U.K.). All mammalian cell culture reagents including media, phosphate buffer saline (PBS), trypsin, foetal calf serum and

glutamine were purchased from Invitrogen (Paisley, U.K.). Primary cultures of smooth muscle cells were maintained in medium 231 supplemented with smooth muscle growth supplement (consisting of hormones, growth factors and proteins) purchased from Cascade Biologics (Nottingham, U.K.). Poly-D-lysine was obtained from Sigma-Aldrich (Poole, U.K.).

The protein kinase inhibitor H89 and myristolated peptide PKA 14-22 amide inhibitor was obtained from Calbiochem (Nottingham, U.K.). Pluronic acid F-127 and fluo-4-AM (fluo-4-acetoxymethylester) were from Molecular Probes (Eugene, U.S.A. and Cambridge, U.K. respectively). Agarose powder was purchased from BiozymT BV (Landgraaf, The Netherlands). Recombinant human TNF-α was purchased from RαD systems (Abingdon, U.K.). TRIzol was purchased from Invitrogen (Paisley, U.K.). The α-actin antibody (A2547), phalloidin antibody, FITC-conjugated goat anti-mouse antibody, anti-rat IgG HRP-conjugated and anti-mouse IgG HRP-conjugated (A9037) secondary antibodies were from Sigma-Aldrich (Poole, U.K.). pERK antibody (NEB9102) and the anti-rabbit IgG HRP-linked antibody were from New England Biolabs (Hereford, U.K.). Total ERK antibody was supplied by Santa Cruz (Heidelberg, Germany, SC-93) and the anti-HA antibody was from Roche (Basel, Switzerland, 3F10). Polyvinylidene fluoride (PVDF) membrane was supplied by Millipore (Hereford, U.K.). Kodak hyperfilm was supplied by G.R.I. (Rayne, U.K.). Pre-stained molecular weight markers used for Western blotting (10-250kDa) were purchased from Fermentas (York, U.K.). ECL+ was from G.E. Healthcare (Little Chalfont, U.K.). Lipofectamine 2000^{TM}, dNTPs mixture and DNA ladder (1kb plus, 100bp-12kp range) were purchased from Invitrogen (Paisley, U.K.). GelRed^{TM} was purchased from Cambridge Bioscience (Cambridge, U.K.). Bradford reagent was purchased

from Sigma-Aldrich (Poole, U.K.). Plasmid kits and QIAquick gel extraction kits were supplied by Qiagen (Crawley, U.K.). Midiprep kits were supplied by Macherey-Nagel (Dűre, Germany). Myo-[^{3}H]-inositol (81Ci/mmole) was purchased from GE Healthcare (Little Chalfont, U.K.). Scintillation vials and SafeFluor scintillation cocktail were obtained from PerkinElmer (Aylesbury, U.K.). 1,1,2-trichlorofluoroethane and tri-n-octylamine were purchased from Merck Bioscience (Nottingham, U.K.). Dowex 1 X8 (8% cross linkage, 200-400 dry mesh, chloride form) anion exchange columns were from Sigma-Aldrich (Poole, U.K.). XL1-Blue Subcloning-grade competent cells were obtained from Stratagene (San Diego, U.S.A.). All restriction enzymes, DNA ligase, Vent, Phusion high-fidelity polymerase for cloning PCR and appropriate buffers were purchased from New England Biolabs (Hitchin, U.K.). All oligonucleotides were synthesised by Invitrogen (Paisley, U.K.).

2.1.4 Bacterial strains

XL1-Blue from Stratagene (Amsterdam Zuidoost, The Netherlands) was the bacterial strain used as the host for generating the HA-Epac1-DS-red2 gene.

2.2 Cell culture and transfection procedures

2.2.1 Cell line culture

HEK 293, HEK GLP-1R and HBSM were maintained at 37ºC and 5% CO_2 in a humidified environment. The HEK cell lines were subcultured every 2 days when confluent and maintained in Dulbecco's modified eagles medium (DMEM) (Gibco 41966) supplemented with 10% foetal bovine serum. The HASM was subcultured every two days and maintained in DMEM (Gibco 42430) supplemented with 10% FBS. All cells were washed with

Dulbecco's phosphate buffered saline (DPBS) (without Ca^{2+}/Mg^{2+}) and detached using trypsin (0.5g L^{-1}). The trypsin was neutralised by adding medium and the cells were centrifuged for 3 minutes at 400g, being resuspended in media post-centrifugation and diluted at 1:5-1:10 to re-seed flasks. The number of cells plated is shown below and were counted using a haemocytometer.

96 well plate $2x10^4$ cells per well

24 well plate $2x10^5$ cells per well

6 well plate $2x10^6$ cells per well

2.2.2 Smooth muscle cell culture

Male wistar rats (<300g) were culled by concussion followed by cervical dislocation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. An incision was made in the midline of the animal and the trachea isolated. The trachea was immediately placed in ice-cold Ca^{2+}-free isolation buffer (137mM NaCl, 5.4mM KCl, 1mM MgCl2, 10mM glucose, 10mM HEPES, 0.44mM Na_2HPO_4, 0.42mM NaH_2PO_4). The trachea was placed in fresh Ca^{2+}-free isolation buffer and any fat and connective tissue removed. The tissue was cut into 1-2mm pieces and enzymatically digested with papain (0.75mg/ml) in the presence of 1,4-dithiothreitol (DTT; 0.5mg/ml) for 1h at 35°C. The smooth muscle cells were washed in a low [Ca^{2+}] isolation buffer (Ca^{2+}-free solution with the addition of 0.1mM $CaCl_2$) and then placed in medium 231, supplemented with growth factors. The tissue was dissociated by mechanical sheer using repeated titration through a sterile plastic Pasteur pipette. Cell suspensions were plated onto 25mm diameter sterile glass coverslips. Rat tracheal smooth muscle (RTSM) cells were maintained in medium 231

(M231500) supplemented with smooth muscle growth supplement and maintained up to eight days after initial isolation.

2.2.3 Transfection of plasmids into HEK 293 cells

Transfections of HEK 293 cells were carried out in 25cm^2 flasks. Cells were placed in Opti-MEM® I Reduced Serum Medium (Gibco 51985-026) (Invitrogen, Paisley, UK,) when approximately 50% confluent and transfected using Lipofectamine 2000™. After 12h the cells were placed back into media containing serum and plated in poly-D-lysine coated multi-well dishes 6-8h later. (Where required, cell culture-ware was poly-D-lysine-coated using a 0.01% solution (in H_2O), which was added to for 10-15 minutes. The solution was then removed and washed with DPBS prior to plating with cells.) HEK 293 cells were transfected with: the nuclear factor of activated T-cells (NFAT) tagged with the fluorescent tag, EGFP (kindly donated by Professor Gerald Crabtree, Stanford University School of Medicine) (Arron *et al.,* 2006); luciferase constructions for NFAT, pGL4.30 *luc2P*/NFAT-RE/Hygro (Promega, Madison, USA, E8481); nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pGL4.32 *luc2P*/NF-κT-RE/Hygro (Promega E8491); the β-galactosidase plasmid, pSV-β-Galactosidase (Promega E1081) or; the pIRES2-DsRed2/HA-Epac1 plasmid (Section 2.3.4). For transfection of a 25cm^2 flask containing HEK 293 cells, 5µg DNA and 10µl Lipofectamine 2000™ were used. To transfect the cells with EGFP-NFAT, 2.5µg DNA and 5µl Lipofectamine 2000™ were used.

2.2.4 Titrating buffer to a [Ca^{2+}] of 100nM

Fluorescence was recorded on a PerkinElmer spectrofluorometer. Free [Ca^{2+}] levels were controlled by EGTA buffering. Fura-2 free acid (final concentration of 2µM) was added to Krebs'-HEPES buffer (composition: 118.6mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM

KH_2PO_4, 4.2mM $NaHCO_3$, 10mM HEPES, 11.7mM glucose, 1.3mM $CaCl_2$, pH 7.4) and fluorescent readings taken at excitation wavelengths of 340nm and 380nm with emission recorded at 510nm. Maximal and minimal fluorescent readings were obtained by the addition of excess Ca^{2+} followed by excess EGTA. The free [Ca^{2+}] was then determined using the calculation shown below.

[Ca^{2+}] nM = (R_T-R_{TMIN})/(R_{TMAX}-R_T) x Kd fura-2 (nM) x SFB

Where: R_T = ratio 340nm/380nm; R_{TMIN} = ratio in presence of excess EGTA; R_{TMAX} = ratio in presence of saturating [Ca^{2+}]

SFB = Max380 (EGTA value)/Min380 ($CaCl_2$ value)

2.3 Molecular biology

2.3.1 Bacterial cell culture

Escherichia coli (E. coli) can easily be grown in a nutrient media. These bacteria contain plasmids that can be used to carry genetic information that can be isolated from chromosomal DNA making *E.coli* suitable for amplifying plasmid DNA. The *E.coli* strain used in this study was XL1-Blue (Stratagene, California, USA).

2.3.1.1 Growth and multiplication of bacteria

E.coli were grown at 37ºC either in Lutria-Bertani (LB) broth (1% *w:v* tryptone, 0.5% *w:v* yeast extract and 1% *w:v* NaCl) or on LB agar plates (1% *w:v* tryptone, 0.5% *w:v* yeast extract, 1% *w:v* NaCl and 1.5% *w:v* agar). Ampicillin (100µg.ml^{-1}) or kanamycin (50µg.ml^{-1}) were used for selection.

2.3.1.2 *E.coli* stock

To make the *E.coli* stock, 600µl fresh, overnight culture and 300µl of 50% (*v:v*) glycerol were mixed gently in sterile 2ml cryovials. Vials were stored at -80ºC. To grow cells from a frozen stock the cells were scraped with a sterile loop and streaked onto an LB agar plate containing the required antibiotic. The plate was then incubated overnight at 37ºC and stored at 4ºC until required for growth.

2.3.1.3 Bacterial transformation

This process allows the uptake of DNA into bacterial cells. If the DNA taken up by the bacteria has an origin of replication recognized by the host cell DNA polymerases, the DNA will be replicated. The bacteria containing the DNA of interest were selected using antibiotic resistance.

2.3.1.4 Transformation procedure

A 50µl aliquot of competent cells was thawed on ice for 10min and 5-10ng of plasmid DNA or 7.5µl of ligation reaction was then added and incubated on ice for a further 10min. The mixture was heat-shocked for 90s at 42ºC and then placed on ice for 2min. The mixture were then incubated at 37ºC in 1ml of SOC medium (0.5% *w:v* yeast extract, 2% *w:v* trytone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose). Transformed cells (50µl) were spread on a 10cm LB agar plate containing appropriate antibiotic and incubated at 37ºC overnight. The plate was then kept at 4ºC until required.

2.3.1.5 Preparation of plasmid DNA

Plasmids were used to express and multiply the Epac1 gene (Section 2.3.3.3). Qiagen plasmid kits were used to isolate and purify DNA from *E.coli*. Nucleobond Macherey-Nagel midipreps were used to amplify the expression plasmid for transfections.

Midipreps were performed with slight modifications to the manufacturer's protocol. A single colony from the LB plate was placed in 50ml of sterile LB broth containing the appropriate antibiotic. After shaking (220rpm) overnight at 37ºC the cells were centrifuged at 3500g for 15min at 4ºC. The pellet was resuspended in 6ml resuspension buffer containing RNase A. The next step was to add 6ml lysis buffer along with 6ml neutralisation buffer and the suspension was filtered through an anion-exchange column. The DNA was eluted using 5ml elution buffer. Isopropanol was added to precipitate the DNA and the sample was centrifuged at 15,000g for 30min at 4ºC. The pellet was washed with 70% ethanol, centrifuged and dried at room temperature (RT). Once completely dry the pellet was reconstituted in Tris (supplied with the kit) and the DNA concentration measured.

2.3.1.6 Quantification of DNA

The yield and quality of the plasmid DNA was determined by measuring absorbance at the two wavelengths of 260nm and 280nm. An absorbance value of 1 at 260nm corresponds to a concentration of 50µg.ml^{-1} of double-stranded DNA. A 260/280 ratio of 1.8-2.0 demonstrates a relatively pure DNA without protein contamination.

2.3.2 Polymerase Chain Reaction (PCR)

2.3.2.1 RNA extraction

The cells were grown to 90% confluency on 10cm diameter tissue culture dishes. The media was removed and cells were washed with ice-cold PBS and processed for RNA extraction using TRIzol reagent (Invitrogen 15596-026). After adding 1ml of TRIzol reagent to each plate, the cells were transferred to 2ml microcentrifuge tubes and

homogenised by drawing up and down through a 23 gauge needle. The homogenised samples were incubated at RT for 5min to allow complete dissociation of the nucleo-protein complexes. Chloroform (0.2ml) was then added and the tubes were shaken vigorously for 15s. Following incubation at RT for 2-3min, tubes were centrifuged at 12,000g for 15min at 4°C. The RNA was precipitated from the colourless upper aqueous phase by addition of 0.5ml isopropyl alcohol. The sample was incubated at room temperature for 10 minutes and centrifuge at 12,000g for 10 min at 4°C. The RNA pellet was washed with 1ml of 75% (*v;v*) ethanol and collected by centrifugation at 7,500g for 5 min at 4°C. After briefly air drying the sample and redissolving in 50µl H_2O, the RNA was treated with 10µl DNase I (final concentration at 1000units/ml) for 30min at 37°C. The RNA was purified by repeating the whole process from the addition of 1ml of TRIzol and finally resuspended in 100µl H_2O.

2.3.2.2 Reverse transcription polymerase chain reaction (RT-PCR)

Following the manufacturer's instructions, 3µg of total RNA from each extraction was used as the template for reverse-transcription with Transcriptor Reverse Transcriptase using oligo $dT_{12–18}$ in a total reaction volume of 20µl. PCRs were then performed in a total volume of 50µl containing 0.5units of Taq DNA polymerase.

Oligos for the human and rat muscarinic M_3 receptors were designed (as experiments were carried out on both RTSM cells and human bronchial smooth muscle cell line), to clone the gene encoding the receptor, using the following primers for the 3rd intracellular loop:

Human muscarinic M_3 receptor

Forward primer

5'-CGA GAC GAG AGC CAT CTA CTC C-3'

Reverse primer

3’-GAC CAG GGA CAT CCT TTT CCG C-5’.

Rat muscarinic M_3 receptor

Forward primer

5’-TGC CTC TGT CAT GAA TCT GC-3’

Reverse primer

3’-TCC TCC TGG ATG ATC GTT TC-5’.

Oligos for the human and rat β_2-adrenoceptor were designed (as experiments were carried out on both RTSM cells and HBSM cell line), to clone the gene encoding the receptor, using the following primers:

Human β_2-adrenoceptor

Forward primer

5’-ACGCAGCAAAGGGACGAG-3’

Reverse primer

3’-CACACCATCAGAATGATCAC-5’

Rat β_2-adrenoceptor

Forward primer

5’-ACCTCCTTCTTGCCTATCCA-3’

Reverse primer

3’-TAGGTTTTCGAAGAAGACCG-5’

The RT-PCR was carried out using the following parameters. The denaturation step occurred at 95°C for 30s followed by 25 cycles at 95°C for 30s. The annealing step was carried out at 55°C for 90s, and the DNA extension step occurred for 3min at 72°C, with a

final extension step of 10 min. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with GelRed™.

2.3.2.3 Reaction mixture

The total volume of the PCR reaction was 50µl. For cloning Epac1, the mixture contained 1µl thermostable DNA polymerase, 5µl reaction buffer, 1µl (1µg) of forward and reverse primers, 1µl of 10mM dNTPs, 0.4µg plasmid DNA and was made up to volume with H_2O.

Primers for Epac1:

Forward – introducing NheI without a start codon

5' -CTA GCT AGC GTG TTG AGA AGG ATG C- 3'

Reverse – introducing EcoRI with stop codon (TCA)

3' –CGG AA TTC TCA TGG CTC CAG CTC TCG- 5'

2.3.2.4 Programming thermocycler for PCR

All PCRs proceeded for 30 cycles with denaturation, annealing and extension in each cycle. The denaturation occurred at 95°C for 1min in the first cycle and for 30s in subsequent cycles. The temperature used for the annealing step was 54°C and was performed for 1min in each cycle. The extension temperature was 72°C for a duration of 3min in the cloning cycle and 10min for the last cycle of PCR.

2.3.2.5 Purification of PCR product

All PCR products were purified using agarose gel electrophoresis followed by DNA extraction.

2.3.3 Vector plasmids

2.3.3.1 pHA-GLP-1R-EGFP

This is a pre-made plasmid pHA-GLP-1R-EGFP containing an N-terminal HA tag on the GLP-1 receptor and C-terminal EGFP tag. It contains restriction sites NheI (between HA and GLP-1R) and EcoRI (between GLP-1R and EGFP), which were used to generate an HA-tagged Epac1 insert. The Kozak sequence and start codon have been inserted before the HA-tag. The stop codon is after the EGFP.

2.3.3.2 pIRES2-DsRed2

pIRES2-DsRed2 (BD Biosciences Clontech, Saint-Germain-en-Laye, France, #6990-1) contains the internal ribosome entry site (IRES) between the multiple cloning site (MCS) and the coding sequence for *Discosoma sp.* red fluorescent protein (DsRED2). This allows the Epac1 gene (cloned into the MCS) and the DsRed2 gene to be translated together in a single bicistronic mRNA. This allows efficient selection of transiently transfected cells expressing Ds-Red2 and Epac1.

2.3.3.3 Making Epac1 constructs

Step 1 – Replace the GLP-1R with Epac1. The human Epac1 gene in pCFP-Epac1(δDEP)-YFP was used as the starting template for generating the Epac1 construct and was a kind gift from Johannes L. Bos (Department of Physiological Chemistry and Centre of Biomedical Genetics, UMCU, Utrecht, The Netherlands)

This step produces N-terminal HA-tag Epac1.

CTA GCT AGC GTG TTG AGA AGG ATG C (Forward primer for Epac1)

CGG AA TTC TCA TGG CTC CAG CTC TCG (introduce EcoRI)

Step 2 - Using this as template to clone the N-terminal HA-tagged Epac1 into Nhe1-EcoRI digested Ds-red vector by PCR.

5'~primer, to introduce SpeI, Kozak sequence and start codon (34bp) (for cloning N-terminal HA tagged constructs)

5'-GACTAGT GCC ACC ATG CGC CCC CAG GGT GCC ACT-3'

2.3.4 DNA ligation

To construct the HA/Epac1-DsRed2 plasmid, the Epac1 PCR product and the DsRed2 vector had to be combined. The pIRES2-DsRed2/HA underwent a digest between Nhe1-EcoR1. A ligation was carried out to join the linear DNA fragments. The total reaction mixture was 20µl and included 0.05µg of vector DNA, 1µg of Epac1 DNA, 1µl T4 DNA ligase, 2µl 10xT4 ligation buffer made up to volume with H_2O. The mixture was vortexed and collected at the bottom of the tube by brief centrifugation and incubated for 1h at RT. A volume of 10µl was then transformed directly into XL1-Blue cells followed by selection using antibiotic resistance contained in the vector (see section 2.3.1.1).

2.4 Measuring elevations in intracellular Ca^{2+}

2.4.1. Measuring $[Ca^{2+}]_i$ in single cells

Cells were plated onto 25mm, sterile, borosilicate glass coverslips coated with 0.01% *w:v* poly-D-lysine. Cells, at 70-80% confluency, were loaded with 2µM fluo-4 AM and pluronic F-127 (0.036% *w:v*) in Krebs'-HEPES buffer for 40min at 37°C. Cells were washed once and imaged using confocal microscopy. The temperature of the coverslip was maintained at 37°C using a Harvard Apparatus temperature controller (Harvard Applications Inc, Kent, UK). Agonist was added at a volume of 50µl, by bath addition, to a chamber volume of

450µl. Cells were imaged using Ultra*View 4.0* confocal microscopy (PerkinElmer Life and Analytical Sciences, Beaconsfield, Bucks., U.K.) with x60 oil-immersion objective lens and a 488nm Kr/Ar laser line. Emitted light was collected above 510nm and images captured at a rate of approximately 3 frames per minute using a CCD camera. Fluorescence prior to addition of agonist was regarded as baseline.

A region of interest was marked within the cytoplasm of each cell using the Ultra*View* software. The change in cytosolic fluorescence was taken as an index of a change in $[Ca^{2+}]_i$. The data obtained for each cell were expressed as the change in cytosolic fluorescence (F/F_0 or F/F_0-1) relative to the basal reading (an average of 16 cells in the field of view).

2.4.2 Measuring $[Ca^{2+}]_i$ in populations of cells using a NOVOstar 96-well microplate reader

Changes in $[Ca^{2+}]_i$ were measured using cells loaded with the fluorescent Ca^{2+} indicator, fluo-4AM in a NOVOstar microplate reader (BMG LABTECH Gmbh, Offenburg, Germany). HEK 293 were cultured, to 100% confluency, on poly-D-lysine coated 96-well plates and HEK GLP-1R cells were cultured, to 100% confluency, on ELISA strip plates (Greiner, Gloucestershire, U.K.) coated with poly-D-lysine for 48h at 37ºC in a 5% humidified environment. For the assay, the cells were washed with 100µl Krebs'-HEPES buffer and loaded with 50µl 2µM fluo-4 AM in Krebs'-HEPES buffer containing pluronic F-127 (0.036% *w:v*) for 1h at 37°C. The cells were washed once with Krebs'-HEPES buffer and assayed at 37°C in 100µl of buffer. Agonist, at a volume of 30µl, was added to the cells from a reagent plate by automated addition.

2.5 Western blotting

2.5.1 ERK

Cells were cultured on 6, 12 or 24-well plates, at a plating density of $1x10^6$, $3x10^5$ or $1.5x10^5$ cells per well respectively, for 2 days to give 100% confluency. Media was removed from the wells, cells were washed with Krebs'-HEPES buffer and then equilibrated in this buffer for 30min at 37ºC prior to agonist stimulation. The cells were stimulated with agonist for the times indicated in the figure legends. A volume of 150-200µl of solubilisation buffer (10mM Tris, pH 7.4; 10mM EDTA, pH 8.5; 1% (v/v) NP40; 0.1% (w/v) SDS; 150mM NaCl; 12mM deoxycholic acid; 0.5mM phenylmethylsulfonyl fluoride) was added to each well and the plate was left on ice for 20min. The samples were transferred to cold microcentrifuge tubes and centrifuged for 5min at 15,700g, 4ºC.

A volume of 8µl of 5x sample buffer (10% SDS, 50% glycerol, 200mM Tris pH 6.8, 0.5M bromophenol blue and 5% β-mercaptoethanol) was added to 32µl of each sample. The samples were boiled for 5min at 100ºC and once cooled, 25µl of each sample was added to wells in the gel. These samples were separated by size on an SDS-polyacrylamide gel consisting of a 10% resolving gel and 5% stacking gel. A volume of 4µl pre-stained molecular marker (10-250kDa) was added in the first well. The samples and markers were electrophoresed at a constant voltage between 90 and 140V for ~1.5h. The proteins were then transferred onto PVDF membrane using a semi-dry transfer method. The PVDF was prepared for transfer by soaking in methanol for 15s, H_2O for 2min and then equilibrated in transfer buffer (40mM glycine, 48mM Tris, 0.0375% *w:v* SDS, 20% methanol) for 5min. Once transfer was complete, the PVDF was quickly washed in methanol and allowed to dry

for 15min to increase attachment of protein to the membrane. After this time the membrane was washed in methanol, then H_2O and blocked for 1h in a TBST solution containing 5% *w:v* skimmed milk powder. After the washing step, the membrane was incubated with anti-pERK rabbit polyclonal antibody at a concentration of 1:1000 (final concentration 0.2µg/1ml) in TBST containing 5% *w:v* BSA, overnight at 4°C. After 3x10min washes, the membrane was incubated for 1-2h with horseradish peroxidise (HRP) conjugated anti-rabbit antibody at 1:1000. The membrane was washed, dried and developed using ECL+ reagents and exposed to film.

To determine whether protein had loaded equally in each well the membranes were incubated in stripping buffer (0.7% 2-β-mercaptoethanol, 2% SDS and 62.5mM Tris-HCl (pH 6.8)) for 30min at 50ºC, while being agitated. Membranes were washed with 3x10min washes with TBST and then blocked and re-probed for total ERK. This antibody was used at 1:1000 in 5% *w:v* skimmed milk powder.

2.5.2 Immunoblotting for the HA-tag

To immunoblot for the HA-tag in cells transfected with HA/Epac1-DsRed2, the pERK protocol was applied with some changes. Cells were cultured in 6 well plates with a plating density of $1x10^6$ cells per well. Once 100% confluent, cells were lysed in solubilisation buffer and mixed with 5xsample buffer. The samples were run on an 8% SDS-PAGE gel but without prior boiling. A rat IgG monoclonal anti-HA antibody, used at 1:1000 and anti-rat IgG, HRP-linked antibody was used at 1:1000.

2.5.3 Protein determination using the Bradford method

Protein determination of cell samples was carried out using the Bradford method (Bradford, 1976). The cells were lysed and digested with 0.1M NaOH and protein standards were prepared by diluting BSA (2mg/ml) in 0.1M NaOH such that the final standard concentrations were 40, 20, 15, 10, 8, 6, 4 and 2µg/ml. To analyse the standards and samples, 50µl was added along with 900µl of water. To each solution, 1ml Bradford's reagent was added, mixed and left for 5min. The absorbance of the standards and samples were measured on a spectrophotometer (Beckman, Fullerton, USA) at 595nm. Standard curves were fitted with GraphPad Prism 5, using standard linear regression.

2.6 Immunocytochemistry (ICC)

Cells were grown on 25mm glass coverslips for between 2 and 8 days, depending on the cell type. Monolayers (~70% confluent) were washed with 500ml DPBS twice and fixed with 4% paraformaldehyde in PBS for 10min at RT. Following 3x5min washes with DPBS, cells were permeabilized (1% BSA *w:v* in PBS-Triton X-100 (0.01%)) for 1h at RT. The cells again underwent 3x5min washes with DPBS and were then incubated overnight at 4°C with the muscarinic M_3 antibody or monoclonal anti-α-actin, both at a dilution of 1:400 in DPBS. The cells were washed 3x5min with fresh DPBS and incubated with Alexa-Fluor 594 donkey anti-rat IgG antibody at 1:1000. The cells being stained for the muscarinic M_3 receptor were also incubated with a FITC conjugated phalloidin antibody. After washing twice with DPBS, coverslips were mounted on slides using a 70% glycerol solution in H_2O, sealed with clear nail varnish and analysed by confocal microscopy.

2.7 Luciferase activity of reporter constructs

Cells were transfected in 25cm^2 flasks as described in section 2.2.3. Twenty-four hours after plating, cells were serum-starved for a further 24h. Following serum-starvation, cells were incubated with the test compound for 5.5h, washed once with DPBS and lysed in 250µl luminometry lysis buffer (0.025% (*w:v*) dithiothreitol (DTT), 1% (*w:v*) BSA, 25mM Tris-phosphate, 1% Triton X-100, 15% (*v:v*) glycerol, 0.1mM EDTA, 8mM $MgCl_2$) per well. Samples were collected in microcentrifuge tubes and 100µl of each sample placed into an opaque-white 96 well plate (PerkinElmer). Each sample was supplemented with 10µl of ATP (final concentration 2.5mM) and shaken for 15min using an Orbital Shaker SO3 (Stuart Scientific, Staffordshire, U.K.). The 96 well plates were loaded into a NOVOstar microplate reading luminometer (BMG, Germany). The NOVOstar was programmed to add 100µl of D-luciferin (final concentration 2µM) (Biosynth AG, Staad, Switzerland) into the well, take photon counts at 0.1 second intervals for a total of 5 seconds then move to the next well.

2.8 Data analysis

Concentration-response curves were fitted using Prism (GraphPad Software Inc,. San Diego, CA, U.S.A). All data shown are expressed as mean of three experiments (unless otherwise stated)±s.e.m.. Where representative data are presented, experiments were performed to n of at least 3. Where statistical analysis has been used, the format of the data was taken into account when selecting a test and the analysis was carried out on the raw data. Statistical analysis between several data sets was carried out by a one- or two-way analysis of variance (ANOVA) followed by an appropriate post-hoc test as indicated. An

unpaired Student's t-test was used for direct comparison of a control and test data. Statistical significance was considered at $p< 0.05$. Where FU is used as the y-axis in Ca^{2+} experiments this is defined as arbitrary fluorescence units produced by cells loaded with fluo-4. In single-cell experiments a response is define as a peak ≥ 0.1 F/F_0.

Chapter 3: Characterisation of crosstalk between GPCRs

3.1 Overview of chapter

This chapter examines the relationship between muscarinic M_3 receptor and β_2-adrenoceptor signalling, both receptors of which are expressed on the HEK 293 cells available in the laboratory (both receptor types have an expression level of ~40 fmol/mg protein) (Tovey and Willars, 2004). Activation of muscarinic M_3 receptors leads to an increase in intracellular Ca^{2+} (Caulfield and Birstall, 1998). The β_2-adrenoceptor is normally unable to elicit a Ca^{2+} response, being preferentially coupled to $G\alpha_s$-proteins and therefore activating the cAMP pathway (Strosberg and Nahmias, 2007). However, priming the cells with a muscarinic agonist results in a β_2-adrenoceptor-mediated increase in intracellular Ca^{2+} (Kurian *et al.,* 2009).

3.2 Introduction

Crosstalk between GPCRs coupled to distinct signalling pathways, resulting in an increase in Ca^{2+} signalling, has previously been reported (Section 1.5) (see Werry *et al.,* 2003 for full review). In many examples, receptors which couple to either $G\alpha_s$ or $G\alpha_i$ do not cause an increase in intracellular Ca^{2+} when activated alone. However, when these receptors are activated in the presence of an activated $G\alpha_{q/11}$-coupled GPCR they are able to increase $[Ca^{2+}]_i$. Crosstalk has been shown to enhance Ca^{2+} signalling, however the influence on the pattern of Ca^{2+} signalling and other pharmacological aspects such as the effects of partial agonists has not been well explored. Crosstalk between muscarinic M_3 receptors and β_2-

adrenoceptors has previously been demonstrated in HEK 293 cells and may potentially be important both physiologically and therapeutically as these receptors are often co-expressed in cells and are targeted by drugs to treat a variety of disorders (Kurian *et al.,* 2009). The pattern of intracellular Ca^{2+} signalling is important in the activation of relevant downstream effector molecules and alteration in cellular mechanisms such as gene transcription (Dolmetsch *et al.,* 1997, Dolmetsch *et al.,* 1998, Li *et al*, 1998, Tomida *et al.,* 2003). The pattern of intracellular Ca^{2+} signalling, mediated by the activation of the receptor, is also dependent on agonist efficacy and therefore differs between full and partial agonists of the receptor. For all of these reasons it is important to study both the intracellular Ca^{2+} signalling of populations and single cells.

This chapter explores the crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors leading to a β_2-adrenoceptor-mediated Ca^{2+} response in HEK 293 cells and also between the $G\alpha_s$-coupled GLP-1 receptor and $G\alpha_{q/11}$-coupled P2Y receptor in HEK cells stably expressing the human GLP-1 receptor. The effect of both a partial and full muscarinic agonist on crosstalk was investigated with particular interest in any changes in the pattern of intracellular Ca^{2+} signalling at the single cell level.

3.3 Results

3.3.1 Full and partial agonists of muscarinic receptor-mediated Ca^{2+} responses expressed in populations of HEK 293 cells

Stimulation of populations of HEK 293 cells with a maximal concentration (1mM) of methacholine produced a rapid, transient intracellular Ca^{2+} peak 5s after addition (Figure 3.1a), which sub-sided to a slow decreasing response over the remainder of the experiment. At lower concentrations of agonist, the time taken to reach the peak response increased (Figure 3.1a and Table 1a). Stimulating the cells with 1mM (a maximal concentration) of oxotremorine produced a response that peaked at approximately 12s after addition of oxotremorine (Figure 3.1b and Table 1b) and was very similar in profile to that produced by sub-maximal concentrations of methacholine. The responses to both methacholine and oxotremorine were concentration-dependent (Figure 3.1c and d) with pEC_{50} values for the peak responses of 6.05±0.12 (~1μM, n=3) for methacholine and 5.96±0.12 (~1μM, n=3) for oxotremorine. The pEC_{50} values for the change in $[Ca^{2+}]_i$ measured 25s after agonist addition, denoted plateau responses from here on, were 5.88±0.19 (~2μM) for methacholine and 4.68±0.71 (30μM) for oxotremorine (n=3 for both).

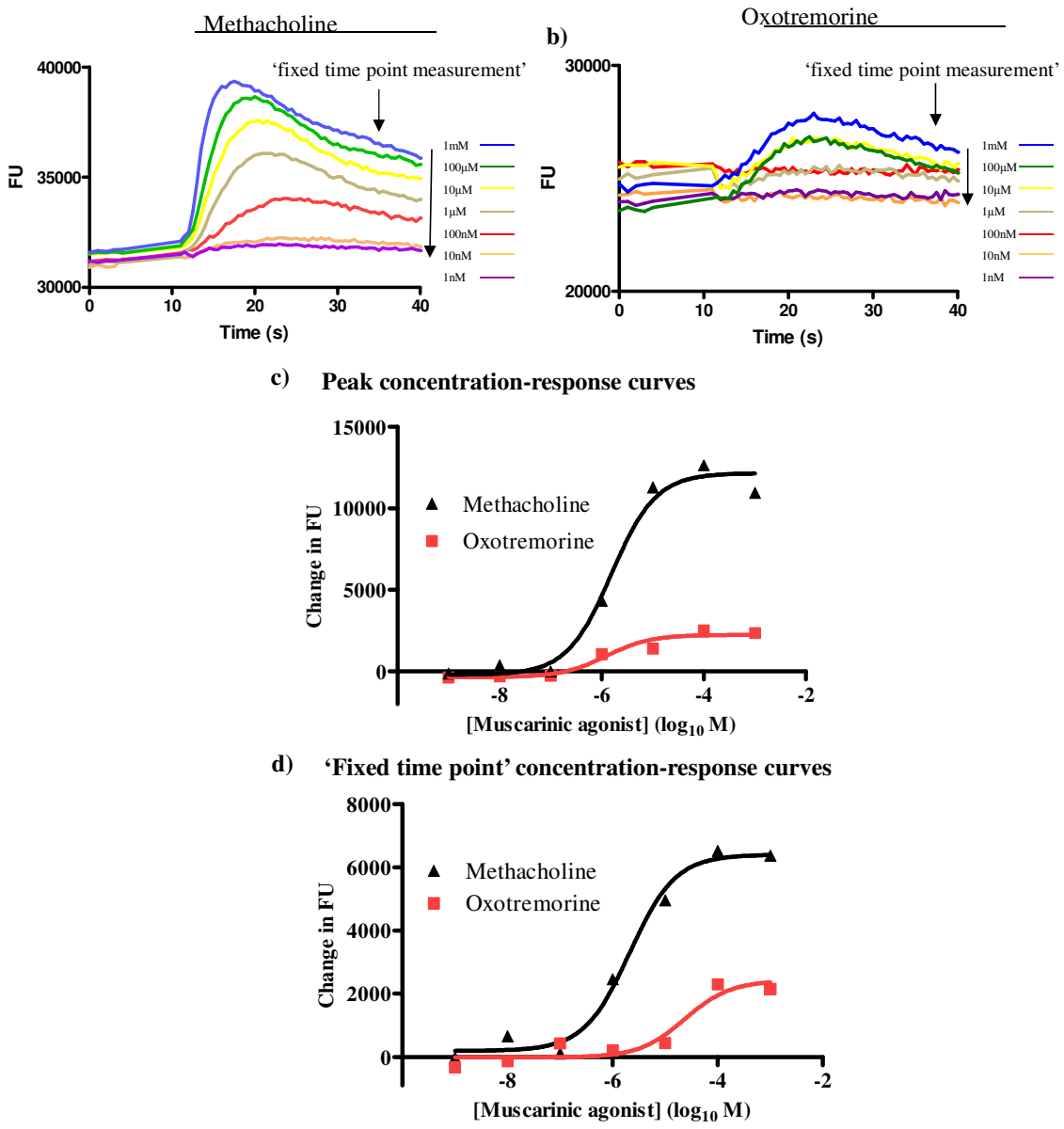


Figure 3.1. Muscarinic receptor agonist-mediated Ca^{2+} responses in HEK 293 cells. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and challenged with a range of concentrations of either methacholine or oxotremorine using a NOVOstar fluorescence plate reader. Representative families of responses over the range of agonist concentrations are shown for methacholine (a) and oxotremorine (b). Concentration-response curves were constructed for the maximal (peak) change in fluorescence (c) and the change in fluorescence 25s after agonist addition (fixec time point measurement) (d). Traces and concentration response curves are representative of 3 experiments. The pEC_{50} values (mean±s.e.mean., n=3) for the peak responses were: methacholine 6.05±0.12 (~1µM); oxotremorine 5.96±0.13 (~1µM). The pEC_{50} values for the plateau responses were: methacholine 5.88±0.19 (~2µM); oxotremorine 4.68±0.71 (30µM).

a) Time to peak and peak height of Ca^{2+} response for different concentrations of methacholine

Concentration of methacholine	1mM	100µM	10µM	1µM	100nM	10nM	1nM
Peak height of response (FU)	9125 ±2581	9939 ±1269	9257 ±1467	4424 ±1408	244 ±187	429 ±118	125 ±179
Time to peak after agonist addition (s)	5 ±0.4	5 ±1	5 ±1	7 ±2	10 ±3	16 ±2	12 ±1

b) Time to peak and peak height of Ca^{2+} response for different concentrations of oxotremorine

Concentration of oxotremorine	1mM	100µM	10µM	1µM	100nM	10nM	1nM
Peak height of response (FU)	1390 ±436	1475 ±307	968 ±190	541 ±319	439 ±191	269 ±222	82 ±165
Time to peak after agonist addition at 10s (s)	12 ±1	16 ±4	14 ±2	21 ±3	23 ±7	25 ±2	28 ±5

Table 1. Time taken to peak height of intracellular Ca^{2+} response mediated by methacholine and oxotremorine. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and challenged with a range of concentrations of either a) methacholine or b) oxotremorine. Peak height and time to peak are demonstrated in the tables. Mean±s.e.mean., n=3

3.3.2 Sub-maximal but not maximal concentrations of methacholine mediate crosstalk

Addition of a maximal concentration of noradrenaline (10μM; see Figure 3.12) alone to HEK 293 cells, produced no elevation in $[Ca^{2+}]_i$ (Figures 3.2a and 3.3). Challenging the cells with a maximal concentration of methacholine (100μM) alone produced a rapid transient peak of intracellular Ca^{2+} but addition of a maximal concentration of noradrenaline (10μM) in the continued presence of methacholine did not produce a second Ca^{2+} response (Figure 3.2b and 3.3). Co-addition of maximal concentrations of methacholine and noradrenaline produced a response not significantly different to methacholine alone (Figure 3.2c and 3.3).

Stimulation of the cells with an EC_{50} concentration of methacholine (1μM; see figure 3.4a) produced a robust intracellular Ca^{2+} signal, which consisted of a transient peak followed by a decline over a few seconds. Addition of noradrenaline (10μM) alone did not cause a Ca^{2+} response. However, in the continued presence of 1μM methacholine, a second Ca^{2+} response was observed (Figure 3.4a and 3.5). This second Ca^{2+} response was again rapid and transient, sub-siding after a few seconds. Co-stimulating the cells with methacholine (1μM) and noradrenaline (10μM) (Figure 3.4b and 3.5) did not appear to produce a significantly greater response compared to stimulation with 1μM methacholine alone (Figures 3.4a and 3.5). However, the error bars on the graph are large and therefore make it difficult to draw complete conclusions. Further experiments must be carried out to confirm this outcome.

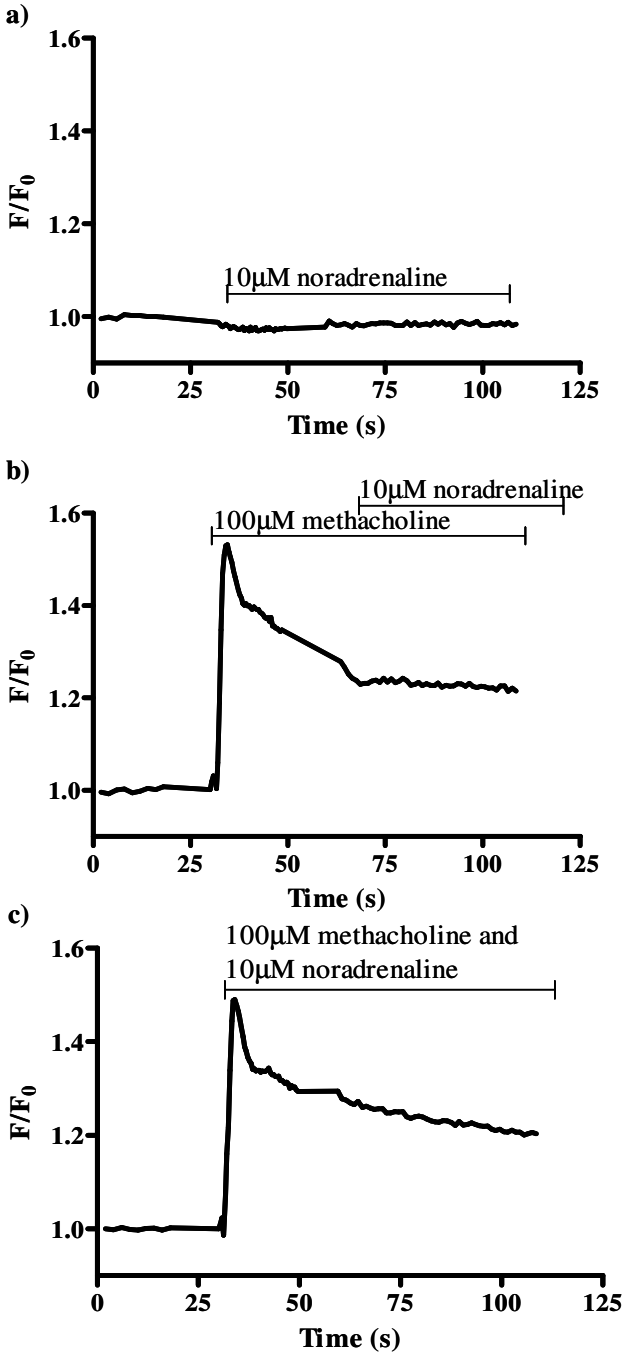


Figure 3.2. Population measurements of Ca^{2+} responses to 100µM methacholine (E_{max} concentration) and 10µM noradrenaline in HEK 293 cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence measured on a NOVOstar fluorescence plate reader. Cells were challenged at 30s with 10µM noradrenaline (a), 100µM methacholine followed by 10µM noradrenaline at 60s (b) or simultaneously with 100µM methacholine and 10µM noradrenaline (c). Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative of n=3.

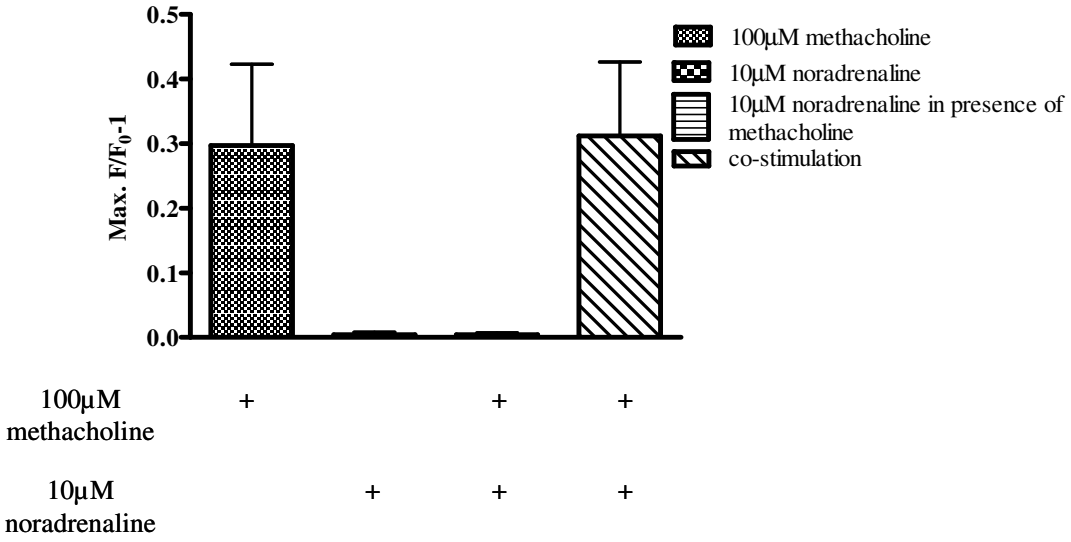


Figure 3.3. Mean peak Ca^{2+} responses elicited by 100µM methacholine (E_{max} concentration) and 10µM noradrenaline. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and changes in fluorescence measured on a NOVOstar fluorescence plate reader. Cells were challenged with either 100µM methacholine, 10µM noradrenaline, 10µM noradrenaline in the presence of 100µM methacholine or co-stimulated with the two agonists. Data are mean+s.e.mean., n=3. The data demonstrate no significant difference between the noradrenaline response in the absence and presence of methacholine or between methacholine alone and co-addition of the two agonists.

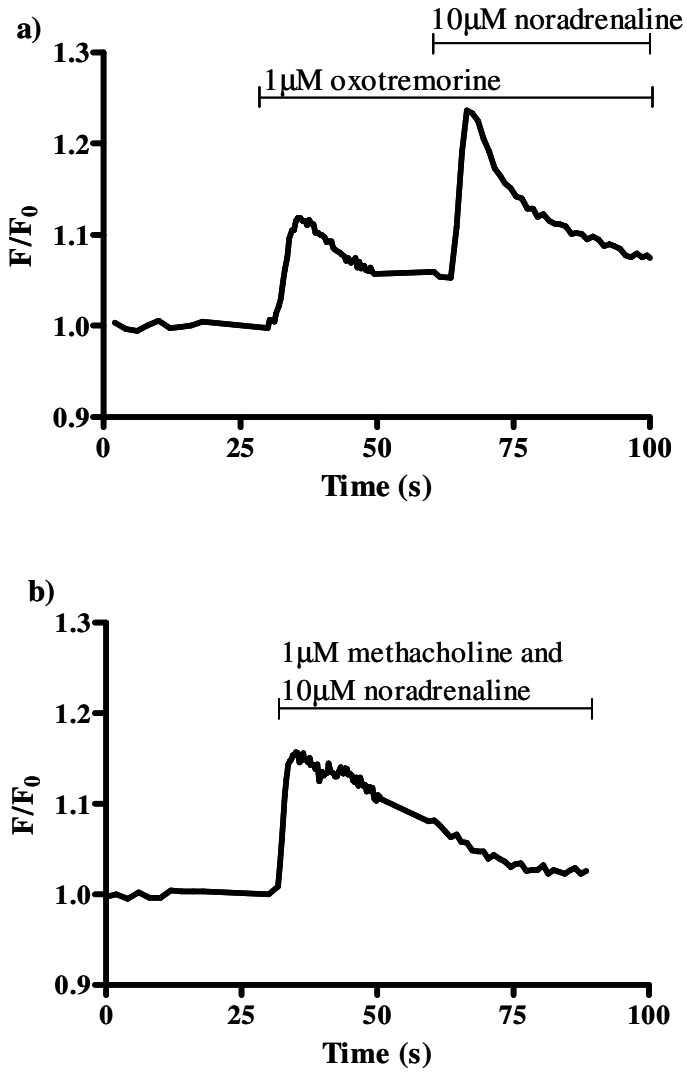


Figure 3.4. Population measurements of Ca^{2+} responses to 1μM methacholine (EC_{50} concentration) and 10μM noradrenaline in HEK 293 cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence were measured on a NOVOstar fluorescence plate reader. Cells were challenged at 30s with 1μM methacholine followed by 10μM noradrenaline at 60s (a) or simultaneously with 1μM methacholine and 10μM noradrenaline (b). Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative of n=3.

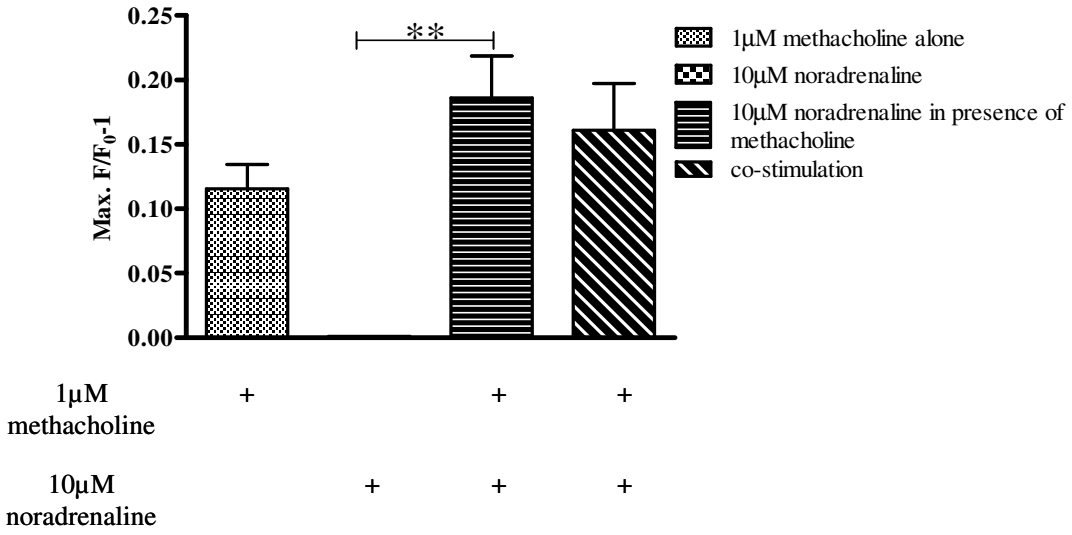


Figure 3.5. Mean peak Ca^{2+} responses elicited by 1µM methacholine (EC_{50} concentration) and 10µM noradrenaline. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and changes in fluorescence measured on the NOVOstar fluorescent plate reader. Cells were challenged with either 1µM methacholine, 10µM noradrenaline, 10µM noradrenaline in the presence of 1µM methacholine or co-stimulated with the two agonists. Data are mean+s.e.mean., n=3. ** $p<0.01$ by Bonferroni's post-hoc test following one-way ANOVA . For clarity not all the statistical significances are shown.

3.3.3 A partial agonist of the muscarinic receptor is able to mediate crosstalk

On addition of a maximal concentration of oxotremorine (100µM) to the cells a rapid, robust intracellular Ca^{2+} response was observed (Figure 3.6a). Challenging the cells with noradrenaline (10µM) in the presence of oxotremorine produced a second intracellular Ca^{2+} response (Figure 3.6a). This second Ca^{2+} response was 46.8±17.6% (n=3, Figure 3.7) of the initial oxotremorine response and significantly greater than addition of noradrenaline alone to the cells, which produced no Ca^{2+} signal (Figure 3.6a). Co-stimulating the cells with oxotremorine (100µM) and noradrenaline (10µM) did not produce a significantly greater response when compared to stimulation with 100µM oxotremorine alone (Figures 3.6b and 3.7).

When cells were stimulated with an EC_{50} concentration of oxotremorine (1µM) an initial increase in $[Ca^{2+}]_i$ was observed (Figure 3.8a and 3.9). When the cells were stimulated with noradrenaline (10µM) in the continued presence of 1µM oxotremorine, a second Ca^{2+} response was produced by the cells (Figures 3.8 and 3.9), whereas the cells did not respond to noradrenaline alone (Figures 3.9). There was a significant difference between the noradrenaline peak produced in the presence of oxotremorine and when added alone. Co-stimulating the cells with 1µM oxotremorine and 10µM noradrenaline produced a peak that was significantly greater than addition of oxotremorine alone (Figure 3.9).

Oxotremorine (1µM) and noradrenaline (10µM) produced the most robust results in terms of crosstalk and these conditions were used to further explore this crosstalk.

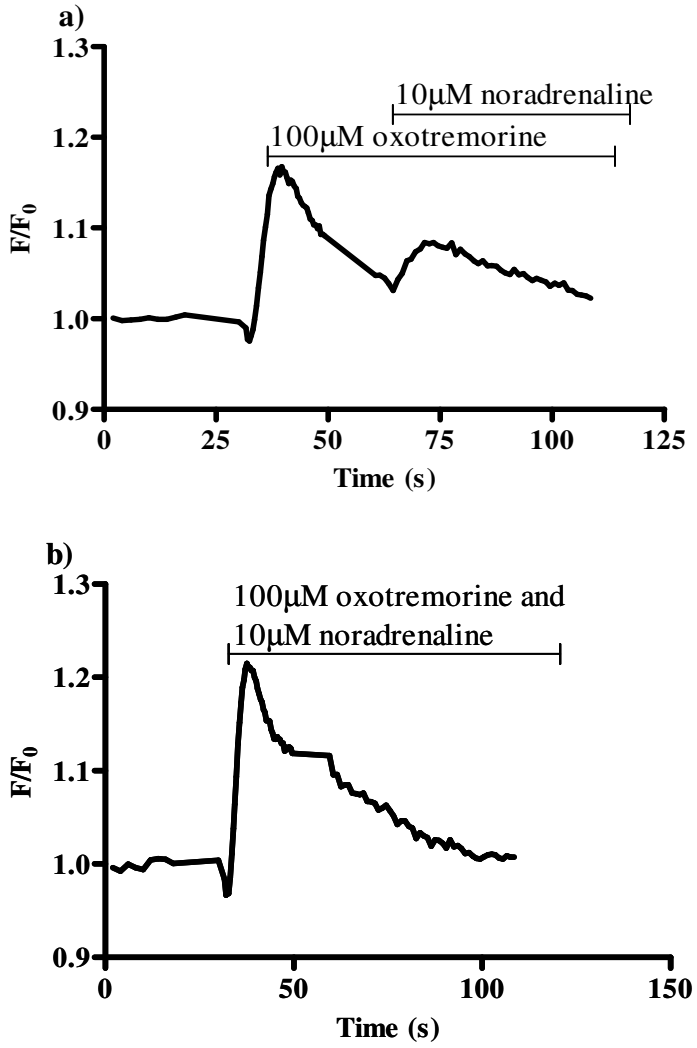


Figure 3.6. Population measurements of Ca^{2+} responses to 100μM oxotremorine (E_{max} concentration) and 10μM noradrenaline in HEK 293 cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence were measured on a NOVOstar fluorescence plate reader. Cells were challenged at 30s with 100μM oxotremorine followed by 10μM noradrenaline at 60s (a) or simultaneously with 100μM oxotremorine and 10μM noradrenaline (b). Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative of n=3.

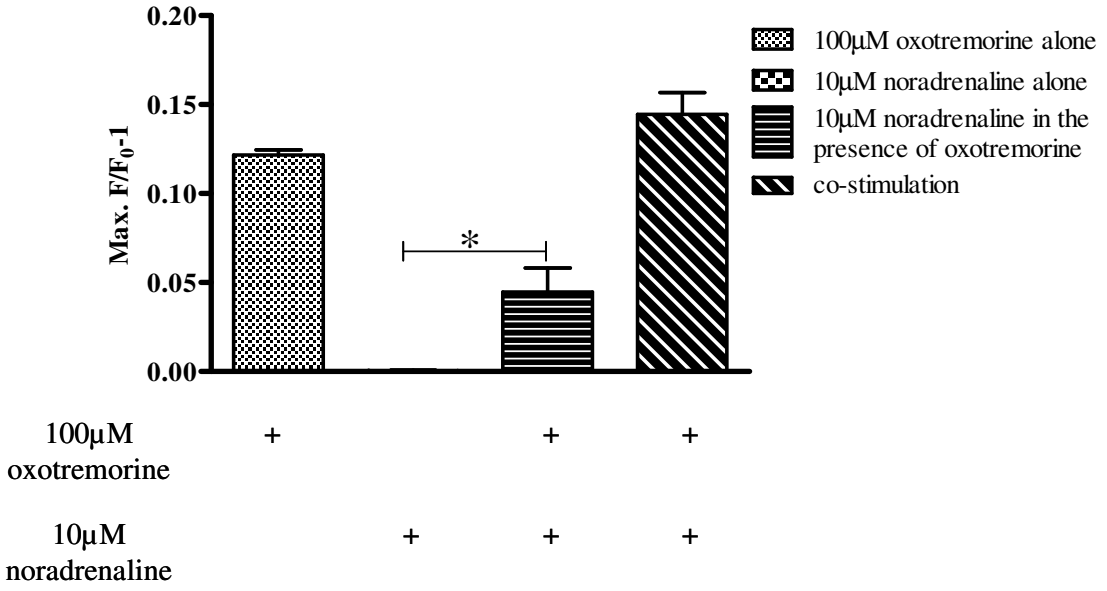


Figure 3.7. Mean Ca^{2+} responses elicited by 100µM oxotremorine (E_{max} concentration) and 10µM noradrenaline. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and changes in fluorescence measured on a NOVOstar fluorescence plate reader. Cells were challenged with either 100µM oxotremorine, 10µM noradrenaline, 10µM noradrenaline in the presence of 100µM oxotremorine or co-stimulated with the two agonists. Data are mean+s.e.mean. n=3. * $p<0.05$ by Bonferroni's post-hoc test following one-way ANOVA. For clarity not all the statistical significances are shown.

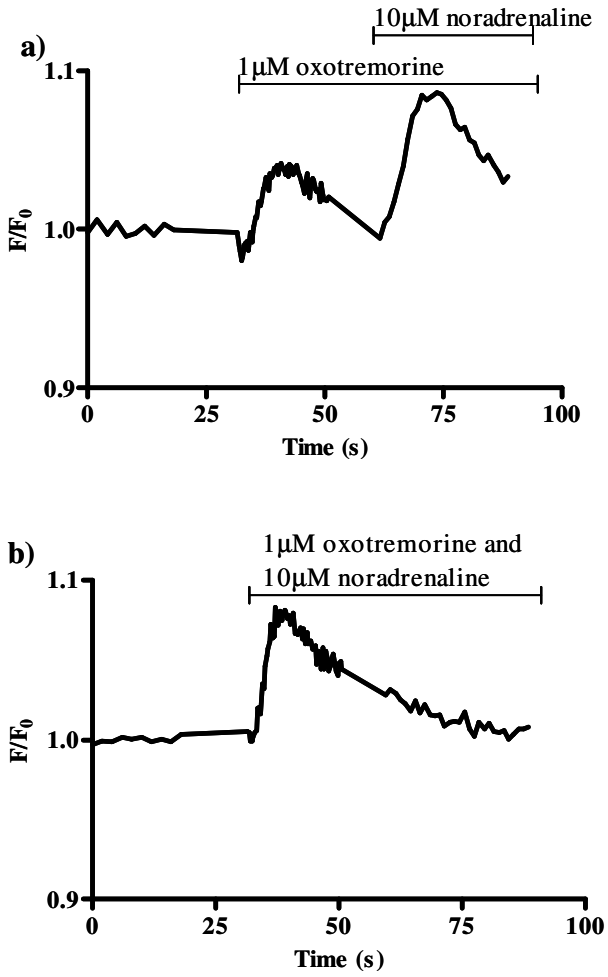


Figure 3.8. Population measurements of Ca^{2+} responses to 1μM oxotremorine (EC_{50} concentration) and 10μM noradrenaline in HEK 293 cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence measured on a NOVOstar fluorescence plate reader. Cells were challenged at 30s with 1μM oxotremorine followed by 10μM noradrenaline at 60s (a) or simultaneously with 1μM oxotremorine and 10μM noradrenaline (b). Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative of n=3.

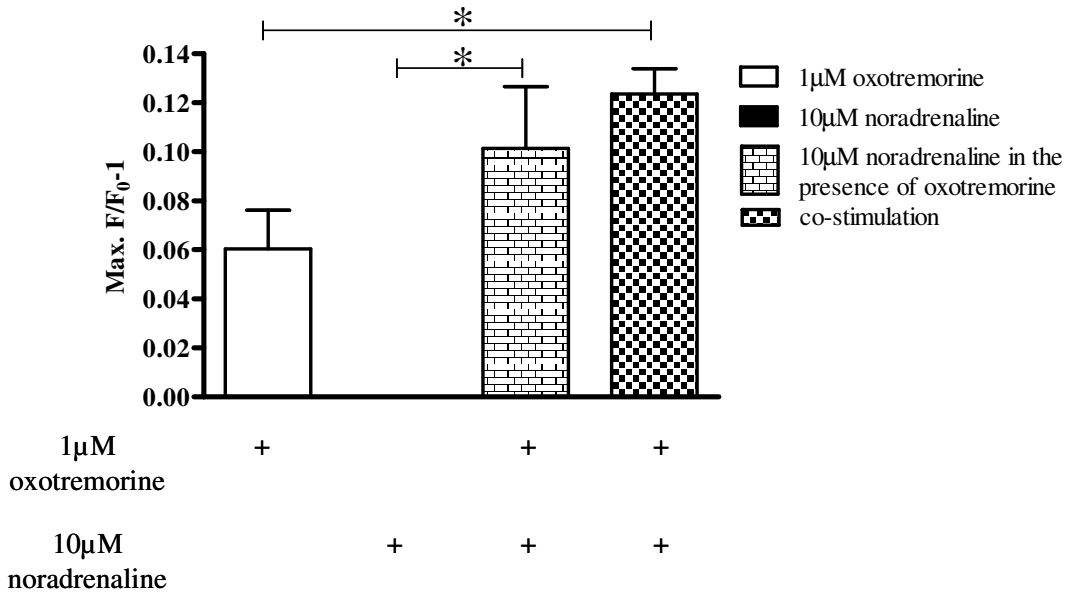


Figure 3.9. Mean Ca^{2+} responses elicited by 1µM oxotremorine (EC_{50} concentration) and 10µM noradrenaline. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and changes in fluorescence measured on a NOVOstar fluorescence plate reader. Cells were challenged with either 1µM oxotremorine, 10µM noradrenaline, 10µM noradrenaline in the presence of 1µM oxotremorine or co-stimulated with the two agonists. Data are mean+s.e.mean., n=3. * $p<0.05$ by Bonferroni's post-hoc test following one-way ANOVA. For clarity not all the statistical significances are shown.

3.3.4 Priming the cells with noradrenaline before addition of oxotremorine also mediates crosstalk

Stimulating cells with oxotremorine (1μM) after addition of buffer (as a control addition) produced an intracellular Ca^{2+} response (Figure 3.10a and 3.11). Addition of noradrenaline (10μM) alone to the cells did not produce an intracellular Ca^{2+} response (Figure 3.10b and 3.11). However, stimulating the cells with oxotremorine (1μM) in the presence of noradrenaline produce an intracellular Ca^{2+} response of significantly greater magnitude then oxotremorine alone (1μM) (Figure 3.10b and 3.11).

3.3.5 The crosstalk elicited by noradrenaline is concentration-dependent

The crosstalk responses elicited by noradrenaline (added 30s after oxotremorine) in the presence of oxotremorine (1μM) were concentration-dependent with respect to noradrenaline with a pEC_{50} value of 6.98±0.22 (approximately 0.1μM) (Figure 3.12).

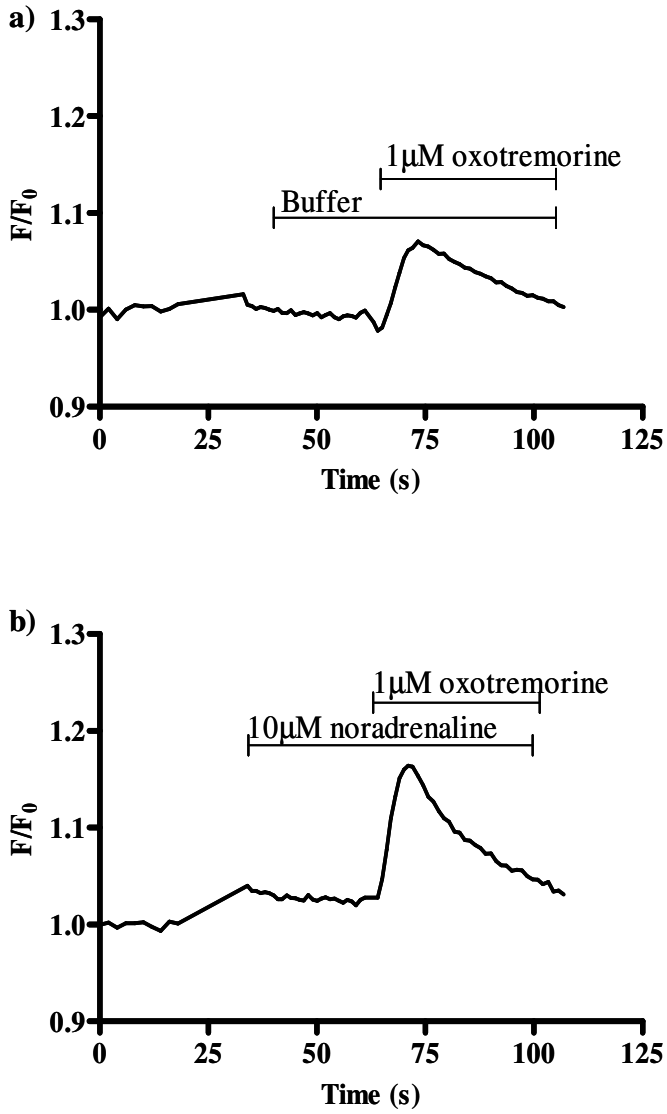


Figure 3.10 Population measurements of Ca^{2+} responses to consecutive additions of 10μM noradrenaline and 1μM oxotremorine in HEK 293 cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence measured on a NOVOstar fluorescence plate reader. Cells were challenged at 30s with buffer followed by 1μM oxotremorine at 60s (a) or 10μM noradrenaline at 30s followed by 1μM oxotremorine at 60s (b). Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative of n=3.

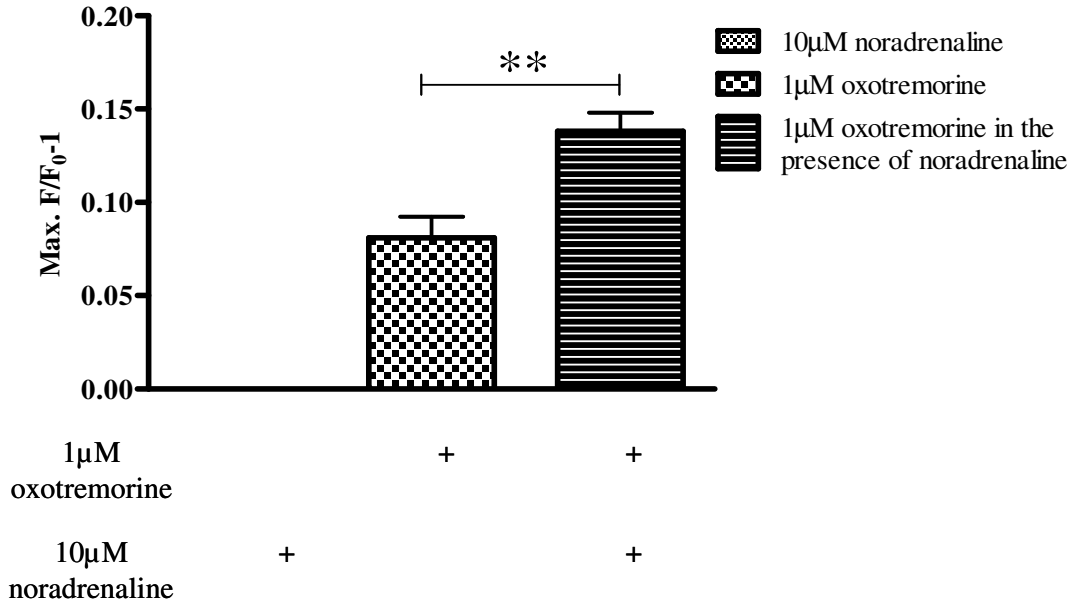


Figure 3.11. Mean Ca^{2+} responses elicited by 10µM noradrenaline and 1µM oxotremorine. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and changes in fluorescence measured on a NOVOstar fluorescence plate reader. Cells were challenged with either buffer followed by 1µM oxotremorine or 1µM oxotremorine in the presence of 10µM noradrenaline. Data are mean+s.e.mean., n=3. ** $p<0.01$ by Bonferroni's post-hoc test following one-way ANOVA. For clarity not all the statistical significances are shown.

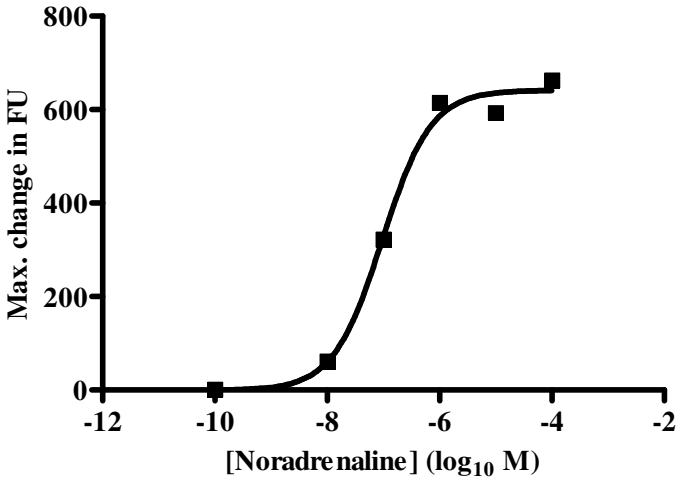


Figure 3.12. Noradrenaline mediated Ca^{2+} responses in HEK 293 cells. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and challenged with a range of concentrations of noradrenaline in the presence of 1μM oxotremorine using a NOVOstar plate reader. A concentration-response curve was constructed for the maximal responses to noradrenaline in the presence of 1μM oxotremorine. The pEC_{50} value for the peak responses to noradrenaline was 6.98±0.22 (approximately 0.1μM; n=6).

3.3.6 Crosstalk still occurs when the interval between addition of oxotremorine (1μM) and noradrenaline (10μM) is increased

As described earlier, addition of noradrenaline (10μM) to HEK 293 cells 30s after oxotremorine (1μM) addition caused a robust intracellular Ca^{2+} response (Figure 3.8 and 3.13). The size of the noradrenaline-mediated response was unaffected with an increase in the time interval, of 30 to 60s, between addition of oxotremorine and noradrenaline (Figure 3.13 and 3.14). Increasing the time interval to 360s still mediated a robust noradrenaline-mediated intracellular Ca^{2+} response (Figure 3.15a and b).

3.3.7 The nature of the β-adrenoceptor subtype responsible for crosstalk

From previous worked carried out in the laboratory it was known β-adrenoceptors elicited the noradrenaline mediated response (Kurian *et al.,* 2009). In order to identify the subtype of β-adrenoceptor involved, experiments were carried out using a β_1-adrenoceptor selective antagonist, atenolol and a β_2-adrenoceptor selective antagonist ICI 118,551. In the presence of atenolol (10μM) no change was seen in the magnitude of either oxotremorine or noradrenaline mediated Ca^{2+} responses, suggesting β_1-adrenoceptor are not involved in the crosstalk (Figure 3.16a). In the presence of ICI 118,551 (1μM) the noradrenaline mediated Ca^{2+} response was abolished implying β_2-adrenoceptors are the subtype involved in the crosstalk (Figure 3.16b). However, at such a high concentration (1μM) ICI 118,551 would have blocked both β_1-adrenoceptor and β_2-adrenoceptor so these experiments are not definitive evidence that β_2-adrenoceptors are involved (ICI 118,551 β_1-adrenoceptor K_i = 49.5nM whereas for β_2-adrenoceptor, K_i = 0.7nM; see Hoffmann *et al.,* 2004).

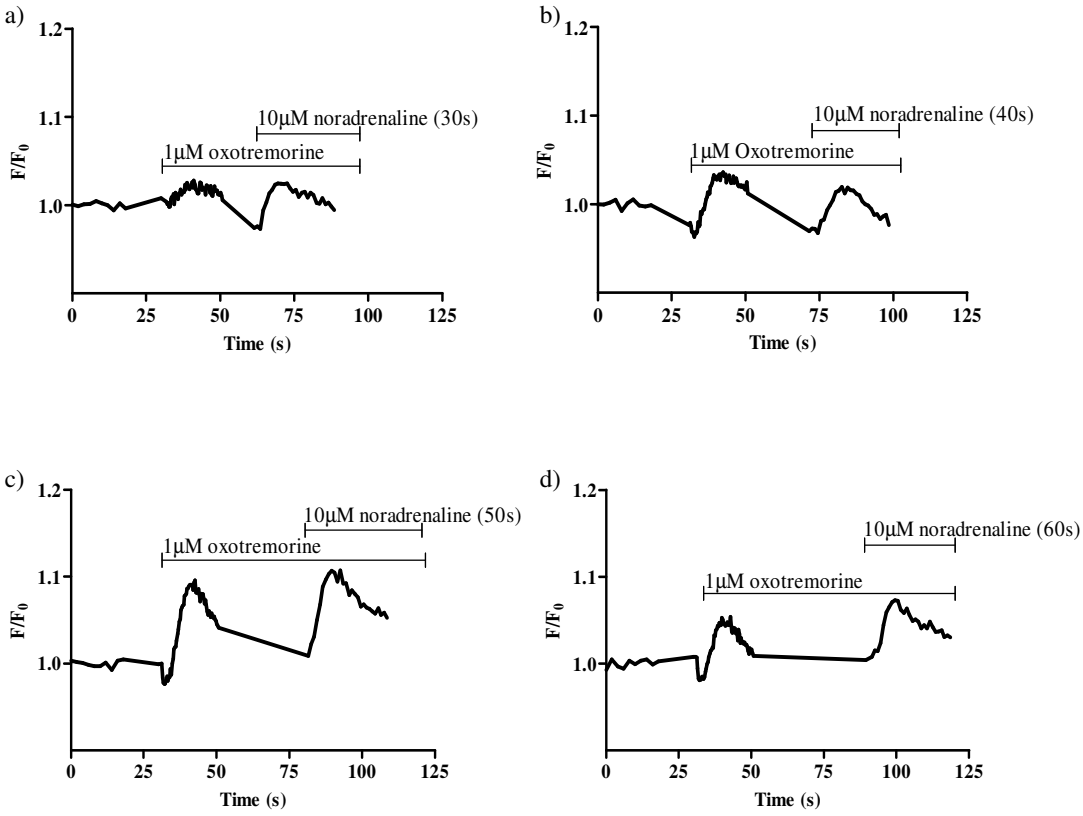


Figure 3.13. Effect of increasing the time between agonist additions on crosstalk. Populations of cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence were measured on a NOVOstar plate reader. Cells were challenged with 1µM oxotremorine at 30s followed by 10µM noradrenaline 30s (a), 40s (b), 50s (c) and 60s (d) after oxotremorine. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative of n=3.

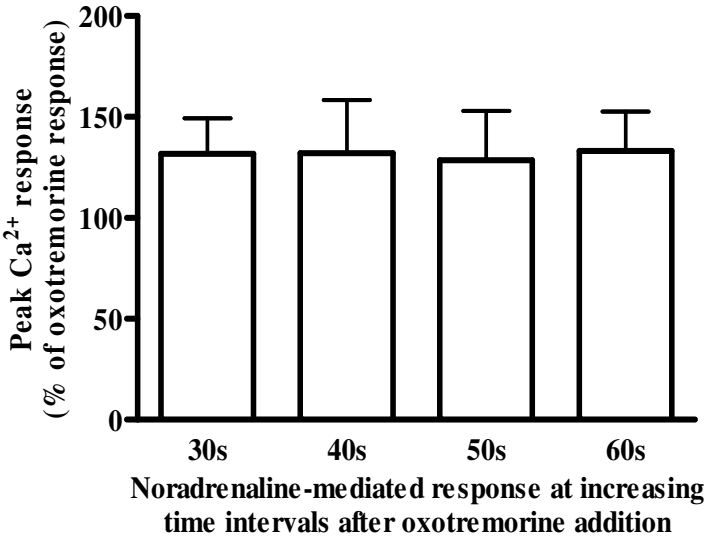


Figure 3.14. Mean noradrenaline-mediated Ca^{2+} responses, in the presence of 1µM oxotremorine, with different times between agonist additions. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and challenged with 1µM oxotremorine at 30s followed by 10µM noradrenaline at either 30s, 40s, 50s or 60s after oxotremorine. Data are mean + s.e.mean, n=4.

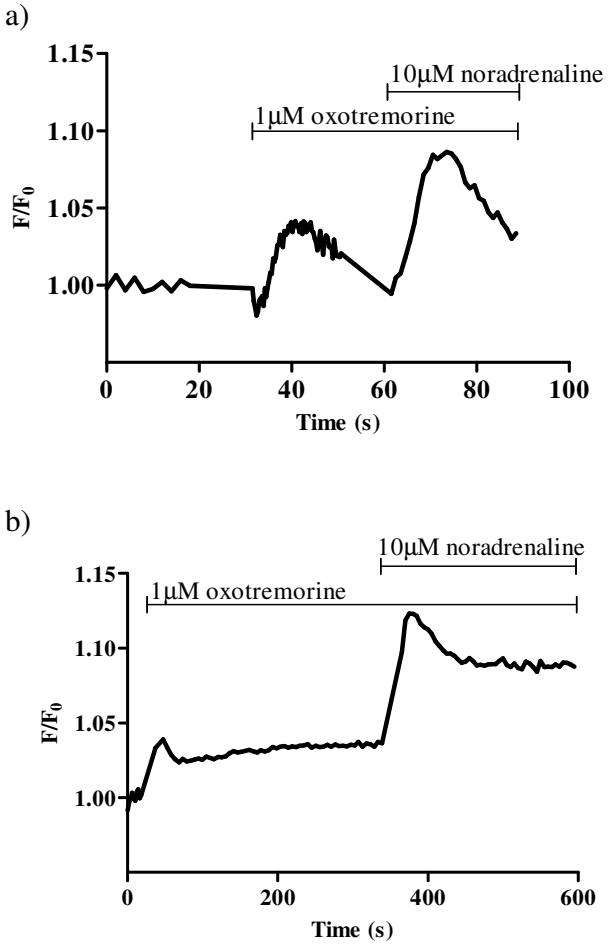


Figure 3.15 Ca^{2+} responses to 1µM oxotremorine and 10µM noradrenaline. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence measured on a NOVOstar fluorescence plate reader. Cells were challenged at 30s with 1µM oxotremorine and 10µM noradrenaline at either 60s (a) or 360s (b). Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative n=3.

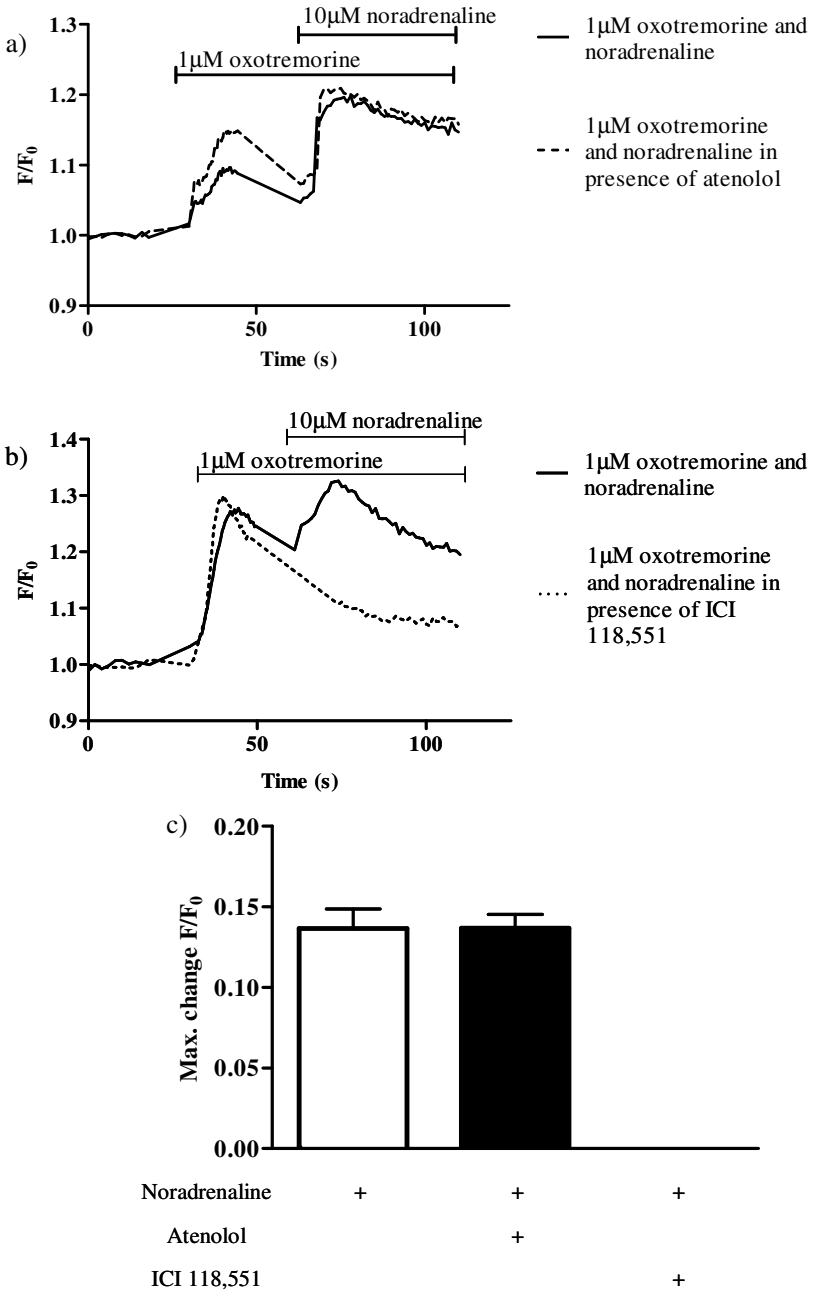


Figure 3.16 Population measurements of Ca^{2+} responses to consecutive additions of 1μM oxotremorine and 10μM noradrenaline in the absence and presence of sub-type selective β-adrenoceptor antagonists. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence measured on a NOVOstar fluorescence plate reader. Cells were challenged at 30s with 1μM oxotremorine followed by 10μM noradrenaline at 60s in the absence (black line) and presence (dashed line) of the β_1-adrenoceptor agonist atenolol (10μM, pre-incubation 30min) (a) or absence (black line) and presence (dashed line) of the β_2-adrenoceptor antagonist ICI 118,551 (1μM, pre-incubation 30min) (b). Mean data in the absence and presence of β-adrenoceptor antagonists is shown in panel (c). Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative of n=3.

3.3.8 Single cell imaging of crosstalk between methacholine- and noradrenaline-mediated Ca^{2+} responses

3.3.8.1 Analysis of single cell experimental results

Agonist-mediated Ca^{2+} responses in single cells grown on coverslips were observed and analysed to determine the pattern of signalling, for a minimum of 16 cells. These experiments were important because changes in the pattern of intracellular Ca^{2+} signals can cause significant effects downstream. For example, oscillation can cause changes in gene transcription. To analyse the responses (for definition of response see Section 2.8) three different parameters were measured: the percentage of cells responding; the frequency of the response (oscillations min^{-1} $cell^{-1}$) and the magnitude of the response (calculated by the area under the 'curve'; Period 2/Period 1). This analysis allows comparisons both within experiments, showing differences between responses on muscarinic agonist addition (Period 1, see Figures 3.18, 3.20, 3.22 and 3.24) and the second part of the experiment where there may or may not be an addition of noradrenaline (Period 2, see Figures 3.18, 3.20, 3.22 and 3.24) and also between experiments. This method therefore allowed analysis of noradrenaline-mediated responses, in the presence of muscarinic agonist compared to experiments where no addition of noradrenaline occurred. Muscarinic agonists were always added 45s into the experiment, after a basal period. The point at which this agonist was added was denoted as zero seconds. For all analyses, from 0s up to 240s was denoted Period 1. If noradrenaline was added to the cells, this occurred at 315s. Therefore Period 2 was taken from 315s to the end of the experiment at 555s. This allowed an equal time frame of 240s to be evaluated in each period (see Figure 3.18).

Addition of 10μM noradrenaline alone did not cause an elevation in $[Ca^{2+}]_i$ in any of the cells imaged (Figure 3.17a). On stimulation with a maximal concentration (100μM) of methacholine, all the cells responded with an elevation in $[Ca^{2+}]_i$ (Figures 3.17b and 3.18). After this initial response, 5±3% of the cells were still oscillating at the end of Period 1 after methacholine addition, whereas the remaining 95% cells had a single initial response (Figure 3.18). When noradrenaline was added at 315s to pre-stimulated cells, no second Ca^{2+} response or alteration in the pattern of signalling compared to the cells left with just methacholine, was observed (Figure 3.17b,c and 3.18). At 315s after addition (Period 2) the percentage of cells responding had significantly decreased when compared to the initial response to methacholine (Period 1) (Figure 3.18).

Stimulating the cells with an EC_{50} concentration of methacholine (1μM) produced an intracellular Ca^{2+} response in all of the cells. Some cells oscillated for up to 255s after agonist addition with the oscillation frequency decreasing over the course of the experiment (Figure 3.19a and 3.20). On addition of noradrenaline (10μM) 315s after methacholine, the number of cells oscillating and the oscillation frequency increased. The magnitude of the oscillatory responses produced, as measured by the area under the responses, significantly increased when cells were stimulated with methacholine and noradrenaline compared to methacholine alone (Figure 3.20).

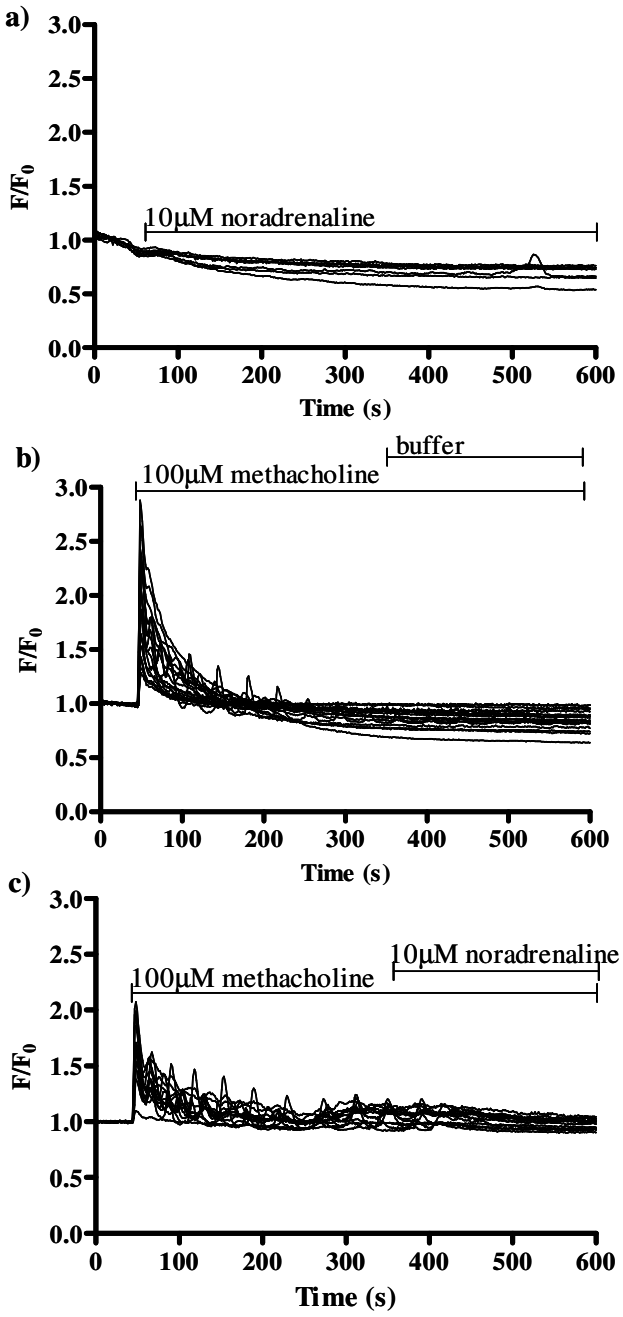
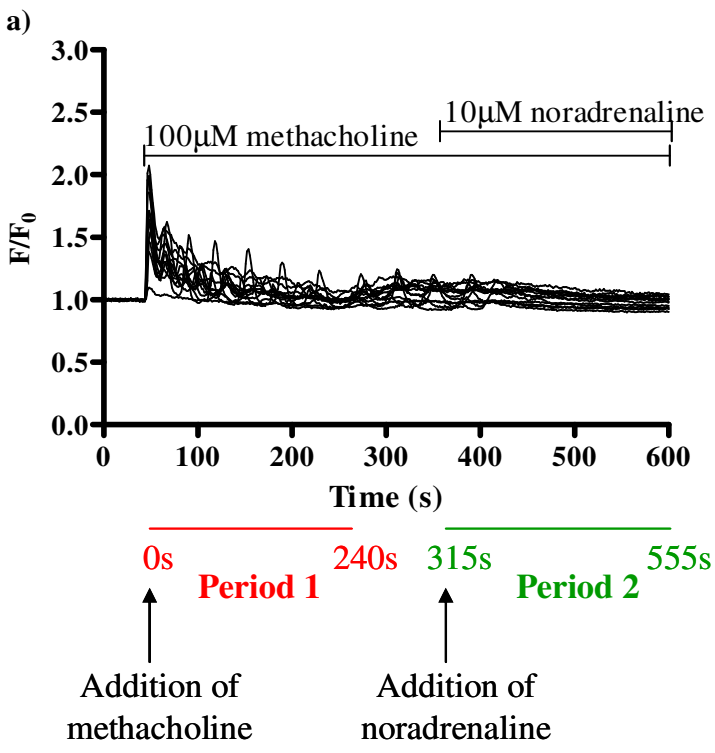


Figure 3.17. Single cell imaging of Ca^{2+} responses to 100µM methacholine (E_{max} concentration) and 10µM noradrenaline in HEK 293 cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods,* then imaged by confocal microscopy. Cells were challenged with 10µM noradrenaline (a), 100µM methacholine (b) or 100µM methacholine and 10µM noradrenaline (c), by bath addition, as indicated. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 16 cells in the field of view. Data are representative of n=4.

a)



b)

	100µM methacholine alone		100µM methacholine and 10µM noradrenaline	
			methacholine alone	methacholine and noradrenaline
	Period 1	Period 2	Period 1	Period 2
% cells responding	100 ± 0	5 ± 3	97 ± 3	23 ± 16
Response frequency (responses min^{-1} cell^{-1})	0.41 ± 0.08	0.15 ± 0.15	0.79 ± 0.07	0.10 ± 0.06
Ratio of area under response (period 2/period 1)	0.2 ± 0.1		0.4 ± 0.2	

Figure 3.18. Analysis of responses to 100µM methacholine (E_{max} concentration) and 10µM noradrenaline. The percentage of cells responding was calculated for Period 1 (from addition of agonist at 45s (taken as 0s of response on panel (a) up to 285s into the experiment (240s into response)) and Period 2 (from addition of noradrenaline at 360s (taken as 315s after initial response) to end of experiment at 600s (555s after initial response)) within each experiment (see (a) and (b)). Panel (a) is for reference only. Oscillation frequency was calculated for Period 1 and 2. Area under the oscillations was calculated for each period, Period 2/Period 1. Data are mean ± s.e.mean, n=4. * $p<0.05$.

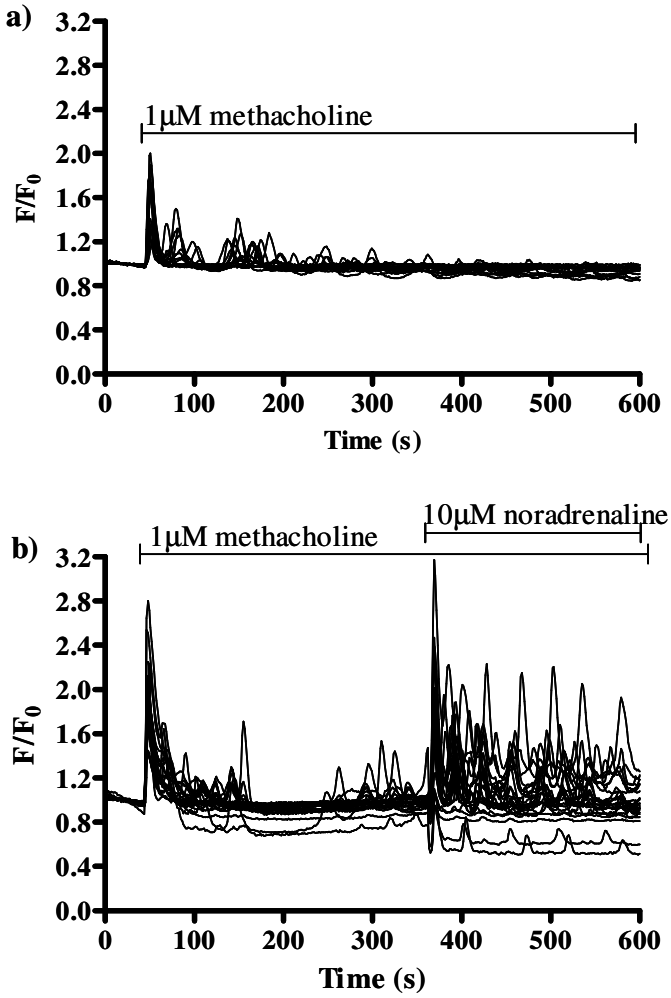
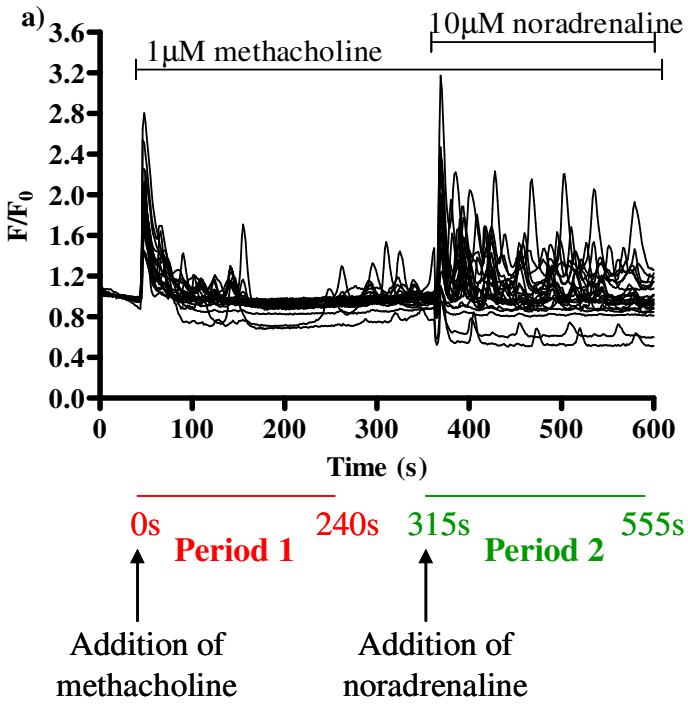


Figure 3.19. Single cell imaging of Ca^{2+} responses to 1μM methacholine (EC_{50} concentration) and 10μM noradrenaline in HEK 293 cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods,* then imaged by confocal microscopy. Cells were challenged with 1μM methacholine (a) or 1μM methacholine at and 10μM noradrenaline (b) by bath addition, as indicated. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 15 cells in the field of view. Data are representative of n=4.

a)



b)

	1µM methacholine alone		1µM methacholine and 10µM noradrenaline	
			methacholine alone	methacholine and noradrenaline
	Period 1	Period 2	Period 1	Period 2
% cells responding	98 ± 2	* 9 ± 5	** 89 ± 5	97 ± 1.3
Response frequency (response min^{-1} cell^{-1})	0.32 ± 0.4	* 0.03 ± 0.02	* 0.60 ± 0.09	0.99 ± 0.3
Ratio of area under response (period 2/period 1)	0.07± 0.06		* 1.7 ± 0.53	

Figure 3.20. Analysis of responses to 1µM methacholine (EC_{50} concentration) and 10µM noradrenaline. The percentage of cells responding was calculated for Period 1 (from addition of agonist at 30s (taken as 0s of response on panel (a)) up to 285s into the experiment (240s after initial response)) and Period 2 (from addition of noradrenaline at 360s (taken as 315s after initial response) to end of experiment at 600s (555s after initial response)) within each experiment (see (a) and (b)). Panel (a) is for reference only. Oscillation frequency was calculated for Period 1 and 2. Ratio of area under the oscillations was calculated for each period, Period 2/Period 1. Data are mean±s.e.mean, n=4. * $p<0.05$ ** $p<0.01$.

3.3.9 Single cell Ca^{2+} imaging analysis of crosstalk using oxotremorine and noradrenaline

On addition of 100µM oxotremorine to the cells, oscillatory intracellular Ca^{2+} responses were observed and whilst the number of cells responding and the oscillation frequency decreased throughout the experiment, with Period 2 < Period 1, the reductions were not significant (Figures 3.21a and 3.22). Noradrenaline addition 315s into the experiment, in the presence of 100µM oxotremorine, caused the cells that initially responded to continue and sustained the frequency of that response at the level seen on initial oxotremorine stimulation, Period 2 = Period 1 (Figure 3.22). In cells treated only with 100µM oxotremorine the percentage of cells responding and the response frequency had decreased by 315s, Period 2< Period 1, into the experiment (Figure 3.22).

When the cells were stimulated with 1µM oxotremorine the number of cells producing an intracellular Ca^{2+} response stayed constant throughout the 600s of the experiment (Figures 3.23a and 3.24). Addition of 10µM noradrenaline after oxotremorine caused a significant increase in the Ca^{2+} oscillation frequency. The percentage of cells responding significantly increased after addition of noradrenaline in the presence of oxotremorine (Period 2>Period 1) when compared to the continued presence of oxotremorine alone (Figures 3.23b and 3.24). The magnitude (area under the curve) of the Ca^{2+} responses was significantly increased upon noradrenaline addition compared to the Ca^{2+} responses from oxotremorine alone (Figure 3.24).

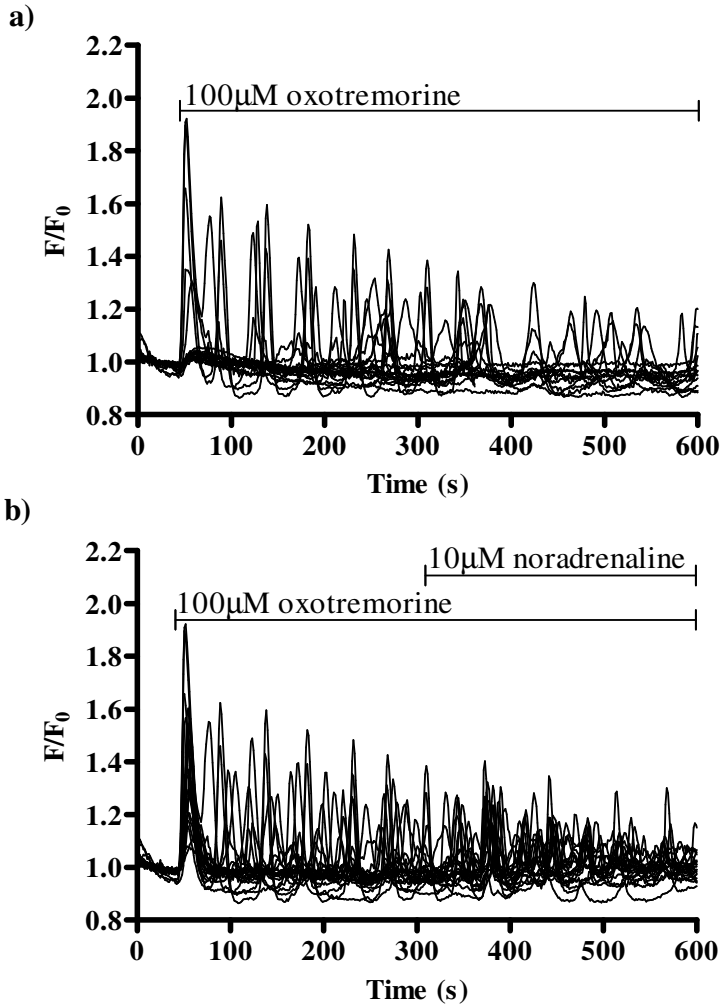
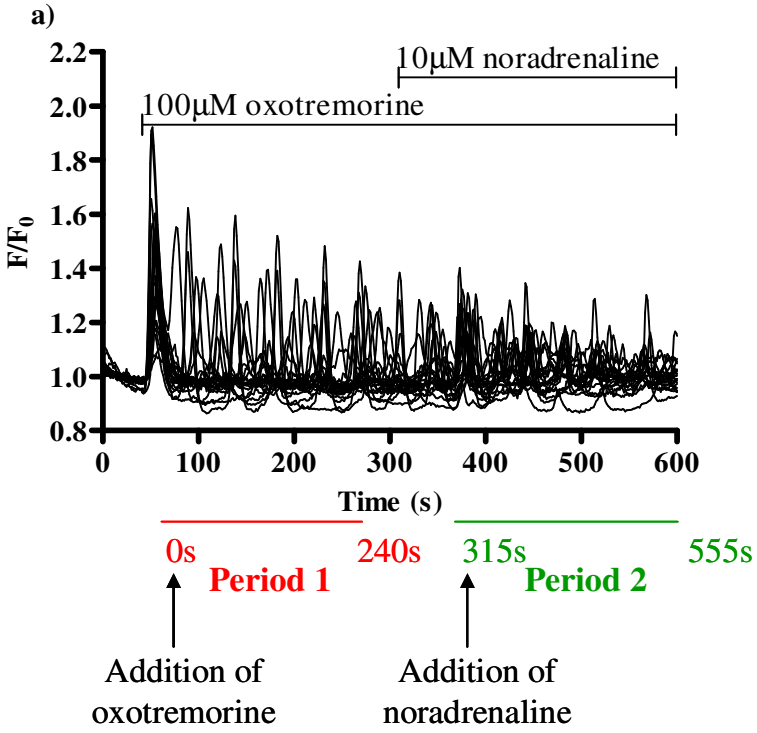


Figure 3.21. Single cell imaging of Ca^{2+} responses to 100µM oxotremorine (E_{max} concentration) and 10µM noradrenaline in HEK 293 cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods,* then imaged by confocal microscopy. Cells were challenged with 100µM oxotremorine (a) or 100µM oxotremorine and 10µM noradrenaline (b), by bath addition. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 16 cells in the field of view. Data are representative of n=4.

a)



b)

	100µM oxotremorine alone		100µM oxotremorine and 10µM noradrenaline	
			oxotremorine alone	oxotremorine and noradrenaline
	Period 1	Period 2	Period 1	Period 2
% cells responding	71 ± 13	37 ± 19	63 ± 20	81 ± 14
Response frequency (oscillations min^{-1} cell^{-1})	0.54 ± 0.19	0.3 ± 0.18	0.60 ± 0.2	0.63 ± 0.31
Ratio of area under response (period 2/period 1)	0.64 ± 0.52		1.1 ± 0.6	

Figure 3.22. Analysis of responses to 100µM oxotremorine (E_{max} concentration) and 10µM noradrenaline. The percentage of cells responding was calculated for Period 1 (from addition of agonist at 30s (taken as 0s of response on panel (a) up to 285s into the experiment (240s into the response)) and Period 2 (from addition of noradrenaline at 360s (taken as 315s after initial response) to end of experiment at 600s (555s after initial response)) within each experiment (see (a) and (b)). Panel (a) is for reference only. Oscillation frequency was calculated for Period 1 and 2. Area under the oscillations was calculated for each period, Period 2/Period 1. Data are mean ± s.e.mean, n=4. *p<0.05.

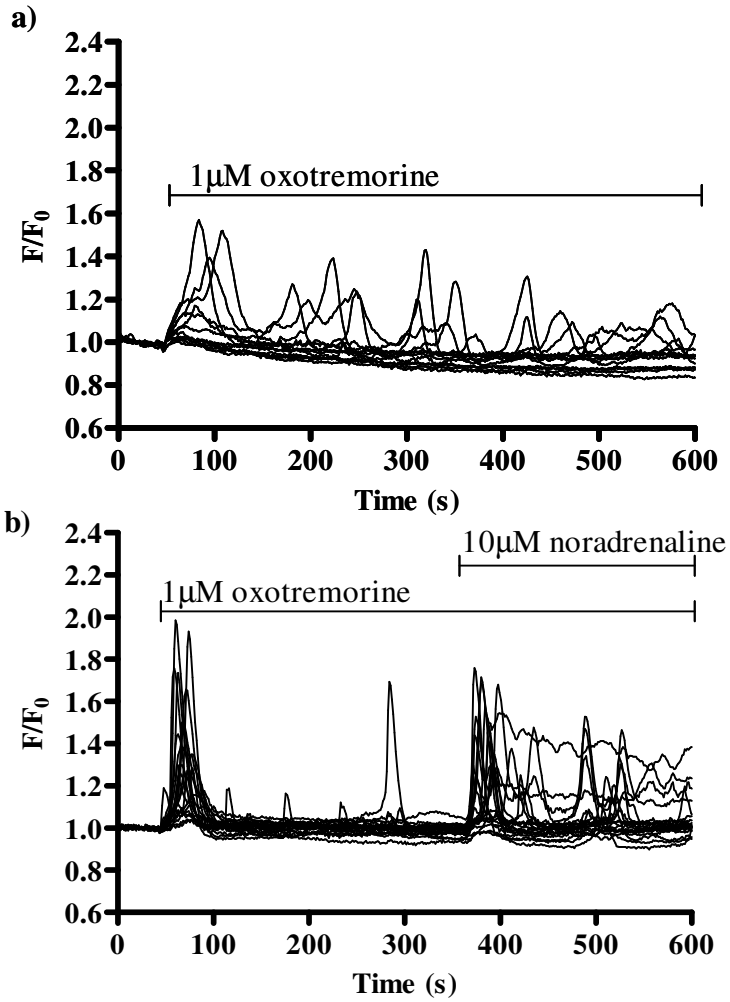
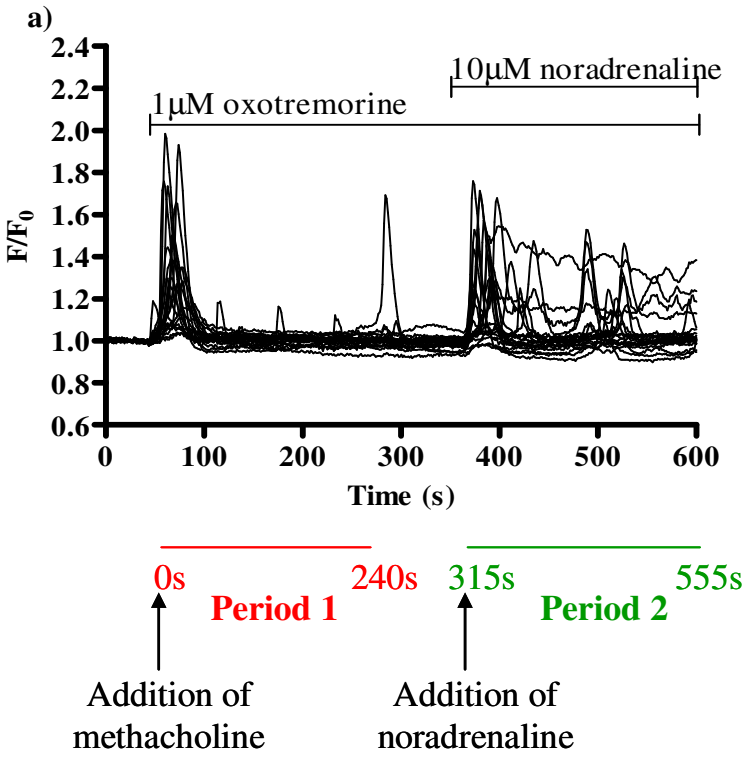


Figure 3.23. Single cell imaging of Ca^{2+} responses to 1µM oxotremorine (EC_{50} concentration) and 10µM noradrenaline in HEK 293 cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* then imaged by confocal microscopy. Cells were challenged with 1µM oxotremorine (a) or 1µM oxotremorine and 10µM noradrenaline (b) by bath addition. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 16 cells in the field of view. Data are representative of n=4.

a)



b)

	1µM oxotremorine alone		1µM oxotremorine and 10µM noradrenaline	
			oxotremorine alone	oxotremorine and noradrenaline
	Period 1	Period 2	Period 1	Period 2
% cells responding	61 ± 14	53 ± 15	62 ± 16 *	89 ± 4 *
Oscillation frequency (oscillations min^{-1})	0.41 ± 0.11	0.34 ± 0.08	0.19 ± 0.04	0.51 ± 0.12
Ratio of area under oscillations (period 2/period 1)	0.57 ± 0.13		2.36 ± 0.29 *	

Figure 3.24. Analysis of responses to 1µM oxotremorine (EC_{50} concentration) and 10µM noradrenaline. The percentage of cells responding was calculated for Period 1 (from addition of agonist at 30s (taken as 0s of response on panel (a)) up to 285s into the experiment (240s after the response)) and Period 2 (from addition of noradrenaline at 360s (taken as 315s after initial response) to end of experiment at 600s (555s after initial response)) within each experiment (see (a) and (b)). Panel (a) is for reference only. Oscillation frequency was calculated for Period 1 and 2. Area under the oscillations was calculated for each period, Period 2/Period 1. Data are mean ± s.e.mean, n=4. *p<0.05

3.3.10 Crosstalk is also mediated by UTP

UTP is able to mediate a Ca^{2+} response in wild-type HEK 293 cells via the endogenous $G\alpha_{q/11}$-coupled $P2Y_2$ receptors expressed within these cells (Werry *et al.,* 2002). Addition of 100µM UTP caused a robust increase in intracellular Ca^{2+} (Figure 3.25a). A concentration-response curve of peak intracellular Ca^{2+} responses was constructed for UTP demonstrating 100µM to be a maximal concentration and a pEC_{50} of 5.19±0.12 (~10µM) (Figure 3.25b).

In the presence of either a maximal or sub-maximal concentration of UTP, noradrenaline (10µM) was able to elicit a second intracellular Ca^{2+} response (Figure 3.26). The noradrenaline-mediated response in the presence of 100µM UTP was 109±22% of the UTP response (Figure 3.26a). In the presence of 10µM UTP the noradrenaline mediated response was 133±24% of the UTP response (Figure 3.26b). Addition of noradrenaline alone to the cells did not elicit an intracellular Ca^{2+} response.

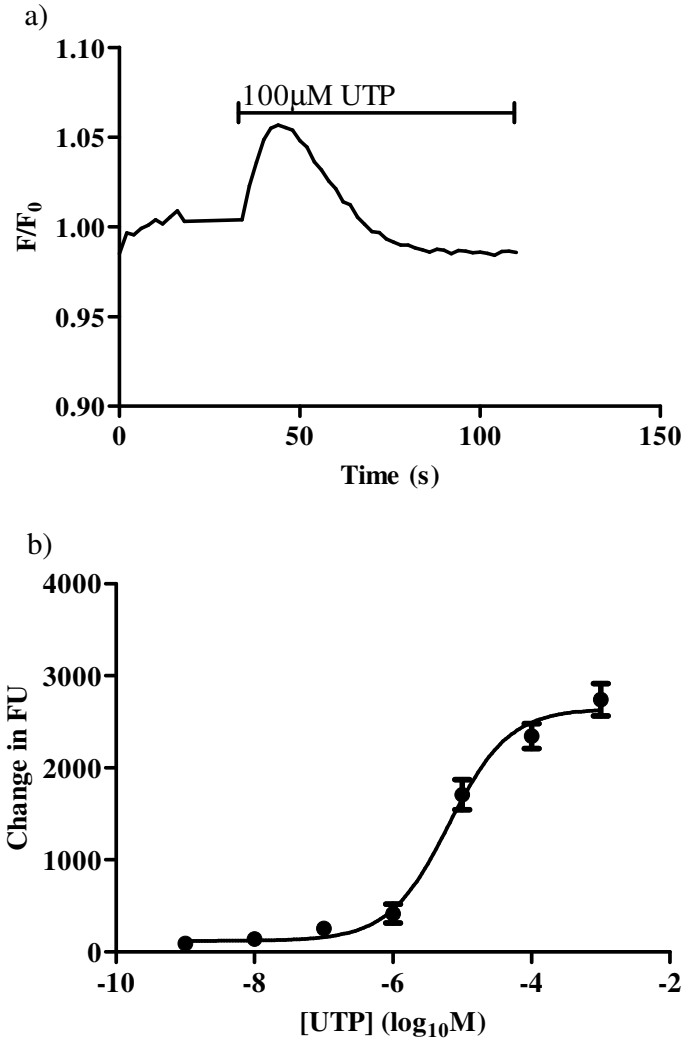


Figure 3.25. Concentration-dependent UTP Ca^{2+} responses in HEK 293 cells are concentration dependent. Populations of cells were loaded with fluo-4 AM as described in *Materials and Methods* and changes in fluorescence were measured on a NOVOstar plate reader. (a) Cells were challenged at 30s with 100µM and changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). (b) A concentration-response curve was constructed for the peak intracellular Ca^{2+} response of a range of UTP concentrations with a pEC_{50} of 5.19±0.12 (~10µM). Data are mean±s.e.mean., n=4.

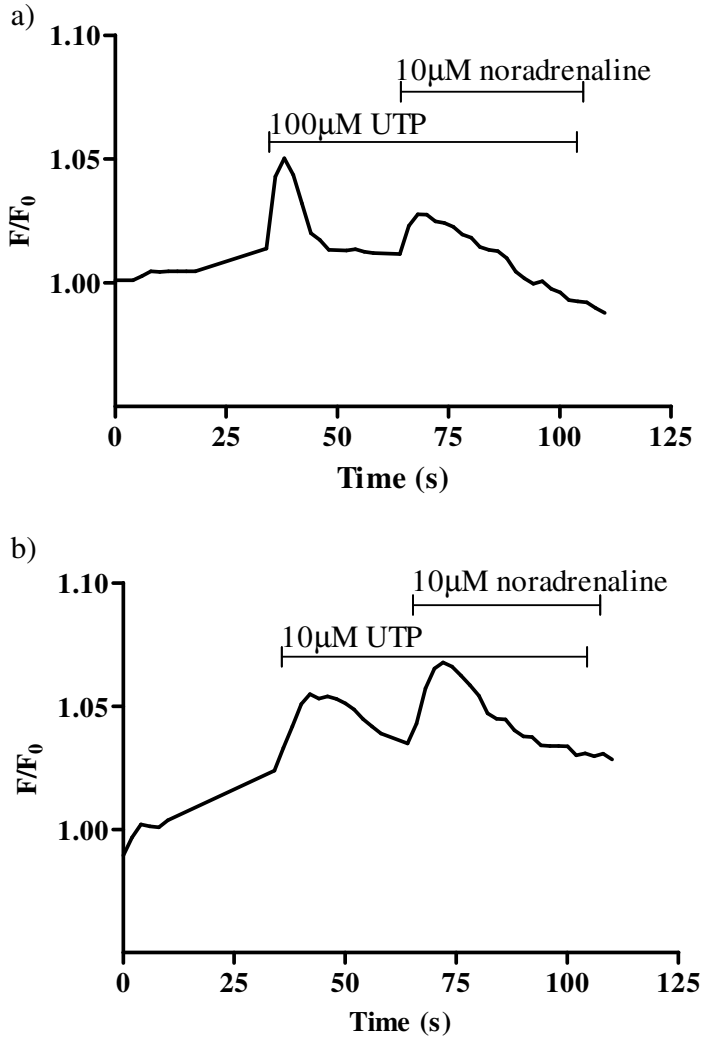


Figure 3.26. Population measurements of Ca^{2+} responses to consecutive additions of 100µM (maximal) or 10µM (sub maximal) UTP and 10µM noradrenaline in HEK 293 cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence measured on a NOVOstar fluorescence plate reader. (a) Cells were challenged with 100µM UTP at 30s and 10µM noradrenaline at 60s or (b) 10µM UTP at 30s and 10µM noradrenaline at 60s. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative of n=3

3.3.11 Concentration-dependent Ca^{2+} responses to GLP-1 and UTP in HEK 293 cells expressing the human GLP-1 receptor

Experiments were carried out in a HEK-Flp-In cell line stably expressing the human GLP-1 receptor (HEK GLP-1R) (1061±223 fmol/mg protein) (Coopman *et al.,* 2010). These experiments allowed contrast between the $G\alpha_s$-coupled GLP-1 receptor, which elicits a Ca^{2+} response on addition of its agonist without priming of the system and the $G\alpha_s$-coupled β_2-adrenoceptor, which cannot elicit a Ca^{2+} response when added alone. The HEK GLP-1R cells were unresponsive to muscarinic receptor agonists and therefore another $G\alpha_{q/11}$-coupled receptor, the P2Y receptor was used in these experiments to explore the possibility of crosstalk.

Addition of maximal and sub-maximal concentrations of either GLP-1 (GLP-1 7-36 amide) or UTP elicited robust intracellular Ca^{2+} responses (Figure 3.27a and b and 3.28a and b). The responses to both GLP-1 and UTP were concentration-dependent (Figure 3.27c and 3.28c) with pEC_{50} values for the peak responses of 5.09±0.35 (10μM) for UTP and 9.84±0.44 for GLP-1 (100pM).

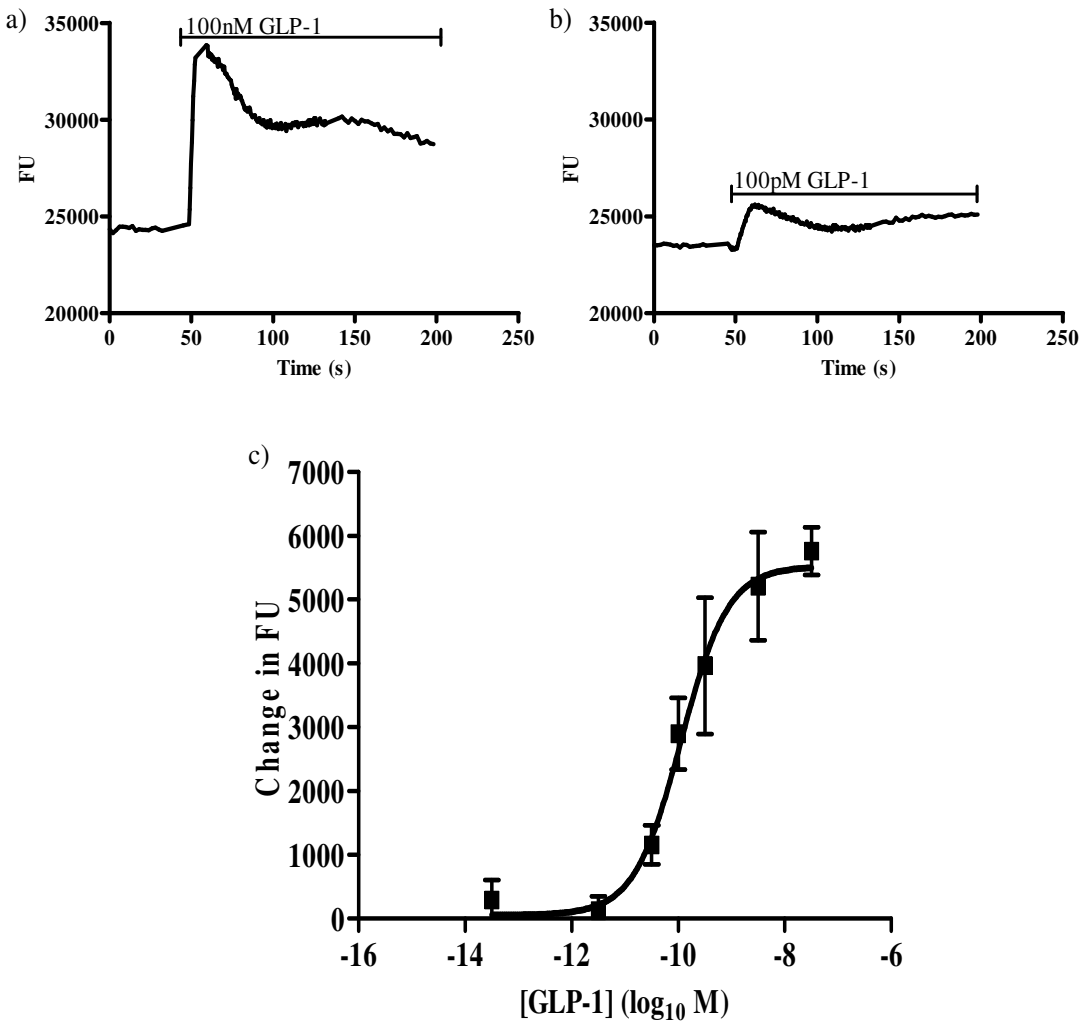


Figure 3.27. GLP-1 mediated Ca^{2+} responses in HEK GLP-1R cells. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and challenged with a range of concentrations of GLP-1 using a NOVOstar fluorescence plate reader. Representative responses are shown to (a) maximal (30nM) or (b) sub-maximal (100pM) concentration of GLP-1. A concentration-response curve weas constructed for the maximal (peak) change in fluorescence. The pEC_{50} value for the peak response was 9.84±0.44 (100pM). Data are representative of GLP-1 responses or mean ± s.e.mean, n=3.

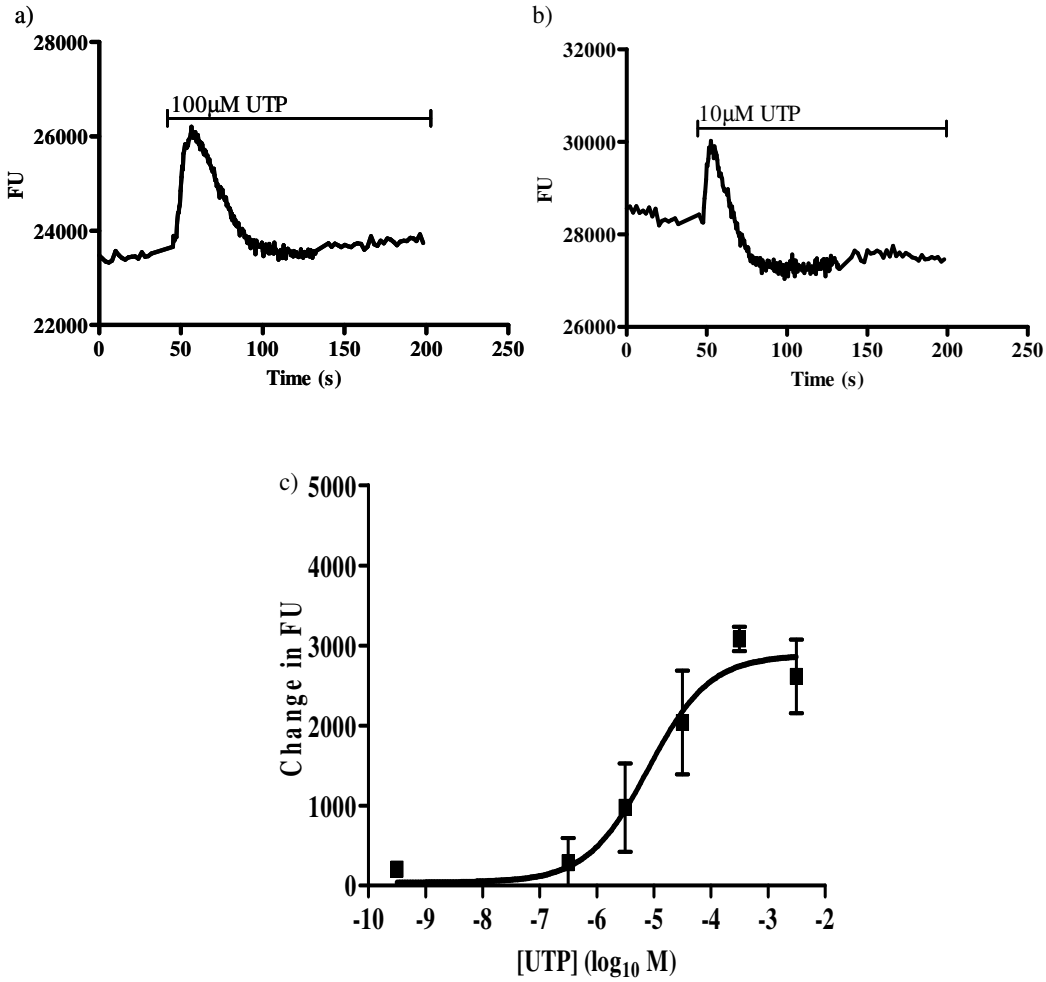


Figure 3.28. UTP mediated Ca^{2+} responses in HEK GLP-1R cells. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and challenged with a range of concentrations of UTP using a NOVOstar fluorescence plate reader. Representative responses are shown to (a) maximal (100µM) and (b) sub-maximal (10µM) concentrations of UTP. (c) A concentration-response curve was constructed for the maximal (peak) change in fluorescence. The pEC_{50} value for the peak responses was 5.09±0.35 (10µM). Data are representative of UTP responses or mean ± s.e.mean, n=3.

3.3.12 Crosstalk does not occur between P2Y receptors and GLP-1 receptors when using maximal or sub-maximal concentrations of UTP and a maximal concentration of GLP-1

On addition of a maximal concentration of GLP-1 (30nM) 45s into the experiment a rapid and robust intracellular Ca^{2+} response was observed (Figure 3.29a and 3.30). Stimulating the cells with a maximal concentration of UTP (100µM) also caused a rapid, transient Ca^{2+} response (Figure 3.28a and 3.29b). Addition of GLP-1 (30nM) 45s after treating the cells with UTP (100µM) produced an intracellular Ca^{2+} response that was not significantly different to the signal elicited by GLP-1 alone, 0.32±0.1F/F_0 and 0.41±0.1F/F_0 respectively (n=5) (Figure 3.29c and 3.30).

Stimulating the cells with a sub-maximal concentration of UTP (10µM) caused a rapid, transient Ca^{2+} response (Figure 3.31a and 3.32). Addition of GLP-1 (30nM) after treating the cells with UTP (10µM) produced an intracellular Ca^{2+} response (0.43±0.02 F/F_0) that was not significantly increased when comparing to GLP-1 alone (0.41±0.03 F/F_0) (Figure 3.32).

3.3.13 Increasing the time interval between addition of UTP and GLP-1 reduces the magnitude of the GLP-1-mediated Ca^{2+} response

GLP-1 (30nM) was added to the cells at 95s, 155s and 215s after UTP (100µM) stimulation (Figure 3.33). The magnitude of the GLP-1 response significantly decreased as the time interval increased with the responses at 95s being 133±6%, at 155s 93±17% and at 215s the response 81±13% of the UTP response (Figure 3.34). The same pattern of decreasing GLP-

1 intracellular Ca^{2+} signalling was also observed when cells had been stimulated with a sub-maximal concentration of UTP (10μM) (Figure 3.35 and 3.36).

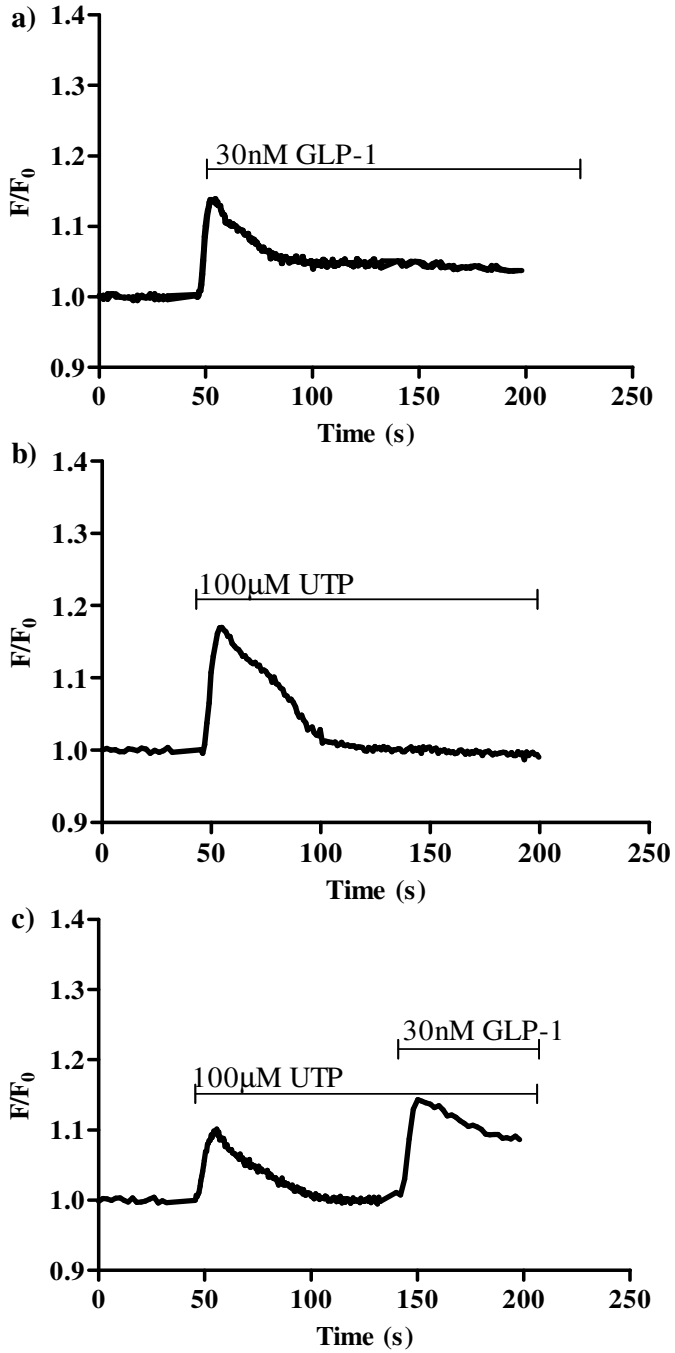


Figure 3.29. Population measurements of intracellular Ca^{2+} responses to 100µM UTP (E_{max} concentration) and 30nM GLP-1 (E_{max} concentration) in HEK 293 stably expressing the GLP-1 receptor. Cells were loaded with fluo-4 AM as specified in *Materials and Methods*. Changes in fluorescence were measured on a NOVOstar plate reader. Cells were challenged with 30nM GLP-1 at 45s (a), 100µM UTP at 45s (b) or 100µM UTP at 45s and 30nM GLP-1 at 95s after UTP (c). Changes in cytosolic fluorescence was calculated compared to the basal (F/F_0). Data are representative of n=3.

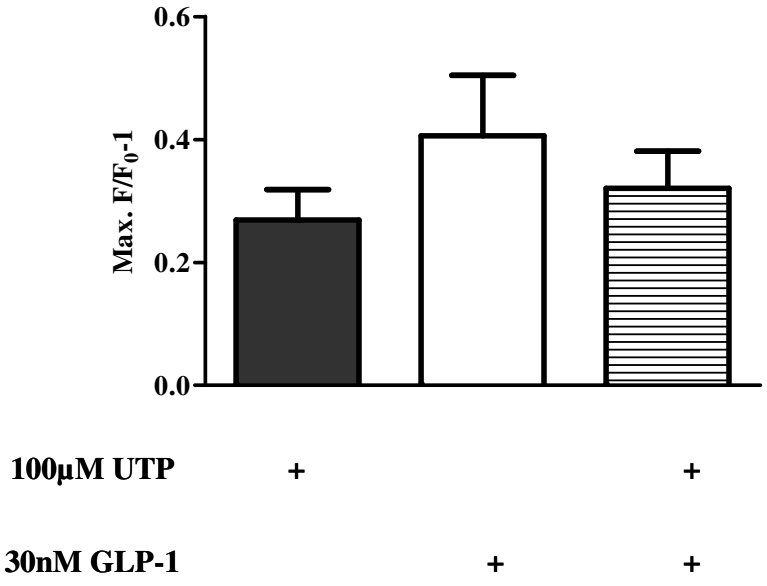


Figure 3.30. Mean peak Ca^{2+} responses mediated by UTP, GLP-1 or GLP-1 in the presence of UTP in HEK 293 cells stably expressing the GLP-1 receptor. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and challenged with either 100µM UTP at 45s, 30nM GLP-1 at 45s or 30nM GLP-1 in the presence of 100µM UTP (as shown in Figure 3.29). Data are mean + s.e.mean, n=5.

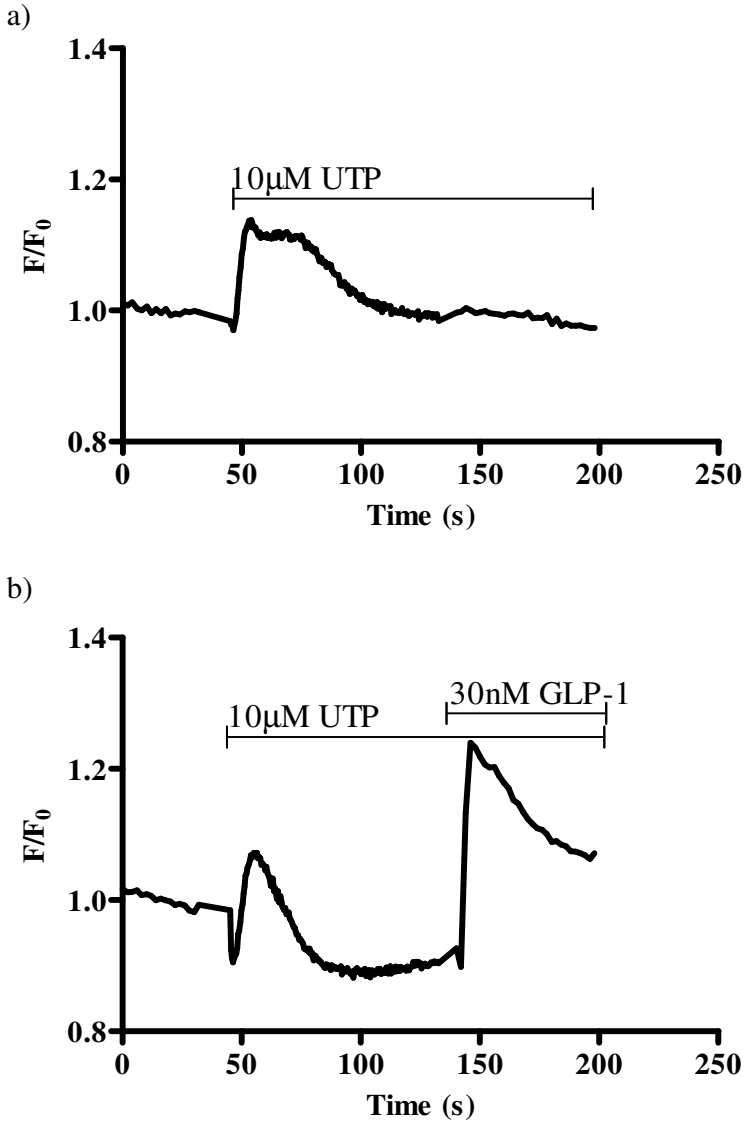


Figure 3.31. Population measurements of intracellular Ca^{2+} responses to 10µM UTP and 30nM GLP-1 in HEK 293 stably expressing the GLP-1 receptor. Cells were cultured and loaded with fluo-4 AM as specified in *Materials and Methods*. Changes in fluorescence were measured on a NOVOstar plate reader. Cells were challenged with (a) 10µM UTP at 45s or (b) 10µM UTP at 45s and 30nM GLP-1 95s after UTP. Change in cytosolic fluorescence was calculated compared to the basal (F/F_0). Data are representative of n=3.

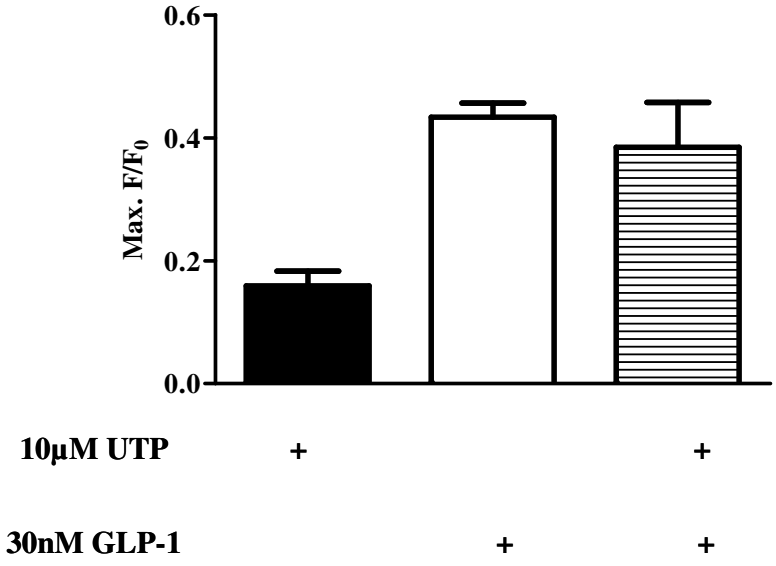


Figure 3.32. Mean Ca^{2+} responses mediated by UTP, GLP-1 or GLP-1 in the presence of UTP in HEK 293 cells stably expressing the GLP-1 receptor. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and challenged with either 10µM UTP at 45s, 30nM GLP-1 at 45s or 30nM GLP-1 in the presence of 100µM UTP (as shown in Figure 3.31). Data are mean+s.e.mean., n=5.

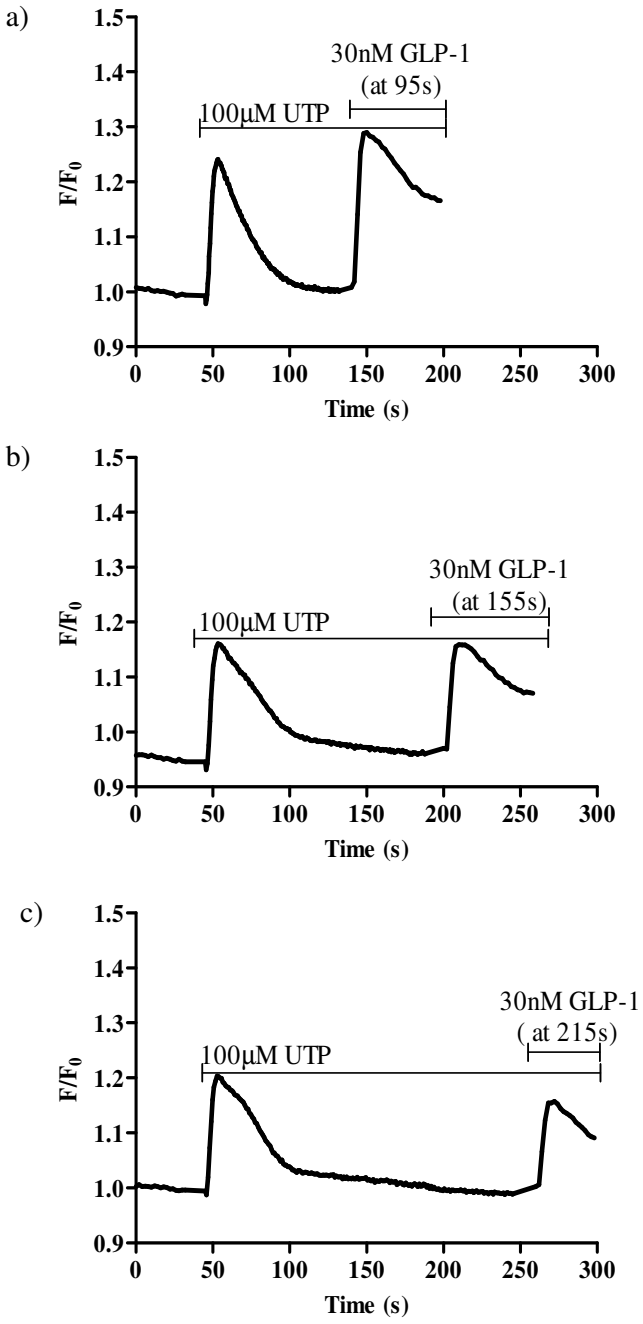


Figure 3.33. Effect of altering the time between agonist additions on the GLP-1 response in HEK GLP-1R cells. Populations of cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence were measured on a NOVOstar plate reader. Cells were challenged with (a) 100µM UTP at 45s followed by 30nM GLP-1 at either 95s, (b) 155s or (c) 215s, after UTP. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative of n=3.

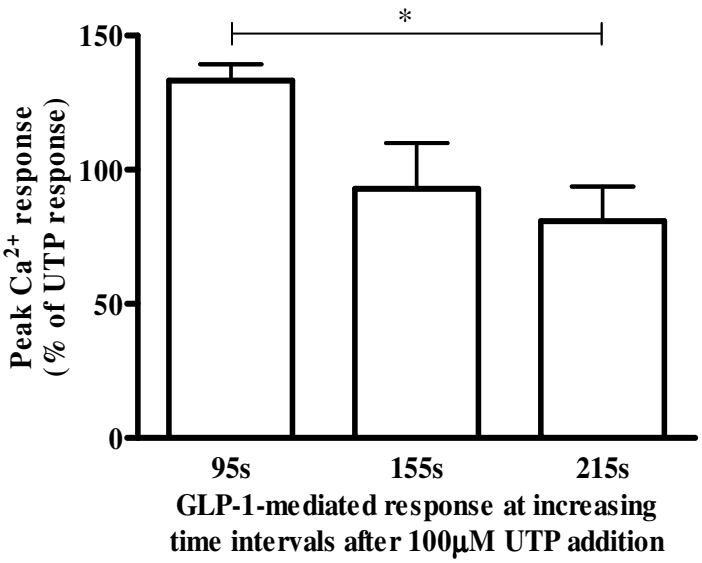


Figure 3.34. Mean peak GLP-1-mediated intracellular Ca^{2+} response at increasing time intervals between initial 100µM UTP and GLP-1 addition. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and challenged with 100µM UTP at 45s followed by 30nM GLP-1 at 95s, 155s or 215s after UTP (as shown in Figure 3.33). Data are mean+s.e.mean, n=5; * $p<0.05$ by one-way ANOVA and Dunnett's pot hoc test (performed on raw data).

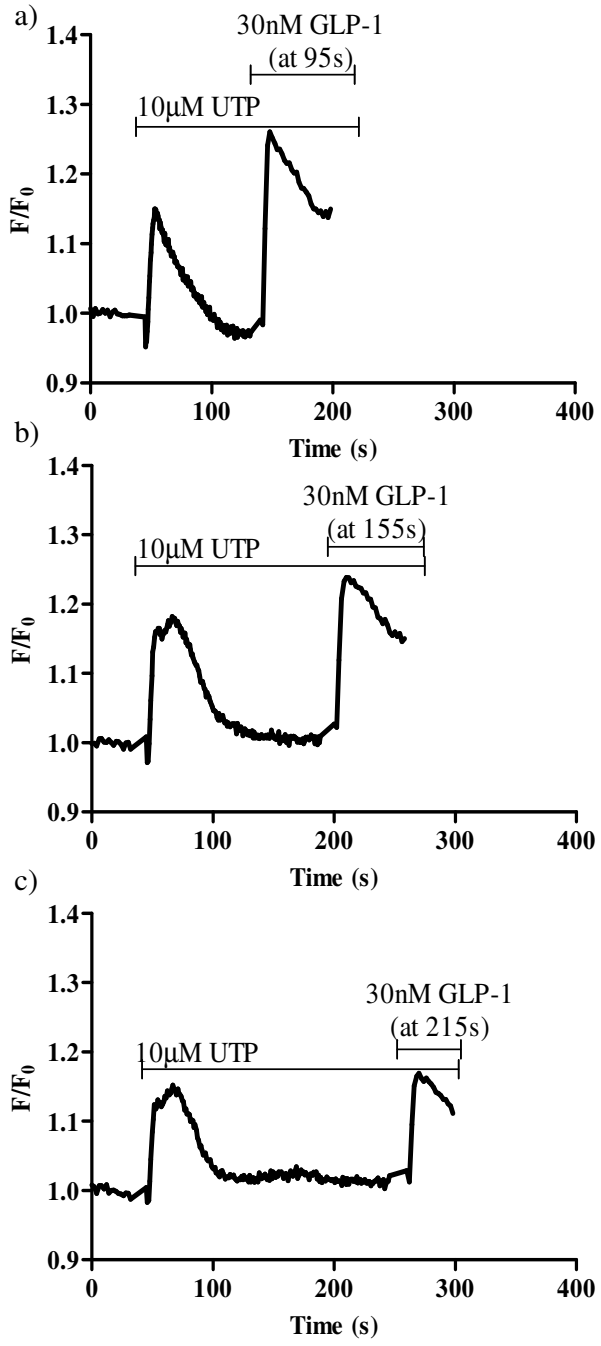


Figure 3.35. Effect of altering the time between agonist additions on the GLP-1 response in HEK GLP-1R cells. Populations of cells were loaded with fluo-4 AM as specified in *Materials and Methods*. Changes in fluorescence were measured on a NOVOstar plate reader. Cells were challenged with 10µM UTP at 45s followed by 30nM GLP-1 at either (a) 95s, (b) 155s or (c) 215s, after UTP addition. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative of n=3.

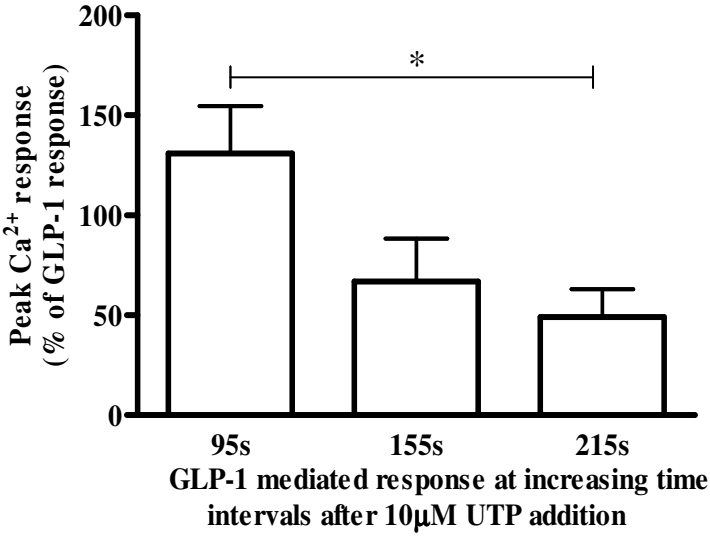


Figure 3.36. Mean peak GLP-1-mediated intracellular Ca^{2+} response at increasing time intervals between initial 10µM UTP and GLP-1 addition. Cells were loaded with fluo-4 AM as described in *Materials and Methods* and challenged with 10µM UTP at 45s followed by 30nM GLP-1 at 95s, 155s and 215s after GLP-1 addition (as shown in Figure 3.35). Data are mean + s.e.mean, n=5; * $p<0.05$ by one-way ANOVA and Dunnett's post hoc test (performed on raw data).

3.4 Discussion

3.4.1 Summary of data

This chapter aimed to explore whether crosstalk occurs between $G\alpha_s$-coupled and $G\alpha_{q/11}$-coupled receptors, specifically whether $G\alpha_s$-coupled β_2-adrenoceptors signal to Ca^{2+} in the presence of either activated muscarinic M_3 or P2Y receptors, both of which are $G\alpha_{q/11}$-coupled. Additional studies were performed to examine the potential of such crosstalk with the $G\alpha_s$-coupled GLP-1 receptor.

Both population and single cell experiments were carried out to observe the differences in the pattern of responses. In single cell experiments it was possible to see oscillatory intracellular Ca^{2+} responses because each individual cell, in the viewing area of the coverslip, was being observed. Therefore although many responses can be observed together, the magnitude of the response will only be equivalent to the response of a single cell. However, when carrying out experiments using populations of cells the response of all the cells is measured, at a particular time point and therefore the sum of the magnitude of all the signals are read. Therefore responses produced by populations of cells appear greater in magnitude than those of single cells because the sum of the responses is detected.

On activation of the muscarinic M_3 receptor an intracellular Ca^{2+} response was seen. In these HEK 293 cells, when observing both the concentration-response curves for peak and 'fixed time point' measurement intracellular Ca^{2+} responses, to methacholine and oxotremorine, it is clear oxotremorine is a partial agonist when compared to the full agonist methacholine. When measuring the 'fixed time point' in comparison to the peak response, a

rightward shift in the pEC_{50} was observed. This shift in pEC_{50} may occur because at the 'fixed time point' measurement, agonist has been bound to the receptors for 25 seconds causing desensitisation. For the 'fixed time point' measurements to gain an equivalent response to the peak response signal a greater number of receptors would need to be activated and therefore a higher concentration of agonist would be needed to produce the same efficacy. Therefore, when comparing the same measurement (EC_{50}) on the graph a higher concentration of agonist would be needed, when measuring the 'fixed time point', to activate 50% of the receptors. The maximum response of the receptors to either the partial of full agonist is also reduced because some desensitised receptors will be unable to elicit a response therefore reducing the overall intracellular Ca^{2+} signal produced by the cells.

On addition of noradrenaline, an adrenoceptor agonist, no intracellular Ca^{2+} response was elicited. However, after priming with a muscarinic agonist, a noradrenline-mediated Ca^{2+} response was elicited. This noradrenaline mediated Ca^{2+} response occurred via crosstalk between muscarinic receptors and β-adrenoceptors and was concentration-dependent with respect to the muscarinic agonist, with higher concentrations unable to elicit crosstalk. The partial agonist, oxotremorine, mediated more robust and consistent crosstalk. Partial agonists are less efficacious than full agonists at the receptor, resulting in a reduced response but also causing reduced receptor desensitization, which can potentially prolong receptor activation (Charlton, 2009, January *et al.,* 1997, Kurian *et al.,* 2009, Lape, *et al*., 2008). The extent of the crosstalk elicited by the full and partial muscarinic receptor agonists was not directly linked to their initial peak Ca^{2+} response. This may be due to a range of factors including store depletion, efficacy of the agonist on both the crosstalk mechanism and Ca^{2+} release. At high concentrations of methacholine the intracellular Ca^{2+}

store is likely to be depleted, leaving little or no Ca^{2+} for noradrenaline-mediated signalling. This could explain why a maximal concentration of methacholine is unable to elicit crosstalk where as a sub-maximal concentration or addition of the partial agonist to the cells mediates robust crosstalk on addition of noradrenaline. Interestingly, concentrations of methacholine and oxotremorine that produced the same magnitude in peak Ca^{2+} response (1µM and 100µM respectively) facilitated very different noradrenaline-mediated Ca^{2+} responses. However, due to differences in the oscillatory Ca^{2+} behaviour and activation rates of these agonists, differences in store depletion are likely to occur. These concentrations of the agonists are also likely to have different levels of receptor occupancy. On addition of noradrenaline to the cells, in the presence of 1µM oxotremorine, both the magnitude and frequency of response and number of cells responding increased. However, when noradrenaline was added in the presence of 100µM oxotremorine the number of cells responding, after addition of noradrenaline, was maintained whereas in the absence of noradrenaline the number of cells responding significantly decreases. The dynamics involved in filling and refilling the intracellular Ca^{2+} stores could differ between the maximal and EC_{50} concentrations of oxotremorine causing different filling and emptying of the stores, causing the differences in signalling observed in the results.

Crosstalk was also elicited when the agonists were added in the opposite order, with noradrenaline priming the cells and then oxotremorine activating the muscarinic receptors. The mechanistic reasons behind this synergistic increase in oxotremorine-mediated Ca^{2+} response is unknown, however there are several possible reasons for its occurrence. The noradrenaline may be accessing additional intracellular Ca^{2+} stores increasing the total amount of available Ca^{2+} for the oxotremorine mediated reponse. There are several

potential mechanisms for this increase in available intracellular Ca^{2+} (explored further in Chaper 4) but could include priming of PLCβ or sensitisation of Ins(1,4,5)P_3 receptors. PLCβ is activated upon stimulation of the muscarinic M_3 receptors, increasing Ins(1,4,5)P_3 production and therefore activation of Ins(1,4,5)P_3 receptors. This hypothesis could be measured using a FRET-based Ins(1,4,5)P_3 biosensor known as FIRE (fluorescent Ins(1,4,5)P_3-response element) (Remus *et al.,* 2010). The biosensor has a CFP group attached to the amino terminus of the binding domain within the Ins(1,4,5)P_3 receptor and a YFP group attached to the carboxyl end. If there was an increase in Ins(1,4,5)P_3 production, due to the crosstalk, then an increase in the fluorescence ration F530/F488 would be seen when compared to cells primed with oxotremorine alone. Experiments expressing the three isoforms of the Ins(1,4,5)P_3 receptors, terminally fused with CFP and YFP, would be required to ensure false negative results were prevented. Another possible mechanism involves Ins(1,4,5)P_3 receptors sensitisation, directly or by a protein downstream of adenylate cyclase including cAMP, PKA or Epac, altering the conformation of the Ins(1,4,5)P_3 receptors allowing further release of Ca^{2+} after activation of the receptors. PKA can also exhibit indirect effects on Ins(1,4,5)P_3 receptors (Charnet *et al.,* 1995, Haase *et al.,* 1999). The protein is able to phosphorylate L-type calcium channels, increasing calcium entry into the cells, activating Ins(1,4,5)P_3 receptors and increasing intracellular Ca^{2+} release. Experiments in this thesis have shown that addition of noradenaline alone to the cells does not cause a intracellular Ca^{2+} response but it is possible that the extracellular Ca^{2+} could sensitise the Ins(1,4,5)P_3 receptors, by altering their conformation, partly ungating the receptor, ready for activation by oxotremorine. Trysinisation is one method which has bee used to measure Ca^{2+} induced conformational changes in Ins(1,4,5)P_3 receptors in rat brains (Anyatonwu *et al.,* 2010). In this study trysinisation of the C-terminal

tail of the receptor, after Ca^{2+} binding, reduced the length of the tail and allowed cleavage by trypsin, which was not possible in the absence of Ca^{2+}. A second possible mechanism for the increase in intracellular Ca^{2+} signalling is that noradrenaline causes a leftward shift in the potency curve of oxotremorine. This would cause the EC_{50} of oxotremorine, in the presence of noradrenaline, to be reduced. In order to examine this possibility oxotremorine concentration response curves should be constructed in the absence and presence of noradrenaline. Any shift in the curve could then be observed to determine any changes in potency. Further experiments with 0.1µM and 100µM oxotremorine could be carried out to see whether, in the presence of the EC_{50} shift, crosstalk occurred still occurred with these concentrations. It would be expected that no crosstalk would be seen with 100µM oxotremorine because co-addition of 100µM oxotremorine and noradrenaline did not produce a synergistic intracellular Ca^{2+} response. However with the potential increase in oxotremorine potency it may be possible that crosstalk could be elicited by 0.1µM oxotremorine.

The pattern of Ca^{2+} signalling produced by the full agonist methacholine and the partial agonist oxotremorine differed both in magnitude and temporal profile. Sub-maximal concentrations of the full muscarinic receptor agonist and maximal and sub-maximal concentrations of the partial agonist caused rapid intracellular Ca^{2+} oscillations. Addition of noradrenaline alone to the cells had no effect on Ca^{2+} oscillatory behaviour. However, addition of noradrenline in the presence of oxotremorine markedly increased oscillatory behaviour. Alterations in the pattern of intracellular Ca^{2+} signalling allow the cells to increase the versatility of its signalling, allowing selective activation of downstream effectors and cell processes (Berridge *et al.,* 2000). Release of intracellular Ca^{2+} occurs via

$Ins(1,4,5)P_3$ and ryanodine receptors and Ca^{2+} is sequestered back into the ER via sarco/endoplasmic reticulum Ca^{2+}-ATPases (Berridge *et al.,* 2003, Skupin *et al.,* 2008). Oscillatory intracellular Ca^{2+} responses may occur via CICR. Oscillatory signalling allows Ca^{2+} to control multiple cellular functions with specificity (see section 1.5). The pattern of Ca^{2+} signalling has been demonstrated to be important in the efficiency and specificity of gene transcription. Changes in intracellular Ca^{2+} oscillations have been shown to alter gene expression controlled by transcription factors including NF-AT, OAP and NF-kB (Dolmetsch *et al.,* 1997, Dolmetsch *et al.,* 1998, Li *et al*, 1998, Tomida *et al.,* 2003). Transcription factors such as NF-kB are of particular interest in the context of the airway because they regulate inflammatory genes important in diseases such as asthma and COPD.

In HEK 293 cells stably expressing the GLP-1 receptor (1061±223 fmol/mg protein), in the presence of UTP, previously shown to activate $P2Y_2$ receptors (Werry *et al.,* 2002), noradrenaline was able to mediate a second intracellular Ca^{2+} response. However, crosstalk between $P2Y_2$ receptors and the $G\alpha_s$-coupled GLP-1 receptors could not be elicited. One explanation for this may be the activation of different signalling pathways activated by the β_2-adrenoceptors and GLP-1 receptors. Although the GLP-1 receptor is $G\alpha_s$-coupled it has the ability to elicit a Ca^{2+} response without prior priming of the cell. This is consistent with its role in pancreatic β-cells where these receptors mediate the incretin effect of GLP-1 (enhanced glucose-dependent insulin release) following the release of GLP-1 from intestinal L-cells following nutrient intake (Flint *et al.,* 1998, Ørskov, 1992). A previous study using HEK 293 cells stably expressing the GLP-1 receptor demonstrated a GLP-1 mediated Ca^{2+} response. The Ca^{2+} response was suggested to occur via a PKA directed event leading to Ca^{2+} induced Ca^{2+} release via ryanodine receptors (Gromada *et al.,* 1995).

To observe whether the GLP-1 receptor could participate in crosstalk, the $G\alpha_{q/11}$-coupled $P2Y_2$ receptor, endogenously expressed in these cells, was stimulated prior to or along with GLP-1. As the time gap between addition of UTP and GLP-1 increased, the GLP-1 mediated Ca^{2+} response was reduced in magnitude. The mechanism for this is unclear but a possibility would be that $P2Y_2$ receptor-mediated activation of PKC phosphorylates intracellular Ca^{2+} release channels such as the ryanodine receptor, thereby inhibiting the release of intracellular Ca^{2+}. This has been shown in canine cardiac microsomes where PKC phosphorylated the ryanodine receptors to regulate the release of Ca^{2+} from the cardiac sarcoplasmic reticulum (Takasago *et al.,* 1991). Ca^{2+}/calmodulin-dependent protein kinases have also been shown to modulate ryanodine receptors giving a second potential mechanism by which P_2Y receptors could reduce the GLP-1 mediated Ca^{2+} response (Lokuta *et al.,* 1995, Takasago *et al.,* 1991, Yan *et al.,* 2007). Alternatively, as short-time intervals between UTP and GLP-1 additions did not reduce Ca^{2+} responses suggesting little store depletion, heterologous desensitisation of the GLP-1 receptor could be occurring.

To identify the β-adrenoceptor involved in the crosstalk, experiments were carried out in the presence of atenolol, a selective antagonist of the β_1-adrenoceptor or ICI 118,551, a selective β_2-adrenoceptor antagonist (Bilski *et al.,* 1983, Emilien and Maloteaux, 1998, Hoffman *et al.,* 2004, O'Donnell and Wanstall, 1980). Atenolol had no effect on the crosstalk whereas ICI 118,551 abolished the Ca^{2+} response elicited by noradrenaline suggesting β_2-adrenoceptors were involved in the crosstalk. Although ICI 118,551 is thought to be a competitive antagonist it has been shown in other to behave in a non-competitive manner. In order to detect which it was in this study concentration-response curves would have to be constructed in the absence and presence of antagonist However,

whether the action of ICI 118,551 is a competitive or non-competitive antagonist does not influence the identification of the receptor as a β_2-adrenoceptor in this study (Arnold *et al.,* 1985, Hopkinson *et al.,* 2000, Ostrom *et al.,* 2001, Tesfamariam and Allen, 1994). The concentration of ICI 118,551 (1μM) used in the experiments was much greater than the K_d for β_2-adrenoceptors and therefore the correct concentration should have been calculated using the Gaddum equation (Lazareno and Birdsall, 1993). This equation (see below) calculates agonist receptor occupancy in the presence of antagonist. If the agonist and antagonist compete for the same receptors, fractional occupancy by agonist (F) equals:

$$F = \frac{[A]}{[A]+K_a\left(1+\frac{[B]}{Kb}\right)}$$

Figure 3.1 Gaddum equation for calculating antagonist concentration

Agonist = A, its concentration = [A] and dissociation constant = Ka.
Antagonist = B, its concentration = [B] and dissociation constant is Kb.

The dose ratio of antagonist can be calculated by $1+\frac{[B]}{Kb}$ and calculates how the EC_{50} is altered in the presence of the antagonist.

The magnitude of the noradrenaline-mediated response elicited by crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors was dependent not only on the concentration of muscarinic agonists added to the cells but on whether a partial or full agonist was used. A maximal concentration of a full agonist may empty the Ca^{2+} store and prevent Ca^{2+} being available following addition of noradrenaline, whereas the nature of partial agonism means that these agents do not fully deplete the store (Charlton, 2009, Werry *et al.,* 2002). This

may allow a greater pool of Ca^{2+} to be available for noradrenaline-mediated signalling when following a partial agonist rather than a full agonist. Using a sub-maximal concentration of a full agonist may also prevent depletion of the intracellular Ca^{2+} store therefore having the ability to elicit crosstalk.

This chapter has demonstrated crosstalk between muscarinic M_3 receptors β_2-adrenoceptors are able to mediate a synergistic Ca^{2+}. Although speculation within this chapter suggests the Ca^{2+} is released from an intracellular store via $Ins(1,4,5)P_3$ this is investigated in the next chapter along with the mechanism of the crosstalk.

Chapter 4: Further explorations of the mechanisms of crosstalk

4.1 Overview of chapter

Further exploration of the mechanism of crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors was performed using oxotremorine (1μM) and noradrenaline (10μM), because these agonists produce the most robust crosstalk. Experiments were performed to examine whether the synergistic Ca^{2+} response elicited by crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors was from an intracellular Ca^{2+} store. Different pharmacological tools were used to manipulate the Ca^{2+} signalling including thapsigargin (a SERCA Ca^{2+} ATPase inhibitor) and xestospongin C (XeC) (a putative Ins(1,4,5)P_3 receptor inhibitor). The concentration of extracellular Ca^{2+} was altered both using nominally Ca^{2+}-free buffer and buffer titrated with EGTA to contain a Ca^{2+} concentration of 100nM. The potential mechanism for crosstalk was also explored by examining the potential roles of cAMP, PKA and Epac.

4.2 Introduction

Calcium (Ca^{2+}) is a versatile second messenger involved in many signalling events (Endo, 2009). When the Ca^{2+} concentration outside the cell is greater than in the cytoplasm, Ca^{2+} ions will cross the plasma membrane through any open Ca^{2+} channels (Putney and McKay, 1999, Putney, 2001). Ca^{2+} concentration in the cytoplasm is also affected by release of Ca^{2+} from intracellular stores, which not only causes the cytoplasmic Ca^{2+} concentration to increase but also leads to Ca^{2+} influx across the cell membrane via capacitative calcium

entry (CCE) (see sections 1.5 and 3.4.1) (Putney and McKay, 1999, Putney, 2001). CCE replenishes intracellular Ca^{2+} stores after depletion and can also mediate sustained elevations in $[Ca^{2+}]_i$ (Putney, 2001).

Many examples of crosstalk between GPCRs have demonstrated a synergistic effect on $[Ca^{2+}]_i$ but not all have studied whether this Ca^{2+} originates from an intracellular or extracellular source (see Section 1.5). Both these sources have been revealed to be important in different examples of potentiation of signalling. For example, in human neutrophils the ability of the $G\alpha_{q/11}$-coupled neurokinin 1 receptors to potentiate Ca^{2+} signalling by the $G\alpha_i$-coupled CXCR1/2 receptors required extracellular Ca^{2+} (Section 1.5.2) (Diansani *et al.,* 2001). $G\alpha_s$-coupled receptors have also been shown to potentiate $G\alpha_{q/11}$-coupled intracellular Ca^{2+} signalling. In neonatal rat dorsal root ganglion cells, activation of the $G\alpha_s$-coupled prostaglandin E_2 receptors potentiate the intracellular Ca^{2+} signalling of the $G\alpha_{q/11}$-coupled bradykinin receptor but only in the presence of extracellular Ca^{2+} (Smith *et al.,* 2000). These examples demonstrate that crosstalk, which synergistically increases the Ca^{2+} signal, can depend on an extracellular source of Ca^{2+}. There are also many examples of crosstalk between GPCRs that require intracellular Ca^{2+} for enhanced signalling. For example, crosstalk between the $G\alpha_i$-coupled adenosine A_1 receptor and the $G\alpha_{q/11}$-coupled bradykinin receptor in DDT_1 MF-2 smooth muscle cells, synergistically increased the production of $Ins(1,4,5)P_3$ and therefore the release of Ca^{2+} from the sarcoplasmic reticulum (Gerwins and Fredholm, 1992). The mechanism of this crosstalk is unknown. However, it does not involve an increase in cAMP production. In neuroblastoma/glioma hybrid cells the pathway of the $G\alpha_{q/11}$-coupled bradykinin receptor interacted with that of the $G\alpha_i$-coupled opioid receptor to increase $Ins(1,4,5)P_3$ production

and the release of intracellular Ca^{2+} (Okajima *et al.,* 1993). Although again the mechanism is unknown the suggestion is that the Gβγ-dimer synergistically enhances the activation of PLC by the $G\alpha_{q/11}$-coupled G-protein. The source of Ca^{2+} for the synergistic crosstalk cannot be assumed as both extracellular and intracellular Ca^{2+} have been shown to contribute (although predominantly intracellular Ca^{2+}) therefore this needs to be define experimentally. If the Ca^{2+} is released from an intracellular store then the type of Ca^{2+} channel mediating the Ca^{2+} release needs to be confirmed (see Section 3.4).

The mechanism of crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors, leading to a synergistic Ca^{2+} response, is unknown. Activation of β_2-adrenoceptors increases AC activity leading to accumulation of cAMP and activation of PKA and Epac (see Section 1.7). Both Epac and PKA have been implicated in crosstalk mechanisms in a variety of cells therefore it is important to establish whether either are involved in this crosstalk (see Section 1.5.1)

This chapter aims to determine whether the synergistic Ca^{2+} response elicited in HEK 293 cells, in response to activation of muscarinic M_3 receptors and β_2-adrenoceptors is from an intracellular Ca^{2+} source with a view to determining the type of intracellular Ca^{2+} channel involved. In addition the mechanism of crosstalk between the two receptor pathways was explored to determine whether PKA or Epac could be transducers of the synergism.

4.3 Results

4.3.1 Removal of extracellular Ca^{2+} has no effect on the crosstalk

In cell imaging experiments, on addition of an approximate EC_{50} concentration of oxotremorine (1μM) in the presence of extracellular Ca^{2+} (Ca^{2+}_e) 61±14% of the cells responded, with all of these cells demonstrating a constant oscillatory frequency and magnitude of response throughout the experiments (Figure 4.1a). This pattern of Ca^{2+} response was also observed in experiments performed in nominally Ca^{2+}-free buffer (Figure 4.1b). When experiments were carried out in the presence of Ca^{2+}_e, addition of noradrenaline (10μM), after the cells had been primed with oxotremorine (1μM), caused most of the cells to respond (89±4%). The frequency of the oscillatory response increased in the presence of oxotremorine and noradrenaline when compared to oxotremorine alone (0.19±0.04 to 0.51±0.12 $min^{-1}cell^{-1}$) (Figure 4.1c). The same increase in both the number of cells responding (from 53±15 to 93±5%) and oscillatory frequency (0.19±0.04 to 0.50±0.10 $min^{-1}cell^{-1}$) was observed in the crosstalk experiments carried out in nominally Ca^{2+}-free buffer (Figure 4.1d). However, when the extracellular Ca^{2+} was removed, the noradrenaline-mediated oscillatory pattern was not sustained after 480 seconds into the experiment and the oscillatory frequency and magnitude significantly decreased (Figure 4.1d).

These experiments were carried out in nominally Ca^{2+}-free buffer to assess the potential role of Ca^{2+} entry across the plasma membrane to the responses. Experiments were also performed using buffer in which the extracellular Ca^{2+} was titrated to approximately

100nM using EGTA (determined using standard techniques with fura-2; Grynkiewicz *et al.,* 1985) (Figure 4.2). When cells were stimulated with oxotremorine in the presence of extracellular Ca^{2+}, a response was observed (Figure 4.2a). In EGTA buffered krebs'-HEPES, the peak height of the oxotremorine-mediated signal was significantly reduced (Figure 4.2c and 4.3). When the cells were stimulated with noradrenaline (10μM) in the continued presence of 1μM oxotremorine, a second Ca^{2+} response was observed (Figures 4.2b and d). The removal of extracellular Ca^{2+} (EGTA buffered) had no effect on the amplitude of the response mediated by noradrenaline in the presence of oxotremorine (Figure 4.2b, d and 4.3).

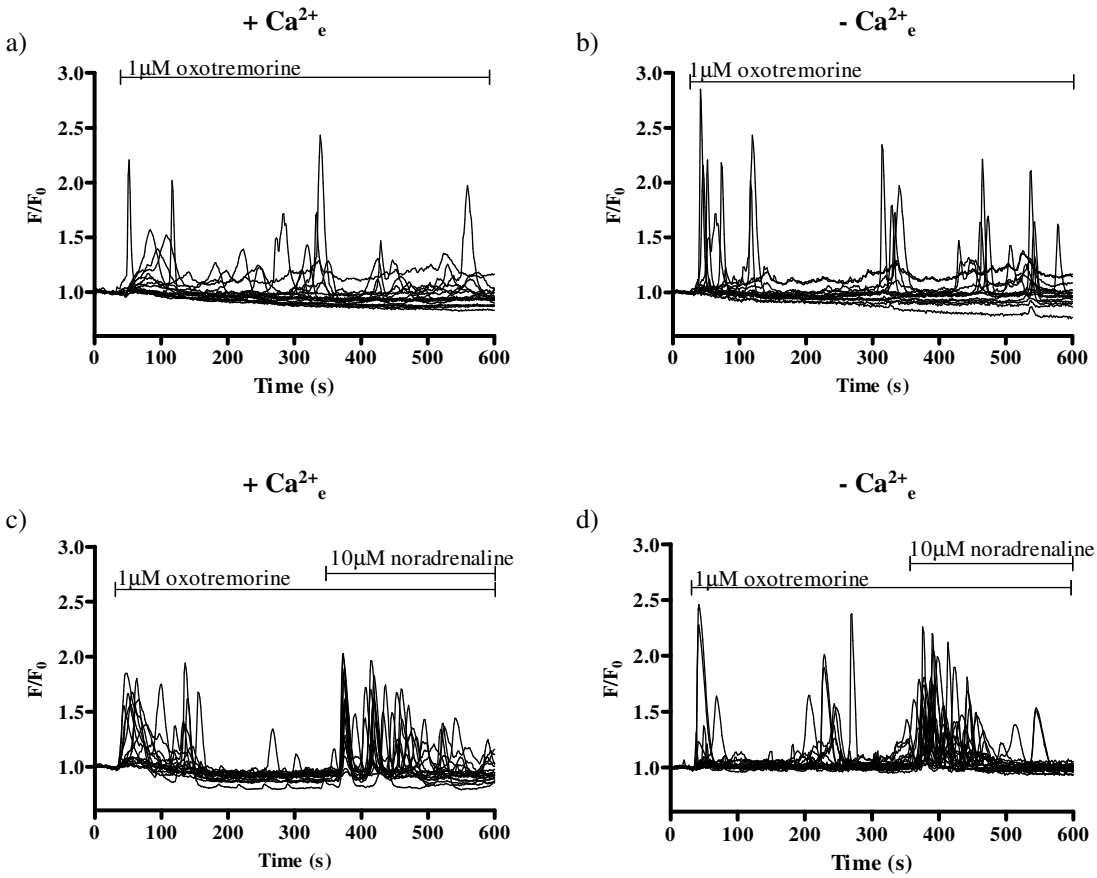


Figure 4.1 Single cell imaging of Ca^{2+} responses to 1µM oxotremorine and 10µM noradrenaline in HEK 293 cells in the presence and absence of Ca^{2+}_e (nominally free). Cells were loaded with fluo-4 AM as specified in *Materials and Methods,* then imaged by confocal microscopy. Cells were challenged with 1µM oxotremorine in the presence (a) or absence (b) of Ca^{2+}_e, or challenged with 10µM noradrenaline, following 1µM oxotremorine, in the presence (c) or absence (d) of Ca^{2+}_e. Fluorescence was calculated compared to the basal (F/F_0) and is shown for approximately 16 cells in the field of view. Data are representative of n=3 experiments, each with ≥ 16 cells.

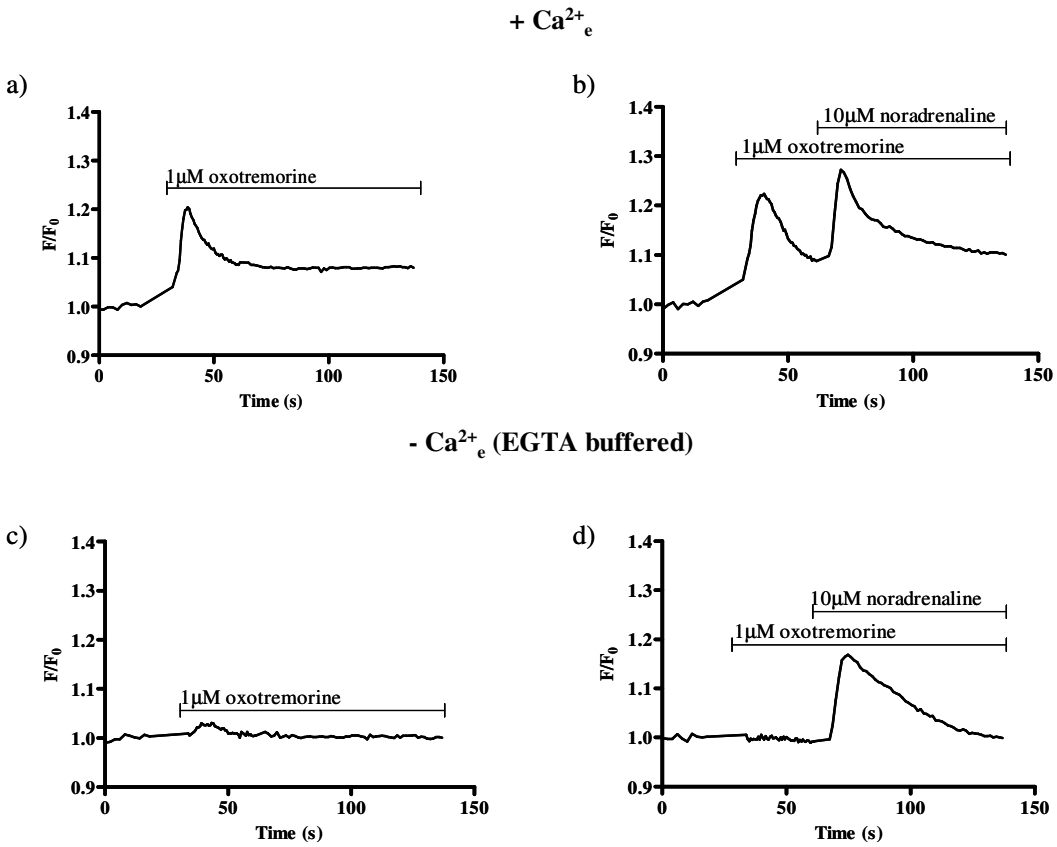


Figure 4.2 Effect of Ca^{2+}_e on intracellular Ca^{2+} responses to 1μM oxotremorine and 10μM noradrenaline. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence were measured on a NOVOstar plate reader. Cells were challenged in the presence (a, b) or absence (c, d) of Ca^{2+}_e with 1μM oxotremorine (a, c), or 10μM noradrenaline following 1μM oxotremorine (b, d). Fluorescence was calculated compared to the basal (F/F_0). Data are representative of n=5.

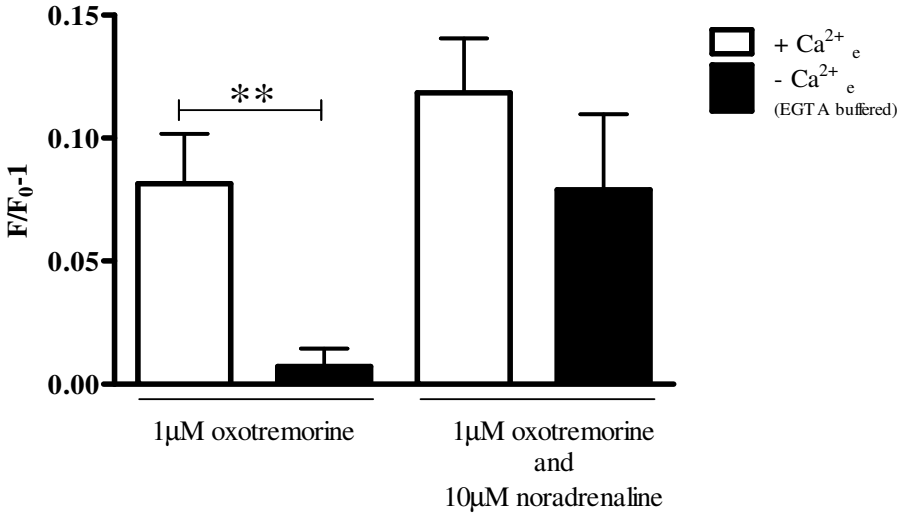


Figure 4.3 Influence of Ca^{2+}_e on mean Ca^{2+} responses elicited by either 1µM oxotremorine or 10µM noradrenaline in the presence of oxotremorine. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence measured in cell populations as an index of changes in $[Ca^{2+}]_i$. Shown are max. responses to addition of 1µM oxotremorine in the presence and absence of Ca^{2+}_e and the responses to 10µM noradrenaline, when cells had been primed with oxotremorine, again in the presence and absence of Ca^{2+}_e. Change in fluorescence was calculated compared to the basal (F/F_0). Data are mean + s.e.mean, n=5; ** $p<0.01$ Student's *t*-test.

4.3.2 Thapsigargin abolished agonist-induced changes in $[Ca^{2+}]i$

Stimulation with an EC_{50} concentration (1μM) of oxotremorine or a maximal concentration of noradrenaline (10μM) in the presence of oxotremorine caused an intracellular Ca^{2+} response in both populations of cells (Figure 4.4a and b) and single cells (4.5a and b). Pre-incubation of cells with the SERCA pump inhibitor, thapsigargin (2μM, 10min) abolished responses both to the muscarinic receptor agonist and the subsequent addition of 10μM noradrenaline in populations of cells (Figure 4.4c and d) and single cells (Figure 4.5c and d). In the single cell experiments, in the absence of thapsigargin, 61±14% of cells initially responded to oxotremorine whereas in the presence of thapsigargin no cells responded (Figure 4.5a and c). This reduction in signal was also reflected in the crosstalk experiments where addition of noradrenaline in the presence of oxotremorine caused 89±4% of the cells to respond but in the presence of thapsigargin this was abolished (Figure 4.5b and d).

4.3.3 Role of $Ins(1,4,5)P_3$-receptors in crosstalk

Pre-incubation of cells with XeC, (10μM, 30min) significantly reduced both the initial muscarinic Ca^{2+} responses to either maximal (100μM) or EC_{50} (1μM) concentrations of either methacholine or oxotremorine (Figure 4.6a) Furthermore, XeC also reduced the crosstalk responses (Figure 4.6b). These agonists were added under conditions where the external $[Ca^{2+}]$ was buffered with EGTA to approximately 100nM. Responses to the subsequent addition of 10μM noradrenaline were also reduced with the exception of addition in the presence of 100μM methacholine (Figure 4.6b). The experiments were carried out in EGTA buffered Krebs'-HEPES because agents such as XeC have been

reported to block store-operated channels, voltage-gated Ca^{2+} and K^{+} channels (Bootman et al., 2002, Liu and Ambudkar, 2001, Ozaki et al., 2002).

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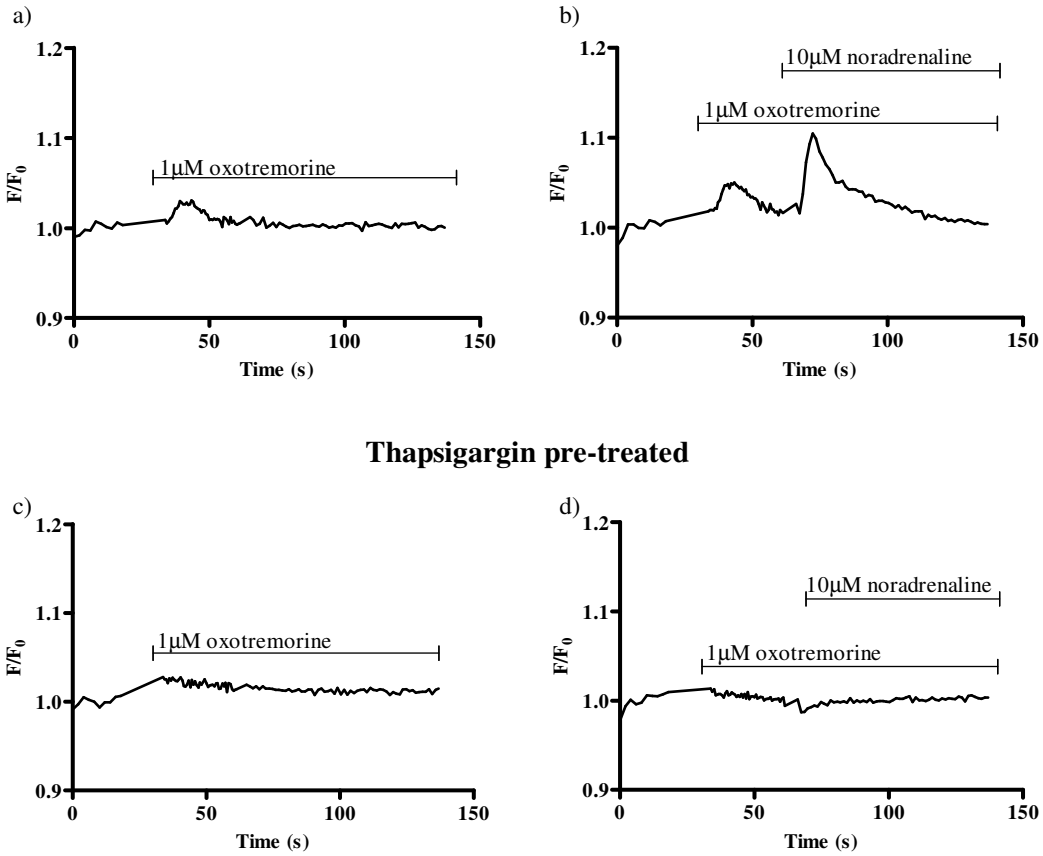


Figure 4.4 Effect of thapsigargin on agonist-mediated Ca^{2+} responses and crosstalk. Populations of cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence measured on a NOVOstar plate reader. Cells were challenged with 1μM oxotremorine (a, c) or 10μM noradrenaline in the presence of 1μM oxotremorine (b, d) either without (a, b) or with (c, d) thapsigargin pretreatment (2μM, 10min). Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0). Data are representative of n=3.

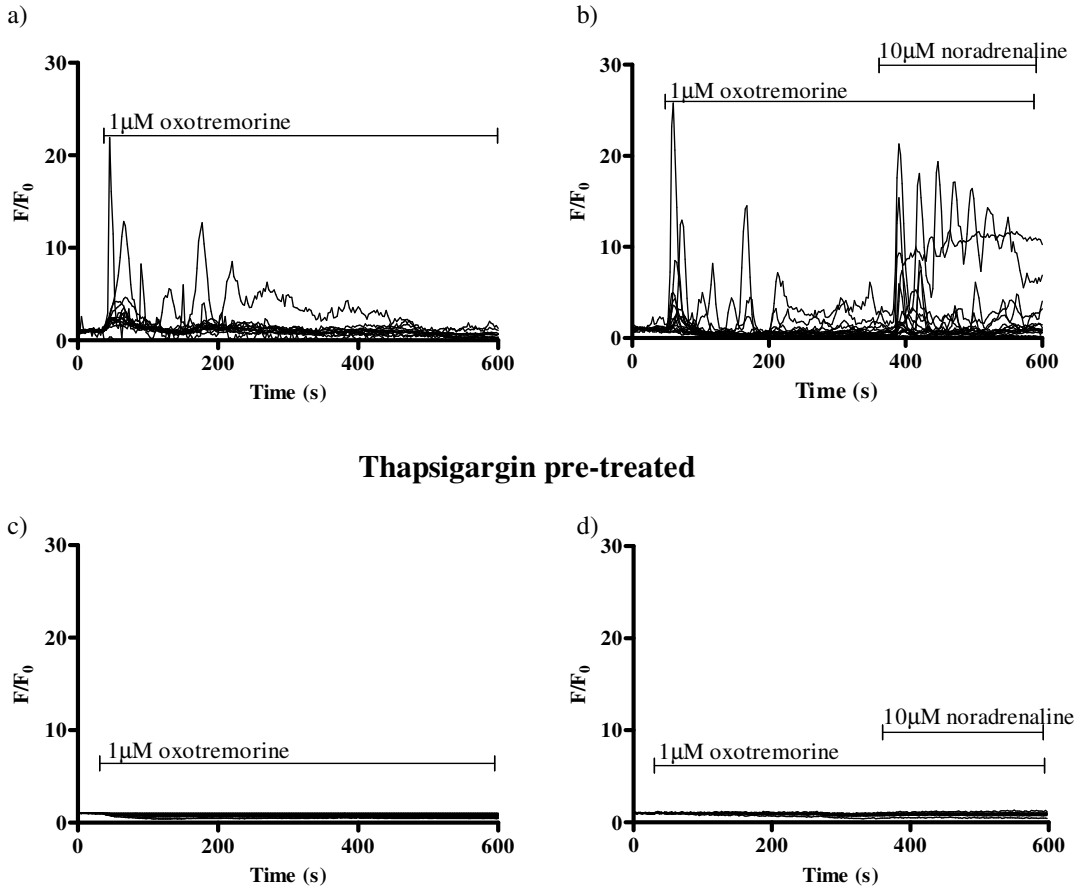


Figure 4.5. Effect of thapsigargin on single cell Ca^{2+} signalling patterns. Cells were loaded with fluo-4 AM as specified in *Materials and Methods,* then imaged by confocal microscopy. Cells were challenged with 1µM oxotremorine (a, c) or 10µM noradrenaline in the presence of 1µM oxotremorine (b, d) either without (a, b) or with (c, d) pretreatment with thapsigargin (2µM, 10min). Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 16 cells in the field of view. Data are representative of n=3.

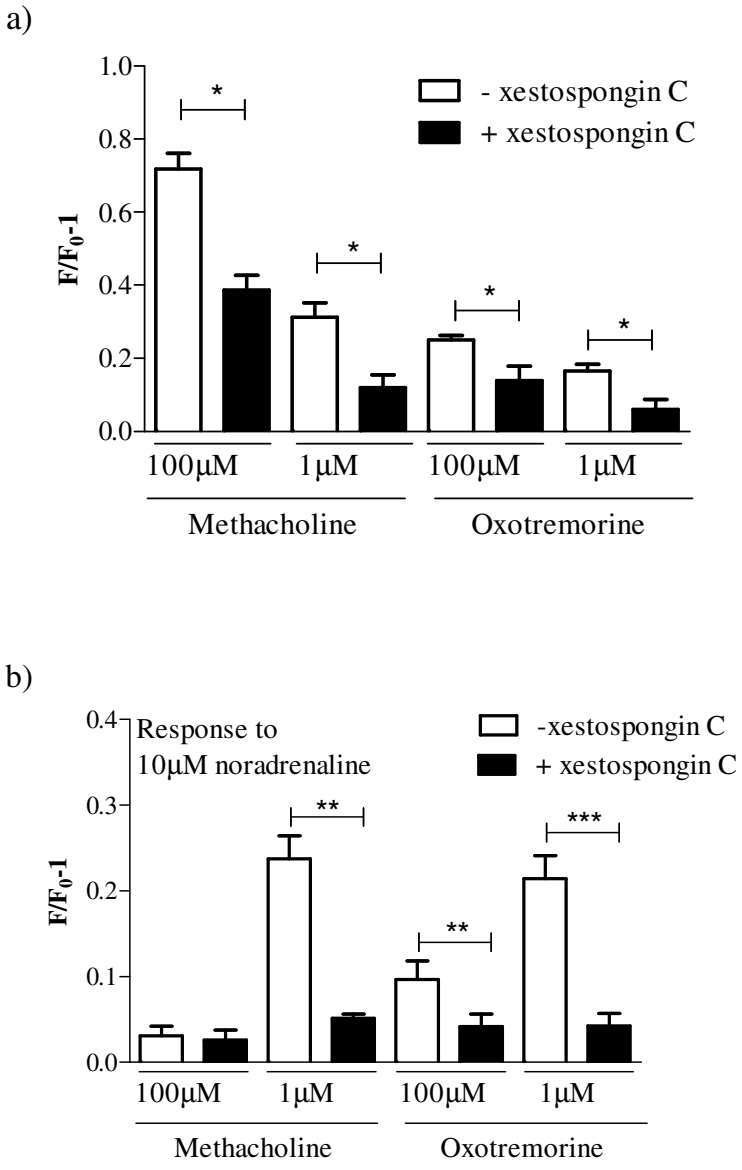


Figure 4.6 Effect of the putative Ins(1,4,5)P_3 receptor Ca^{2+} channel blocker, xestospongin C, on muscarinic receptor-mediated Ca^{2+} responses and crosstalk, in the absence of extracellular Ca^{2+} (EGTA buffered). Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence measured on a NOVOstar plate reader. Cells were incubated with or without xestospongin C (10µM) for 30min and stimulated with a maximal or EC_{50} concentration of muscarinic agonist followed by noradrenaline (10µM). Shown are maximal responses to oxotremorine (a) and noradrenaline (b). Changes in fluorescence were calculated compared to the basal (F/F_0-1). Data are mean + s.e.mean, n=3; *p<0.05, **p<0.01 and ***p<0.001, Student's *t*-test.

4.3.4 Crosstalk is independent of PKA

In the presence of the PKA inhibitors H89 (10μM, 30min pre-incubation) and PKA amide inhibitor 14-22 (25μM, 30min pre-incubation) muscarinic- and crosstalk-mediated Ca^{2+} responses were unaffected (Figure 4.7a to d). The efficacy of the PKA inhibitors was demonstrated by their ability to inhibit forskolin-mediated activation of ERK, as assessed by levels of pERK (Figure 4.7e). Total ERK blots were performed to ensure the differences in results were not dependent on different levels of protein in the blots (Figure 4.7e). Experiments were carried out with other PKA inhibitors, CMIQ and KT5720 but when studying forskolin-mediated phosphorylation of ERK as the outcome, these molecules did not show inhibition. Therefore, functional experiments were only carried out using H89 and PKA amide 14-22.

4.3.5 Construction of pIRES2-DsRed2/HA-Epac1 plasmid

The DNA encoding human Epac1 (exchange protein activated by cAMP) gene was cloned by PCR using the primers shown in *Materials and Methods* (Sections 2.3.15 and 2.3.3). The starting template used in the PCR was pCFP-Epac1(δDEP)-YFP, which contains the full length gene encoding human Epac1 (Figure 4.8a). The PCRs were conducted as described in Section 2.3.2.3**.** The Epac1 was inserted into the pHA/GPL-1R-EGFP vector containing an N-terminal HA-tagged hGLP-1R with a C-terminal EGFP-tag, which was removed at Nhe1 and EcoRI sites and replaced by Epac1. The HA-Epac1 was then cut from the vector at NheI and EcoRI sites and then ligated into a Ds-Red-2 vector, containing the corresponding compatible restriction enzyme (RE) sites, to produce the new plasmid (Figure 4.8b).

4.3.6 Intracellular Ca^{2+} experiments on cells transfected with pIRES2-DsRed2/HA-Epac1 plasmid

The pIRES2-DsRed2/HA-Epac1 plasmid was transiently transfected into wild-type HEK 293 cells to observe whether over-expression of Epac1 had any effect on crosstalk. When the transfected cells were stimulated with either 1μM oxotremorine (Figure 4.9a and 4.9b) or 10μM noradrenaline in the presence of oxotremorine (Figure 4.9a and 4.9b) the Ca^{2+} response was not significantly different to the untransfected cells (91±5% and 89±4% of cells responded respectively to noradrenaline in the presence of oxotremorine) (Figure 4.10).

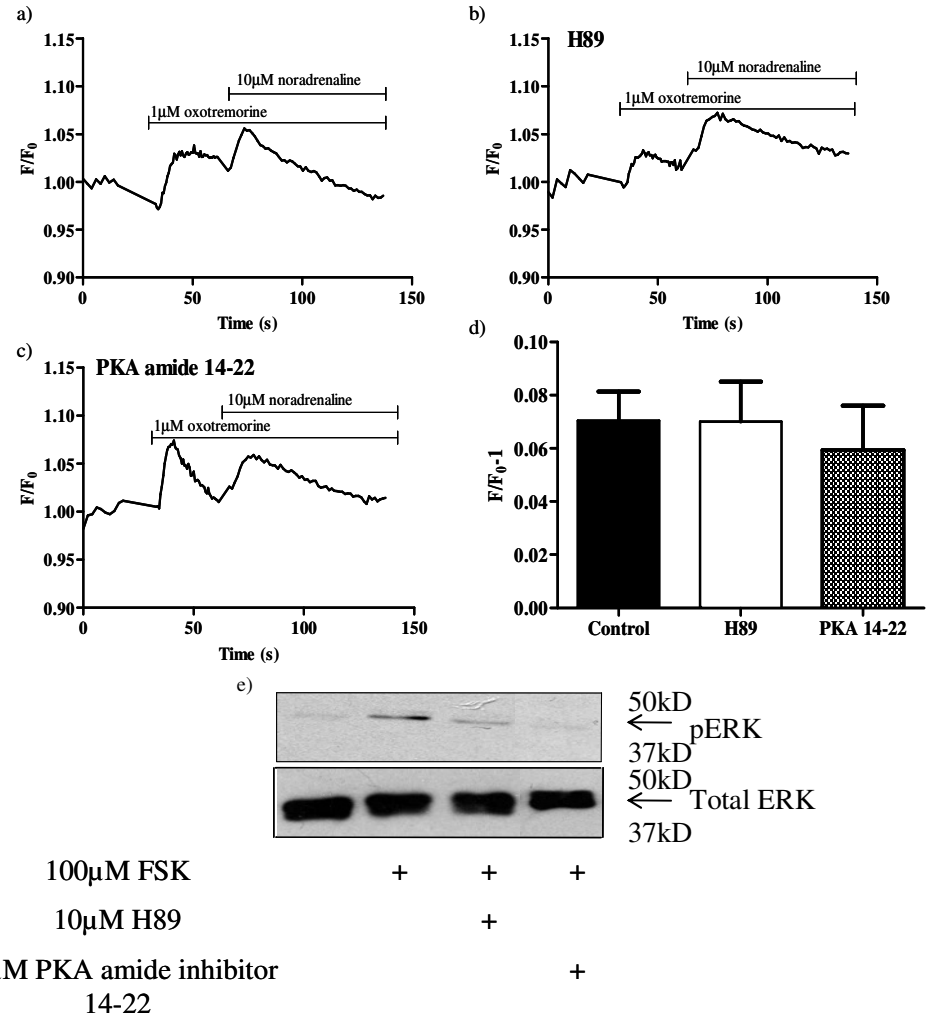


Figure 4.7 Crosstalk is unaffected by inhibition of PKA. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and changes in fluorescence measured on a NOVOstar plate reader (a-d). Cells were stimulated with 1μM oxotremorine followed by 10μM noradrenaline in the absence (a) and presence of PKA inhibitor pre-treatment, H89 (10μM, 30min) (b) or PKA amide 14-22 (25μM, 30min) (c). Maximal responses to noradrenaline in the presence or absence of H89 or PKA 14-22 are shown (d). Immunoblots of either pERK (upper panel) or total ERK (lower panel) from cells either unteated (control) or pre-incubated with the PKA-amide inhibitors H89 (10μM) or PKA amide 14-22 (25μM) and stimulated with forskolin (100μM). Data are either representative of 3 individual experiments (a-c and e) or mean + s.e.mean, n=3 (d).

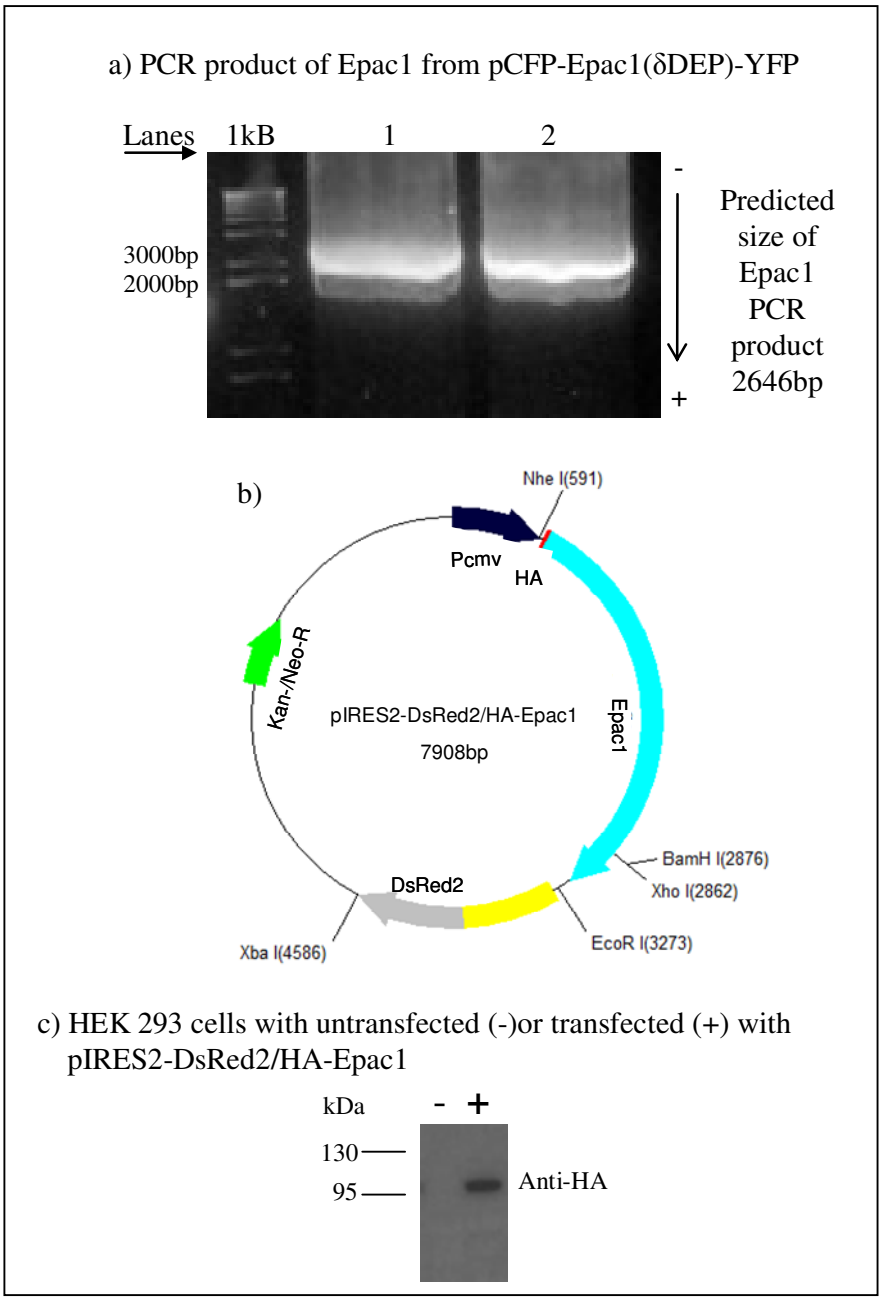


Figure 4.8. Epac1 PCR product, plasmid map and transfection into wild type HEK 293 cells. Epac1 was cloned from pCFP-Epac1(δDEP)-YFP using primers as described in *Materials and Methods* (a). The Epac1 PCR product was inserted into the pHA-GLP-1-EGFP plasmid after removal of GLP-1R-EGFP to produce HA-Epac1. The pIRES2-DsRed2/HA-Epac1 construct was developed by inserting HA/Epac1 into a commercially available Ds-Red2 vector at the restriction sites NheI-Eco-RI (a). Western blots of HEK 293 cells either untranfected (-) or transiently transfected with the pIRES2-DsRed2/HA-Epac1 construct (+) were conducted using an anti-HA antibody (c).

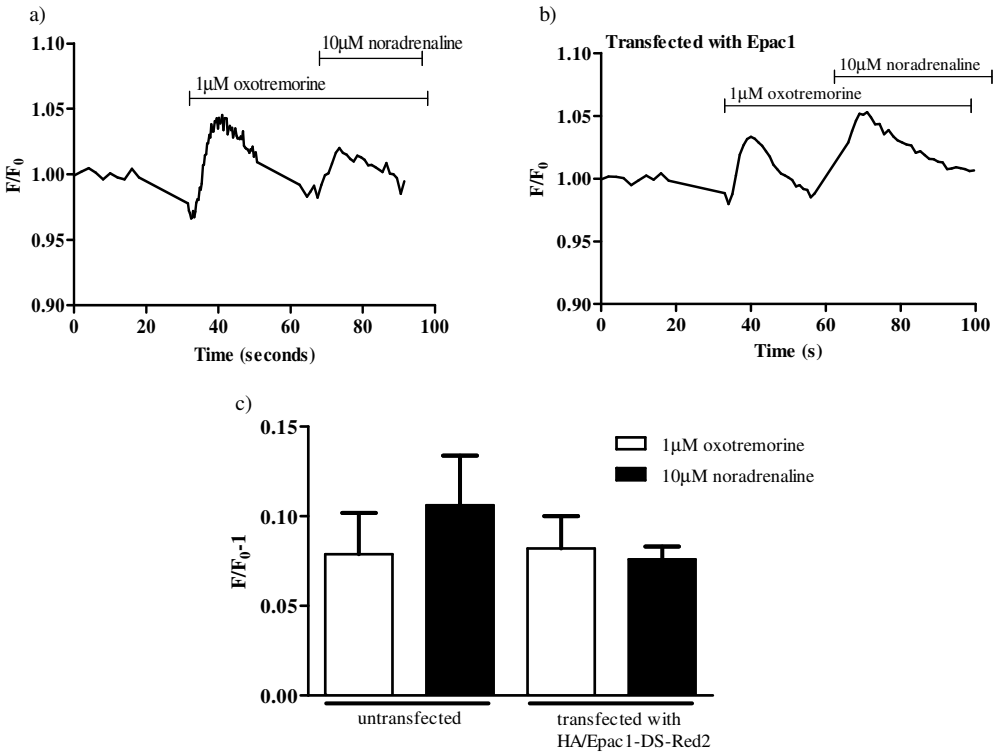


Figure 4.9 Crosstalk is unaffected by expression of HA-Epac1. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* after being cultured in 96-well plates for 24h after being transfected with pIRES2-DsRed2/HA-Epac1 (b) or transfected with the empty DsRed vector (a). Cells were stimulated with an EC_{50} (1µM) of oxotremorine and subsequently noradrenaline (10µM). The average maximal responses to oxotremorine or noradrenaline in the presence of oxotremorine in untransfected and transfected cells are shown (c). Changes in fluorescence were measured on NOVOstar fluorescence plate reader and calculated compared to the basal (F/F_0). Data are either representative of 3 individual experiments (a) and (b) or mean + s.e.mean, n=3 (c).

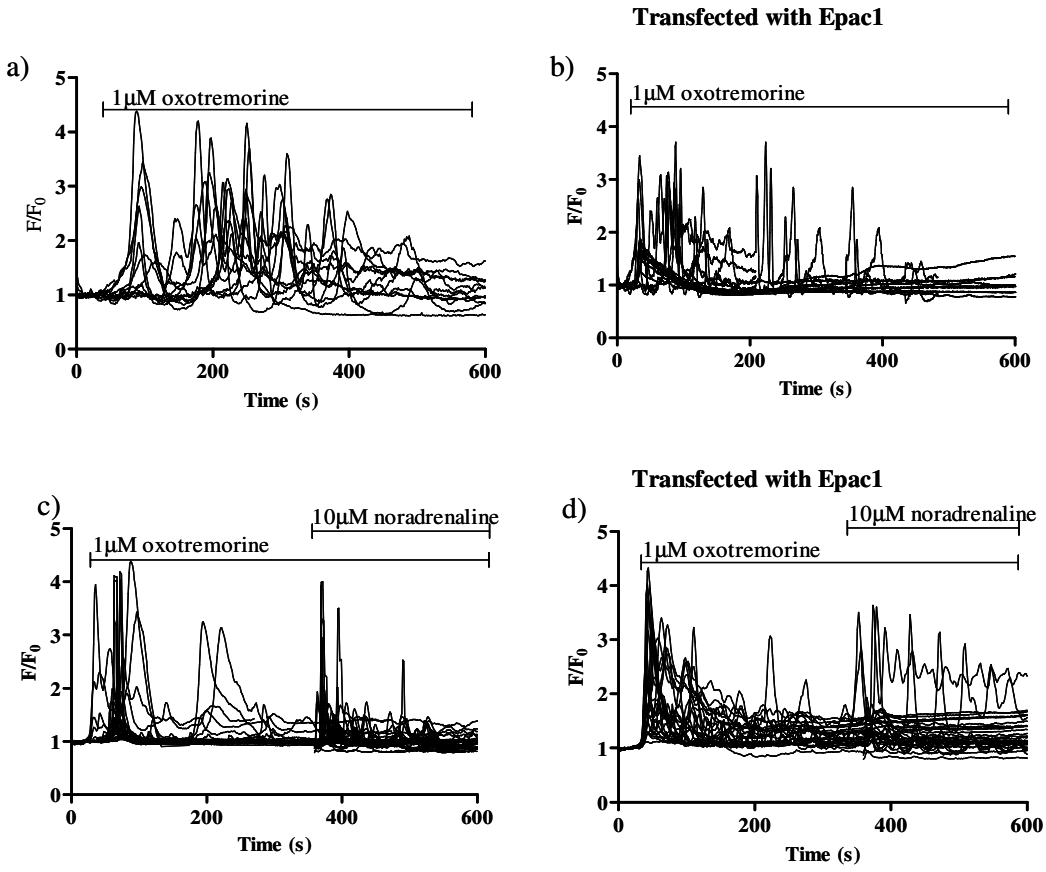


Figure 4.10. Single cell imaging of Ca^{2+} responses in untransfected cells or cells transfected with HA-Epac1. Cells were loaded with fluo-4 AM as specified in *Materials and Methods,* after being cultured for 24h following transfection with pIRES2-DsRed2/HA-Epac1 (b and d) or transfected with empty DsRed vector (a and c), then imaged by confocal microscopy. Cells were challenged with 1μM oxotremorine at 30s (a and b) or 1μM oxotremorine at 30s followed by 10μM noradrenaline at 360s (c and d). Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 16 cells in the field of view. Data are representative of n=3.

4.4 Discussion

4.4.1 Summary of data

The aim of this chapter was to explore the source of Ca^{2+}responsible for the enhanced Ca^{2+} signalling elicited by crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors in HEK 293 cells. Experiments also investigated the potential mechanism of this particular crosstalk studying proteins downstream, particularly in the β_2-adrenoceptor signalling pathway.

To determine the source of Ca^{2+} responsible for the crosstalk, experiments were carried out in Krebs'-HEPES buffer with reduced [Ca^{2+}]. Removal of extracellular Ca^{2+} using either an exclusion of $CaCl_2$ from the buffer (nominally Ca^{2+}-free) or titrating with EGTA ([Ca^{2+}] of approximately 100nM) demonstrated that extracellular Ca^{2+} does not contribute to the noradrenaline-mediated single Ca^{2+} response in the presence of the priming effect of oxotremorine in populations of cells. However, in single cell imaging experiments, removal of extracellular Ca^{2+} prevented the noradrenaline-mediated oscillations from being sustained through the experiment but did not prevent the initial Ca^{2+} response. Oscillatory Ca^{2+} responses can occur after agonist stimulation of GPCRs and can result from the release of Ca^{2+} from intracellular stores via $Ins(1,4,5)P_3$ and ryanodine receptors (see Section 1.5) (Skupin *et al.,* 2008). Release of Ca^{2+} from intracellular stores causes Ca^{2+} to enter the cell through plasma membrane channels including Ca^{2+} release-activated Ca^{2+} (CRAC) channels by CCE (Adler, 2006, Penna *et al.,* 2008, Putney, 2005). The increase in cytosolic [Ca^{2+}] can evoke Ca^{2+}-induced Ca^{2+} release as the Ca^{2+} can sensitise $Ins(1,4,5)P_3$ receptors and act as an agonist at ryanodine receptors thereby increasing Ca^{2+} release from

the intracellular stores (Roderick *et al.,* 2003). The intracellular stores are refilled via Ca^{2+}-ATPases which sequester Ca^{2+} from the cytoplasm back into the endoplasmic reticulum (see Tupling, 2009 for review). Removal of Ca^{2+} prevents CCE via plasma membrane Ca^{2+} channels and therefore prevents refilling of the intracellular stores. This lack of refilling is likely to account for the subsidence of the oscillatory Ca^{2+} signals mediated by crosstalk in the absence of extracellular Ca^{2+}. To further investigate whether the Ca^{2+} signals were from an intracellular store experiments were carried out using thapsigargin to deplete the endoplasmic reticulum stores (Putney, 2001, Rogers *et al.,* 1995). Thapsigargin is a naturally occurring sesquiterpene lactone inhibitor of SERCA, which irreversibly binds to the pump in its Ca^{2+} free form, stabilising the enzyme in an unbound complex. In the absence of refilling, a continual leak of Ca^{2+} from such stores via an $Ins(1,4,5)P_3$-independent pathway leads to store depletion (Kijima *et al.,* 1991, Thastrup *et al.,* 1994, Rogers *et al.,* 1995). Thapsigargin has previously been shown to abolish muscarinic-mediated intracellular Ca^{2+} responses in a range of primary cells and cell lines including the HEK 293 cell line (Ebihara *et al.,* 2006, Kwan, *et al.,* 1990, Tong *et al.,* 1999, Xu *et al*., 1992, Irving and Collingridge, 1998, Zhang and Melvin, 1994). Treatment with thapsigargin abolished the Ca^{2+} signalling, both oxotremorine- and noradrenaline-mediated, by depleting the endoplasmic reticulum stores. These experiments demonstrate that all Ca^{2+} responses in the present study were dependent on an intracellular store. Some aspects of the signalling were, however, dependent on Ca^{2+} entry from outside the cell. Thus, the oxotremorine response was significantly reduced in the absence of extracellular Ca^{2+} and the sustained noradrenaline-mediated oscillations were not maintained.

Although in many examples of crosstalk the source of the Ca^{2+} has not been investigated (Dianzani *et al.,* 2001, Smith *et al.,* 2000), crosstalk enhancing Ca^{2+} signalling independently of extracellular Ca^{2+} has been demonstrated between some $G\alpha_i$- and $G\alpha_{q/11}$-coupled receptors (Tovey *et al.,* 2003, Werry *et al.,* 2002). There are also examples of GPCR crosstalk where extracellular Ca^{2+} is required to potentiate Ca^{2+} signalling demonstrating that the source of Ca^{2+} is not consistent across all crosstalk mechanisms.

To determine the intracellular Ca^{2+} receptors potentially involved in the synergistic Ca^{2+} release, experiments were carried out using XeC, a putative $Ins(1,4,5)P_3$ receptor inhibitor (De Smet *et al.,* 1999, Gafni *et al.,* 1997). In HEK 293 cells XeC (5μM, 30min) has been shown to block the $G\alpha_{q/11}$-coupled αA_1-adrenoceptor from signalling via $Ins(1,4,5)P_3$ receptors (Copik *et al.,* 2009). XeC is selective for $Ins(1,4,5)P_3$ receptors over ryanodine receptors and has several advantages over other methods used to investigate the involvement of $Ins(1,4,5)P_3$ receptors in Ca^{2+} signalling. Heparin, another $Ins(1,4,5)P_3$ receptor inhibitor, has low affinity and selectivity for the receptor and is membrane impermeable (Ghosh *et al.,* 1988, Kobayashi *et al.,* 1988). Heparin has also been shown to uncouple receptors from G-proteins and to activate ryanodine receptors (Bootman *et al.,* 2002). $Ins(1,4,5)P_3$ receptor antibodies are also membrane impermeable meaning specialist techniques such as microinjection must be used (Gafni *et al.,* 1997, Nakade *et al.,* 1991, Sullivan *et al.,* 1995). XeC significantly reduced the muscarinic agonist-mediated intracellular Ca^{2+} responses and the noradrenaline-mediated responses in the presence of a muscarinic agonist. However, there are problems with the selectivity of XeC and at high concentrations XeC interacts with and inhibits ryanodine receptors (Gafni *et al.,* 1997). XeC is also a potent inhibitor of endoplasmic reticulum Ca^{2+} pumps when used at high

concentrations (100μM, which is x10 higher than in the experiments presented here) (De Smet *et al.,* 1999).

Previously our laboratory has performed experiments using 2-aminoethoxydiphenyl borate (2-APB), another Ins(1,4,5)P_3 receptor inhibitor, which again significantly reduced both the muscarinic- and noradrenaline-mediated intracellular Ca^{2+} responses (Kurian *et al.,* 2009). However, again 2-APB has effects other than inhibition of Ins(1,4,5)P_3 including inhibition of store-operated channels that replenish the Ca^{2+} store and has also been shown to potentiate Ca^{2+} signalling via enhanced leak of Ca^{2+} from the stores and inhibition of SERCA (Bootman *et al.,* 2002). In several cell types including platelets, neurons of the suprachiasmatic nucleus, ventricular cardiomyocytes, various types of smooth muscle, pancreatic β cells, fungal growth tips, hippocampal neurons, skeletal myotubes and endothelial cells, 2-APB has been shown to have an inhibitory effect on intracellular Ca^{2+} release (Ascher-Landsberg *et al.,* 1999, Bishara *et al.,* 2002, Bramich *et al.,* 2001, Dobryndeva and Blackmore, 2001, Dyachok and Gylfe, 2001, Estrada *et al.,* 2001, Gysembergh *et al.,* 1999, Hamada *et al.,* 1999, Hirst and Edwards, 2001, Imaeda *et al.,* 2000, Maruyama *et al.,* 1997, Pal *et al.,* 2001, Potocnik and Hill, 2001, Scherer *et al.,* 2001, Silverman-Gavrila and Lew, 2001). However, these studies have not taken into account the other effects of the inhibitor. Furthermore, 2-APB is not equally potent at inhibiting Ca^{2+} release in all cell types, which may be a consequence of the isoform of the Ins(1,4,5)P_3 receptor expressed. The results in this thesis and previous data are consistent with a role for Ins(1,4,5)P_3 receptors in synergistic Ca^{2+} responses as a consequence of crosstalk but some caution is needed when evaluating data using the inhibitors presently available.

The mechanism of crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors, which elicits the synergistic Ca^{2+} response, is unknown. Enhanced PLC activity, generating an increase in Ins(1,4,5)P_3, has been shown to elicit the increase in intracellular Ca^{2+} observed in many examples of crosstalk, although mainly that between $G\alpha_{q/11}$- and $G\alpha_i$-coupled receptors (Katz, *et al.,* 1992, Selbie *et al.,* 1995, Werry *et al.,* 2003, Wu, *et al.,* 1993) (Section 1.5.2). Stimulating HEK 293 cells with muscarinic agonists and/or noradrenaline did not, however, increase inositol phosphate accumulation against a Li^+-block of inositol monophosphate (Kurian, 2006). This would suggest that enhanced PLC activity was not involved in this crosstalk. This is consistent with other examples of crosstalk, indicating that other mechanisms must be considered (Section 1.5.3). Previous work in the laboratory demonstrated the need for $G\alpha_s$-proteins in this crosstalk suggesting it may require increased AC activity and cAMP accumulation. Therefore, with cAMP effects thought to be mediated via cyclic nucleotide-gated channels, PKA and Epac there is potential that PKA or Epac could be involved in the mechanism of crosstalk (Kurian *et al.,* 2009, Ponsioen *et al.,* 2004). PKA consists of two catalytic and two regulatory subunits (RI and RII, of which there are RIα, RIβ and RIIα, RIIβ) (Kim *et al.,* 2007). On binding of cAMP to the regulatory units, the enzyme undergoes a conformational change and releases the active catalytic subunits. PKA is able to phosphorylate a large number of proteins. For instance in airway smooth muscle cells, β_2-adrenoceptor activation leads to the phosphorylation of myosin light chain kinases causing the airway to relax (Billington and Penn, 2002). Intracellular Ca^{2+} channels can be regulated and sensitised by PKA through different mechanisms including interaction of the catalytic region of PKA with the Ins(1,4,5)P_3 binding site of the Ins(1,4,5)P_3 receptor and phosphorylation of certain residues within the channel (Ferris *et al.,* 1991, Tanimura *et al.,* 1999, Volpe and Alderson-Lang, 1990). PKA-

dependent sensitization of $Ins(1,4,5)P_3$ receptors has been demonstrated as a mechanism of crosstalk in rat parotid cells, between $G\alpha_i$-coupled α_2-adrenoceptors and $G\alpha_{q/11}$-coupled muscarinic receptors, leading to an increase in Ca^{2+} signalling (Tanimura *et al.,* 1999).

Stimulation of the β_2-adrenoceptor can also lead to the activation of Epac. There are two closely related Epac proteins (Epac 1 and 2) which act as GEF proteins for Rap 1 and 2 respectively (de Rooij *et al.,* 2000). Epac 1 contains one cAMP-binding domain whereas Epac 2 contains a second in its N-terminus (Bos 2003, Rooij *et al.,* 2000). The cAMP concentration needed to give half-maximal activation of PKA is ~1μM whereas to activate Epac is considerably higher at ~40μM. Therefore Epac is likely to be activated by localised increases in cAMP (Bos, 2003, Kawasaki *et al.,* 1998, Rehmann *et al.,* 2003). Epac 1 contains a C-terminal catalytic domain and an N-terminal domain which includes a cAMP-binding site (Ponsioen *et al.,* 2004). The regulatory domain causes auto-inhibition of the enzyme until cAMP binding causes Epac to adopt an active conformation. Activation of $G\alpha_s$-coupled GPCRs has been shown to mediate a variety of effects through Epac. For example, activation of the β_2-adrenoceptor in human airway smooth muscle (HASM) cells inhibits EGF-stimulated proliferation via an Epac-dependent, PKA-independent pathway (Kassel *et al.,* 2008). This demonstrates that the β_2-adrenoceptor is able to signal through Epac as an alternative to PKA. Epac 1 is ubiquitously expressed in cells whereas Epac 2 is predominantly expressed in regions of the brain and adrenal glands (Bos 2003). Epac has been indicated in a number of mechanisms responsible for crosstalk including Rap-dependent activation of PLCε and sensitisation of intracellular Ca^{2+} release channels by use of the Epac-selective cAMP analogue 8-pCPT-2'-o-Me-cAMP (Kang *et al.,* 2001, Kang *et*

al., 2003, Schmidt *et al.,* 2001). Studies have also shown the ability of Epac to stimulate mitogen-activated protein kinases in endocrine cells and T-cells, via Rap activation (Gerlo *et al.,* 2006, Lofti *et al.,* 2006, Ster *et al.,* 2007, Traver *et al.,* 2006). In several different cell types activation of Epac has been demonstrated as a mechanism by which GPCRs can elicit crosstalk, both by sensitizing intracellular Ca^{2+} channels and by PLCε activation (den Dekker *et al.,* 2002, Kang *et al.,* 2001, Schmidt *et al.,* 2001). In HEK 293, over-expressing the β_2-adrenoceptor, an increase in Ca^{2+} signalling was mediated via Epac-dependent activation of Rap2B, which enhanced PLCε activation (Schmidt *et al.,* 2001). However, the results in this thesis suggest that neither PKA or Epac are involved in the crosstalk although there were short-comings in the Epac experiments carried out, with a low transfection efficiency of the HA/Epac1 plasmid. Ideally endogenous Epac1 should be knocked down, before transient transfection with HA-DsRed2-Epac1. With low transfection efficacy of the plasmid it may appear that overexpressed Epac1 has no role in crosstalk, whereas endogenous Epac1 may be carrying out the required role producing a false negative. Transient tranfection of cells can lead to problems including bio-contamination, immune reactions and cell apoptosis (Li *et al.,* 2001). Biochemical transfection with lipofectamin was used to transfect Epac1 into the HEK 293 cells but to reduce bio-contamination and immune reactions and increase transfecton efficiency electroporation could be used (Li *et al.,* 2001). Electroporation depends on the weak nature of the phospholipid bilayer's hydrophobic/hydrophilic interactions and its ability to spontaneously re-assemble after a quick voltage shock (Purves, *et. al.,* 2001). This shock disrupts areas of the membrane temporarily, allowing polar molecules to pass. The membrane then reseals quickly and leaves the cell intact (Li *et al.,* 2001, Purves, *et. al.,* 2001). This method may increase transfection efficiency, it is versatile, only uses a small quantity of DNA when comparing

to other methods and can be used in intact cells. However, the method has a number of disadvantages with cell damage from colloidal-osmotic swelling and the non-specific transport of material into and out of the cells causing ion inbalance and apoptosis (Li *et al.,* 2001).

From PCR experiments carried out in the laboratory it was shown both Epac1 and 2 are expressed in these HEK 293 and perhaps siRNA knock-out of the Epac proteins would be another way to determine if they are involved in the crosstalk mechanism (de Jesus *et al.,* 2006, Nijholt *et al.,* 2008). This would also determine whether Epac2 has a role in the crosstalk. Both Epac1 and Epac2 have previously been knocked-down successfully in different cells including HEK 293 cells, using siRNAs (Lopez De Jesus *et al.,* 2006, Roscioni *et al.,* 2009, Sehrawat *et al.,* 2008).

Another study, published during the course of this thesis research, demonstrated crosstalk between the $G\alpha_s$-coupled parathyroid hormone and the $G\alpha_{q/11}$-coupled muscarinic M_3 receptor which does not rely on either PKA or Epac (Tovey *et al.,* 2008). cAMP was shown to sensitise $Ins(1,4,5)P_3$ receptors via 'cAMP junctions'. These junctions allowed very high concentrations of cAMP to be produced locally at the site of $Ins(1,4,5)P_3$ type 2 receptors. This directly increases their sensitivity to $Ins(1,4,5)P_3$ via a low affinity cAMP binding site. Specific interactions were shown to occur between adenylate cyclase VI and $Ins(1,4,5)P_3$ receptor 2 accounting for the 'cAMP junctions' (Tovey *et al.,* 2008). This localised cAMP signalling acts as a binary 'on-off' switch system with each activated receptor/AC saturating the local $Ins(1,4,5)P_3$ receptor with high concentrations of cAMP. This mechanism is consistent with the data in this thesis, with co-activation of $G\alpha_{q/11}$- and $G\alpha_s$-

coupled receptors required for the crosstalk. The results of this study suggest Epac and PKA are not involved in the mechanism but that Ins(1,4,5)P_3 receptors release the intracellular Ca^{2+}.

This chapter has demonstrated that the synergistic Ca^{2+} signalling elicited by crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors relies on the released of Ca^{2+} from intracellular Ca^{2+} stores via Ins(1,4,5)P_3 receptors and is independent of Ca^{2+} influx across the plasma membrane. Although the crosstalk is dependent on cAMP (demonstrated in experiments previously carried out in the laboratory) neither PKA or Epac appear to be involved. A potential mechanism could mirror closely work carried out in another study, which demonstrated the ability of cAMP to directly sensitise Ins(1,4,5)P_3 receptors to Ins(1,4,5)P_3 (Tovey *et al.,* 2008).

Chapter 5: Does crosstalk affect activation of transcription factors?

5.1 Overview of chapter

This chapter examines the potential downstream consequences of the synergistic intracellular Ca^{2+} signalling resulting from the crosstalk demonstrated in the previous chapter. The transcription factors extracellular-signal regulated kinase (ERK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) were studied to determine if the synergistic Ca^{2+} response had any affects on the activation of these proteins.

5.2 Introduction

ERK is a mitogen-activated protein kinase (MAPK) that is ubiquitously expressed in eukaryotic cells. The mechanisms of ERK activation are diverse and include many second messengers such as intracellular Ca^{2+} and cAMP (Dumaz and Marais, 2005, Kupzig *et al*., 2005). cAMP has been shown to both cause activation and inhibition of ERK. In Rat1 and NIH3T3 fibroblasts cAMP led to an increase in ERK activity (Cook and McCormick, 1993, Wu *et al.,* 1993, Burgering *et al.,* 1993). In these cells cAMP analogues 8-chloro-cAMP and di-butyryl cAMP blocked growth factor-stimulated ERK activation. Since then, several studies have demonstrated the ability of the cAMP pathway to inhibit ERK activation such as in vascular smooth muscle cells where cAMP inhibits thrombin-activated ERK (Osinski and Schror, 2000). cAMP has also been shown to enhance the activation of ERK in a number of cells including human melanocytes and both Fisher and Wistar rat thyroid cells (Ciullo *et al.,* 2001, De Luca *et al.,* 1993, Miller *et al.,* 1998).

Signalling via ERK involves a cascade of MAPK components which phosphorylate each level of the pathway, after initial activation by a small G-protein (for example Ras or Raf) (Figure 5.1.1 below) (Dumaz and Marais, 2005, Kupzig *et al*., 2005). The main MAPK/ERK kinase kinase (MEKK) components are the Raf family members which activate the next tier of the pathway, MEK, by phosphorylation of two serine residues in their activation loops (Ser218 and Ser222 in MEK1) (Alessi *et al.,* 1994). Once activated, MEKs phosphorylate ERK1/2 within a conserved Thr-Glu-Tyr (TEY) motif in their activation loop, causing ERK to detach from the molecules that maintain it in the cytoplasm and allow translocation to the nucleus, although activation of ERK does not guarantee translocation (Ranganathan *et al.,* 2006, Roux and Blenis, 2004, Wolf *et al.,* 2001). When ERK is dephosphorylated the protein relocates to the cytoplasm. Once activated, ERKs phosphorylate a large number of substrates situated in the cytoplasm and nucleus with transcription factors such as Elk1 and c-Fos being nuclear targets (Gille *et al.,* 1992, Sinnett-Smith *et al.,* 2007). ERK does not have a signal sequence for nuclear import or export and is thought to be small enough (ERK1 44kDa, ERK2 42kDa) to pass through the nuclear pore by passive diffusion or by interaction with nuclear pore proteins (Kondoh *et al.,* 2005). ERK participates in many cell activities including proliferation, differentiation, apoptosis and gene transcription (Shaul and Seger, 2006, Dumaz and Marais, 2005). There are different isoenzymes of ERK (1-8) but only ERK 1 and 2 are studied in this chapter as they are the classic ERK isoenzymes with the others having atypical structure or function (Abe *et al.,* 2001, Lechner *et al.,* 1996, Roux and Blenis, 2004, Schumacher *et al.,* 2004, Yan *et al.,* 2003,).

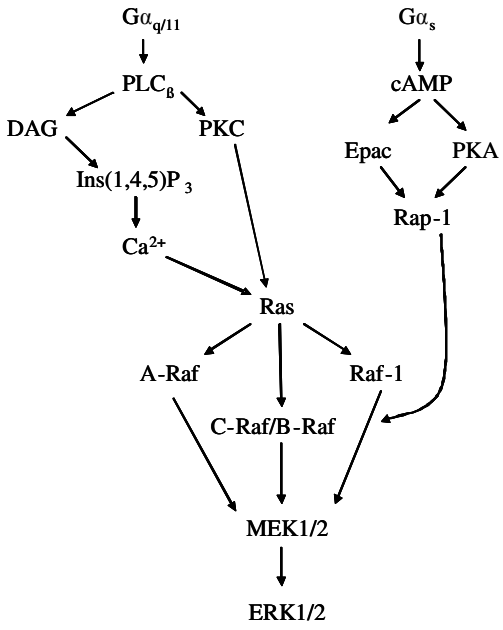


Figure 5.1.1 Pathways of ERK activation. Upon receptor stimulation the G-protein activates a signalling pathway downstream of the GPCR leading to the activation of a small G-protein (Ras or Rap-1) and the MAPK pathway. Activation of a MEKK, e.g. C-Raf or B-Raf, allows phosphorylation of the next tier in the ERK cascade. MEK1/2 is activated via phosphorylation of two serine residues in their activation loops. This allows the MEKs to phosphorylate the TEY motif conserved within ERK1/2 leading to translocation of the MAPK to activate a number of substrates (Alessi *et al.,* 1994, Raganathan *et al.,* 2006, Roux and Blenis, 2004, Wolf *et al.,* 2001).

Both the duration and intensity of ERK signalling is important. Intense short signals may produce different outcomes to longer, weaker signals although the addition of these two outcomes may appear to produce the same overall quantity of signal (Pouyssegur and

Lenormand, 2003). In neuronal precursors, transient ERK signalling stimulates proliferation whereas sustained signalling leads to differentiation (Dumaz and Marais, 2005). In fibroblasts, ERK activation is needed for proliferation but high intensity signals lead to cell cycle arrest and aging (Avruch *et al.,* 2001, Hagemann and Rapp, 1999, Kolch, 2000). The pattern of ERK activation is dependent on the agonist bound to the receptor that is responsible for activating the pathway (Murphy *et al*., 2002, Murphy *et al.,* 2004).

Crosstalk between GPCRs could regulate the activity of ERK, potentially modifying its downstream actions. Gβγ-dimers have been shown to directly activate ERK. MAPKs have specific motifs that bind effector molecules, activators and scaffold proteins with ERK contains a docking groove and common docking site (CDS) (Raman and Cobb, 2003). ERK 1/2 activity can be inhibited by PKA after activation of a $G\alpha_s$-coupled receptor with PKA directly phosphorylating Raf-1 at Ser43 and Ser621 (English *et al.,* 1999, Mischak *et al.,* 1996). This greatly reduces binding to Ras, preventing activation of the lower tiers of the MAPK pathway (Figure 5.1.1.). cAMP-induced inhibition can still be detected when Raf-1 is truncated in the catalytic domain, suggesting phosphorylation at Ser621 is enough to greatly reduce activation of ERK (Mischak *et al.,* 1996). Ras activation, leading to ERK phosphorylation, can be controlled by increases in intracellular Ca^{2+} (Kupzig *et al.,* 2005, Li *et al.,* 1998). The frequency of intracellular Ca^{2+} oscillations affects ERK phosphorylation and the time frame over which this activation occurs. Indeed, Ca^{2+} oscillations have been shown to reduce the effective threshold for the Ras/ERK pathway (Kupzig *et al.,* 2005).

So far this study has demonstrated a synergistic effect on intracellular Ca^{2+} signalling mediated by crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors in HEK 293

cells. One of the proteins activated in the muscarinic pathway is PKC and several studies have demonstrated the ability of both PKC, activated by and independently of Ca^{2+}, and intracellular Ca^{2+} to activate NF-kB. PKCβ, PKCε and PKCζ have all been implicated in the activation of NF-kB in different cells (Guizzetti *et al.,* 2003, Saijo *et al.,* 2002). Increases in intracellular Ca^{2+}, particularly carbachol-mediated Ca^{2+} signals, have also been demonstrated to enhance the transcription of genes via NF-kB (Guizzetti *et al.,* 2003, Li *et al.,* 1996, Todisco *et al.,* 1999, Woods *et al.,* 1999). In canine gastric parietal cells and in COS-7 cells recombinantly expressing human muscarinic M_3 receptors, carbachol induced the phosphorylation of IKKα and β allowing the translocation of NF-kB to the nucleus. This phosphorylation was mediated by both an increase in $[Ca^{2+}]_i$ and activation of PKC (Todisco *et al.,* 1999).

The NF-kB/Rel family of transcription factors is made up of five related proteins p50, p52, RelA/p65, c-Rel and RelB and these proteins normally function as homo- or heterodimers (Basak and Hoffman, 2008, Courtois, 2005, Sun and Andersson, 2002). In most cells these proteins are sequestered in the cytoplasm by association with members of the inhibitor of kB (IkB) family. All of these members contain an N-terminal Rel homology domain (RHD), which binds NF-kB proteins to DNA and inhibitors of NF-kB (IkB) proteins (Perkins and Gilmore *et al.,* 2006). IkB inhibits NF-kB proteins by masking their nuclear localisation signal (NLS) preventing translocation to the nucleus and terminating or preventing signalling. On activation of the NF-kB pathway the IkB protein is phosphorylated by IkB kinases (IKKs) at two important serine residues (serines 32 and 36 for IkBα) causing their degradation by proteasomes and release of NF-kB (Basak and Hoffman, 2008, Karin and Ben-Neriah, 2000, Perkins and Gilmore, 2006). The

transcription factor then translocates into the nucleus and binds to consensus (kB) sites in the promoter sequences of inducible genes (GGGACTTTCC) allowing transcription of many genes that control processes including inflammation, proliferation and apoptosis (Barnes and Adcock, 1997, Edwards *et al.,* 2009, Hayden and Ghosh, 2004, Sun and Andersson, 2002). NF-kB gene targets include IkBα, therefore providing a feedback inhibition cycle allowing self-control of its activation (Basak and Hoffman, 2008).

Altered activation of the NF-kB pathway leads to many downstream effects. For example, activation of the pathway by a high concentration of tumour necrosis factor α (TNF-α) leads to apoptosis in the liver of mouse embryos leading to death (Beg *et al.,* 1995). NF-kB has also been implicated in airway diseases such as asthma and COPD where much of the underlying inflammation is mediated at least in part by NF-kB (Edwards *et al.,* 2009, Barnes, 2006). Many of the pro-inflammatory molecules produced in these diseases activate the NF-kB pathway. Changes in intracellular Ca^{2+} oscillations have also been shown to alter gene expression controlled by transcription factors including NF-AT, OAP and NF-kB (Dolmetsch *et al.,* 1997, Dolmetsch *et al.,* 1998, Li *et al*, 1998, Tomida *et al.,* 2003).

This chapter details exploration of some of the downstream effectors of intracellular Ca^{2+} and whether the synergistic signalling described earlier in this study has an effect on the activation of these proteins. The stimulation of ERK and NF-kB was examined under conditions highlighted earlier, which promoted robust crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors.

5.3 Results

5.3.1 Time-dependent activation of ERK

Stimulation of cells with methacholine (100μM) caused a rapid and transient increase in the activation of ERK (as assessed by phosphorylation) with maximal phosphorylation occurring between 1-2min, followed by a dramatic tail-off over the subsequent 30 minutes (Figure 5.1a and c). Challenging the cells with a lower concentration of methacholine (1μM) also caused a rapid increase in ERK phosphorylation that was maximal at 1-5min, although only approximately a quarter of the maximal response to 100μM (Figure 5.1b and c).

Addition of oxotremorine (100μM) to the cells caused a rapid and transient increase in the activation of ERK with maximal phosphorylation occurring between 1-2min (Figure 5.2a and c). From 5min onwards, ERK phosphorylation returned to basal. Stimulation with oxotremorine (1μM) caused a rapid and transient increase in ERK phosphorylation which was maximal up to 5min but again decreased to basal rapidly (Figure 5.2b and c). The time-course for ERK phosphorylation differed for the two concentrations with 100μM peaking at 1-2min whereas the 1μM peaked at 2-5min with the maximal 1μM response being approximately half of the response to 100μM.

Addition of methacholine (100μM) to the cells caused a significantly greater response than challenging them with either methacholine (1μM) or oxotremorine (100μM or 1μM). This demonstrates that oxotremorine is also a partial agonist for phosphorylation (activation) of ERK.

No ERK phosphorylation could be detected when the cells were exposed to noradrenaline (10μM) for up to 30min (Figure 5.3a and b). Indeed, during this time noradrenaline caused a decrease in phosphorylation. Cells were also stimulated by a co-addition of oxotremorine (1μM) and noradrenaline (10μM) because this showed the most robust crosstalk response when measuring $[Ca^{2+}]_i$ responses. On co-addition of the two agonists, a significant synergistic phosphorylation of ERK was observed with maximal activation occurring at 1min after agonist addition (4.4±0.8 F/F_0, n=3, **$p<0.05$) (Figure 5.4a and b). This co-addition not only caused a synergistic increase in ERK phosphorylation but altered the time-course of the response, with maximal phosphorylation occurring at 1min rather than the 2-5min that occurred on addition of oxotremorine (1μM) alone.

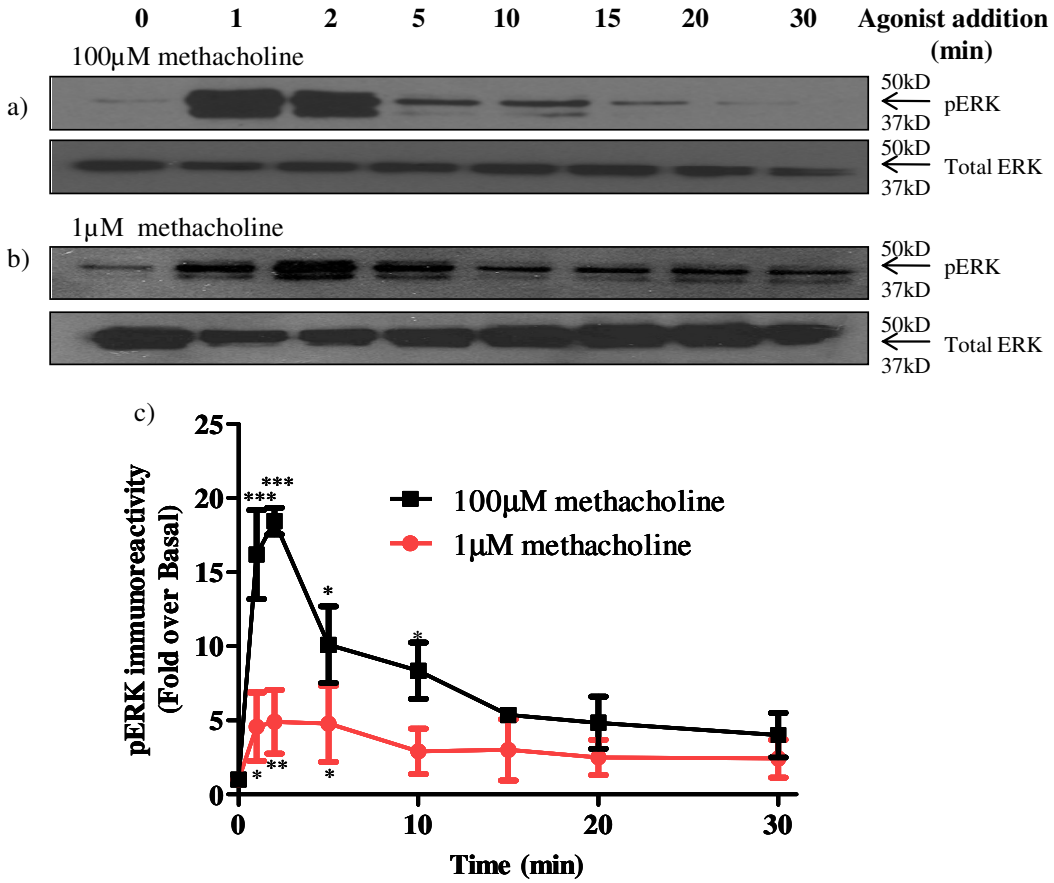


Figure 5.1. Phosphorylation of ERK 1/2 mediated by either 100µM or 1µM methacholine. Cells were cultured for 48h in 12-well plates. The cells were stimulated with 100µM or 1µM methacholine for the indicated length of time and then lysed as described in *Materials and Methods*. Whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with either pERK or total ERK antibodies and visualised with ECL+ and exposure to film. Panels a) and b) are representative of 3 experiments. Optical densitometry was carried out on the blots and values expressed relative to their own basal control (0 min). Data in c) are mean±s.e.mean., n=3. ***p<0.001 and *p<0.05 one-way ANOVA (Dunnett's multiple comparison test).

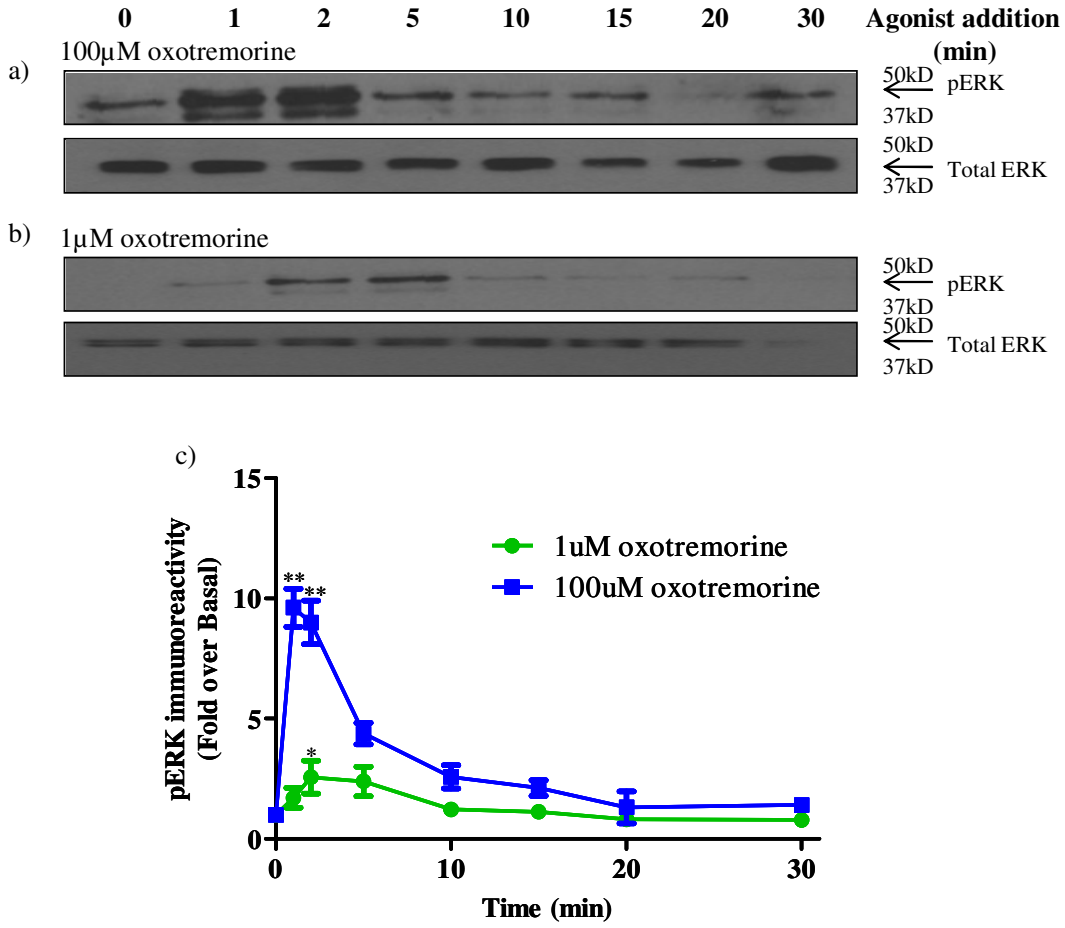


Figure 5.2. Phosphorylation of ERK 1/2 mediated by either 100µM or 1µM oxotremorine. Cells were cultured for 48h in 12-well plates. The cells were stimulated with 100µM or 1µM oxotremorine for the indicated length of time and then lysed as described in *Materials and Methods*. Whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with either pERK or total ERK antibodies and visualised with ECL+ and exposure to film. Panels a) and b) are representative of 3 experiments. Optical densitometry was carried out on the blots and values expressed relative to their own basal control (0 min). Data in c) are mean± s.e.mean., n=3. ***p<0.001, One-way ANOVA (Dunnett's multiple comparison test).

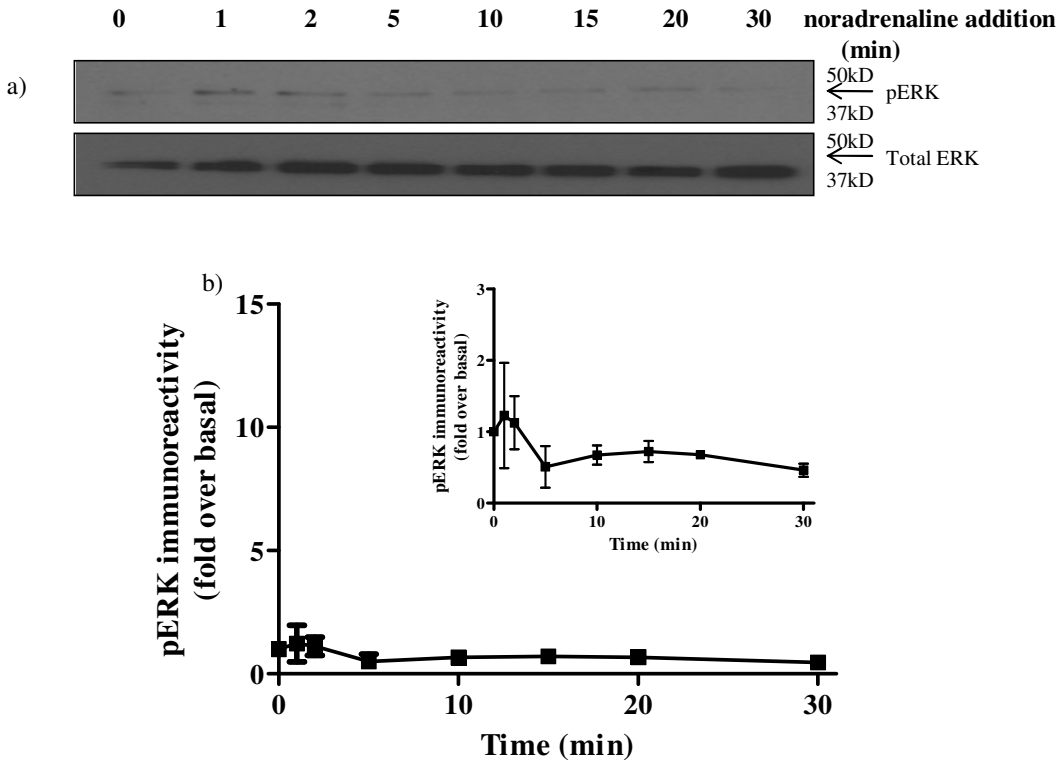


Figure 5.3. Lack of effect of 10µM noradrenaline on the phosphorylation of ERK 1/2. Cells were cultured for 48h in 12-well plates. The cells were stimulated with 10µM noradrenaline for the indicated length of time and then lysed as described in *Materials and Methods*. Whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with either pERK or total ERK antibodies and visualised with ECL+ and exposure to film. Panel a) is representative of 3 experiments. Optical densitometry was carried out on the blots and values expressed relative to their on basal (0 min). Data in b) are mean ± s.e.mean., n=3. Insert graph demonstrates the inhibition of ERK activation from 5 minutes onwards.

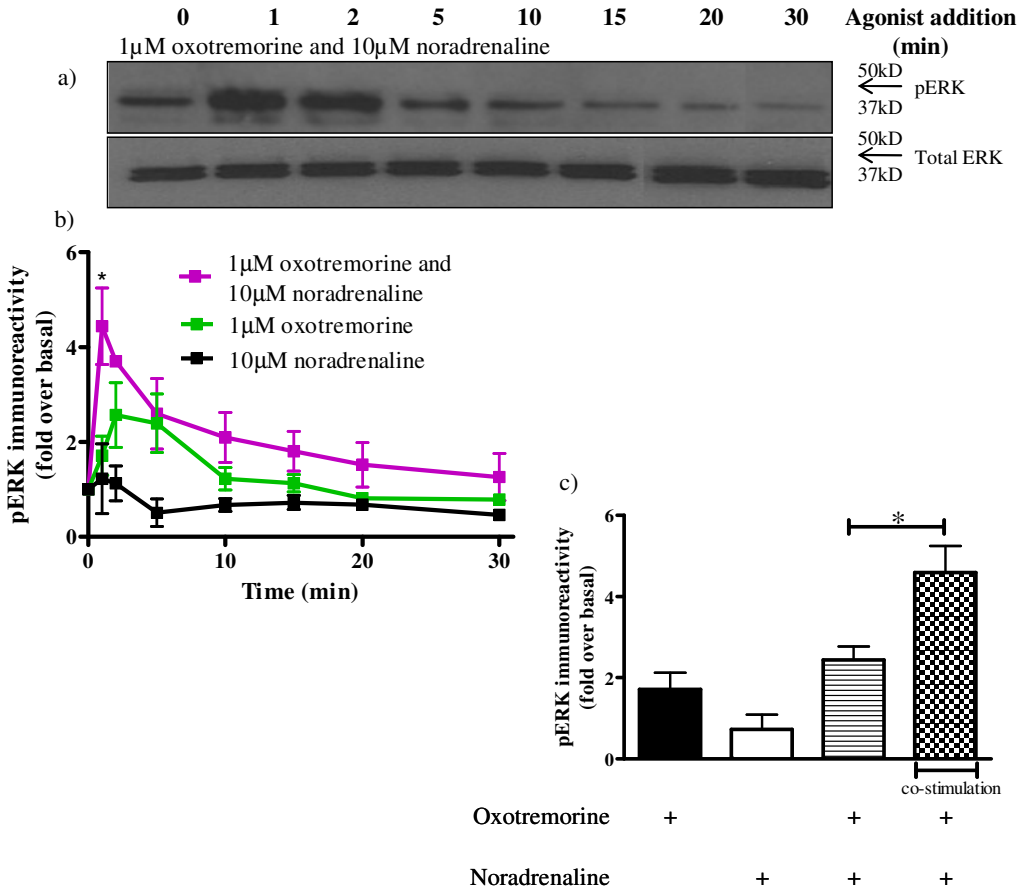


Figure 5.4. Phosphorylation of ERK 1/2 mediated by co-stimulation with 1µM oxotremorine and 10µM noradrenaline. Cells were cultured for 48h in 12-well plates. The cells were co-stimulated with 1µM oxotremorine and 10µM noradrenaline for the indicated length of time and then lysed as described in *Materials and Methods* (Panel A). Whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with either pERK or total ERK antibodies and visualised with ECL+ and exposure to film. Panel a) is representative of 3 experiments. Optical densitometry was carried out on the blots and values expressed relative to the background. Data in b) are mean± s.e.mean., n=3 for co-stimulation (purple) and separate stimulation with oxotremorine (green) and noradrenaline (black), as shown in previous figures. Panel c) shows pERK levels 1 minute after stimulation, with a range of agonists. *p<0.05, one-way ANOVA (Dunnett's multiple comparison test), (Comparison between oxotremorine and noradrenaline co-stimulation and the addition of the two agonists together).

5.3.2 Is ERK phosphorylation a consequence of intracellular Ca^{2+} signalling?

Crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors has shown intracellular Ca^{2+} and pERK synergy but are the two related? When the cells were incubated with the Ca^{2+} chelator BAPTA-AM (100μM for 30min), intracellular Ca^{2+} responses were abolished both to oxotremorine and to noradrenaline, in the presence of oxotremorine (Figure 5.5). BAPTA-AM also significantly reduced synergistic activation of ERK (pERK levels) after co-stimulation of the cells with oxotremorine and noradrenaline (Figure 5.6). However, BAPTA-AM did not have a significant effect on the level of pERK after addition of oxotremorine. BAPTA-AM also abolished UTP- (100μM) and GLP-1- (30nM) mediated intracellular Ca^{2+} responses. Although BAPTA-AM was able to abolish UTP-mediated increases in pERK it had no effect on GLP-1-mediated increases in pERK (Figure 5.7 and Figure 5.8).

5.3.3 Does crosstalk affect gene transcription?

HEK 293 cells transiently transfected with a luciferase reporter of NF-kB activity. To confirm activity of the reporter, cell were treated with increasing concentrations of TNF-α. This treatment elicited a concentration-dependent increase in NF-kB-mediated luciferase expression (Figure 5.9).

Cells transiently transfected with a luciferase reporter of NF-kB activity were stimulated with either oxotremorine (1μM) or noradrenaline (10μM) or the two in combination for 5.5h (Figure 5.10). There was an increase in NF-kB-mediated luciferase expression with either oxotremorine or noradrenaline, although these increases were small and not

statistically significant. However, when the cells were co-stimulated with oxotremorine and noradrenaline there was an increase in NF-kB-mediated luciferase expression that was significantly greater than that mediated by either agonist alone and greater than a simple additive effect of the two individual ligands.

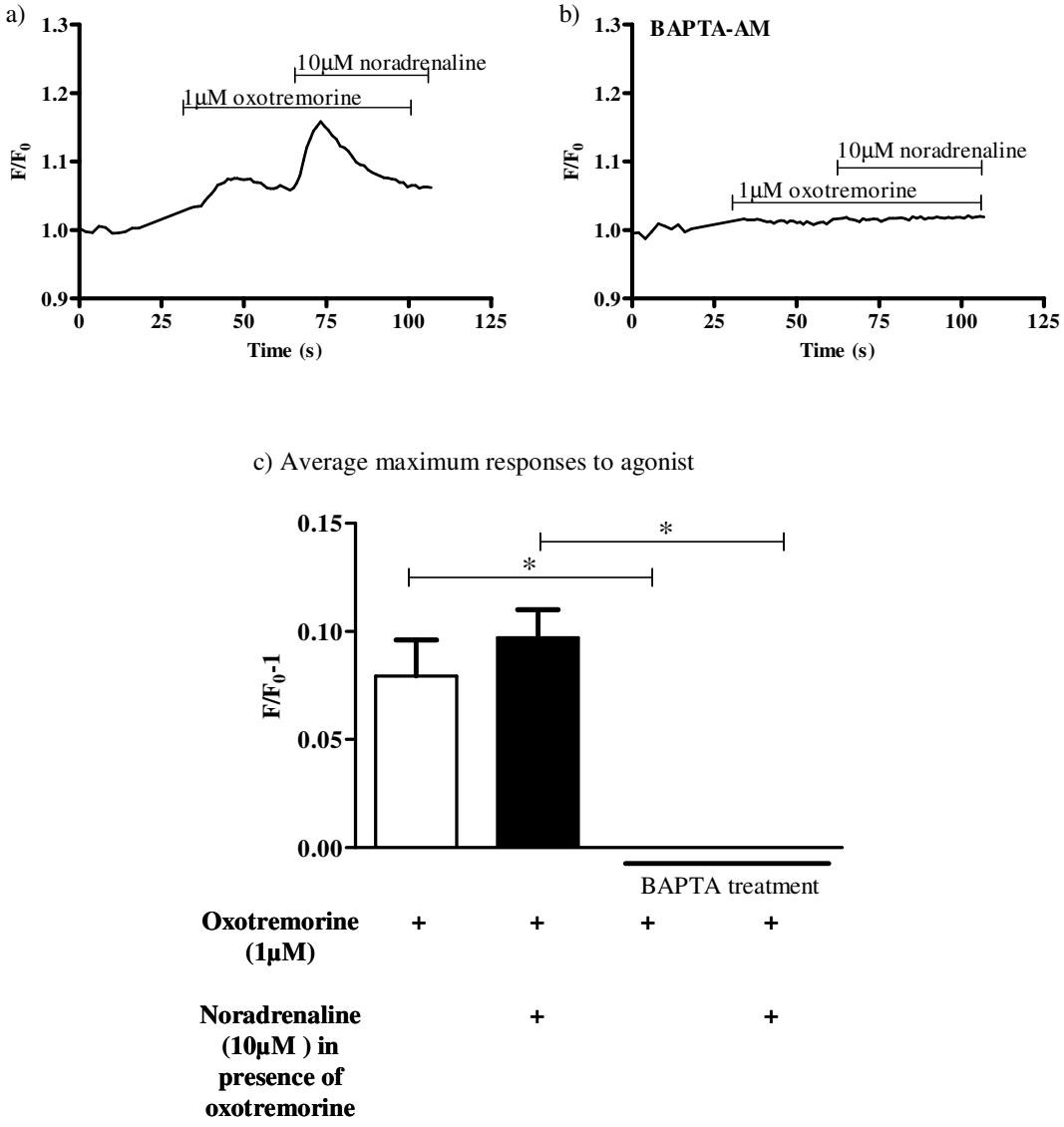


Figure 5.5 Ca^{2+} signalling is abolished by the Ca^{2+} chelator BAPTA-AM. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and stimulated with an EC_{50} (1µM) oxotremorine and subsequently noradrenaline (10µM). Cells were either untreated a) or pre-incubated with BAPTA-AM (100µM) for 30min b). Maximal responses to oxotremorine and noradrenaline in the absence and presence of BAPTA-AM are shown c). Changes in fluorescence were measured on the NOVOstar plate reader and calculated compared to the basal (F/F_0-1). Data are either representative of 3 individual experiments (a and b) or mean+ s.e.mean, n=3 (c). *p<0.05 Student's *t*-test

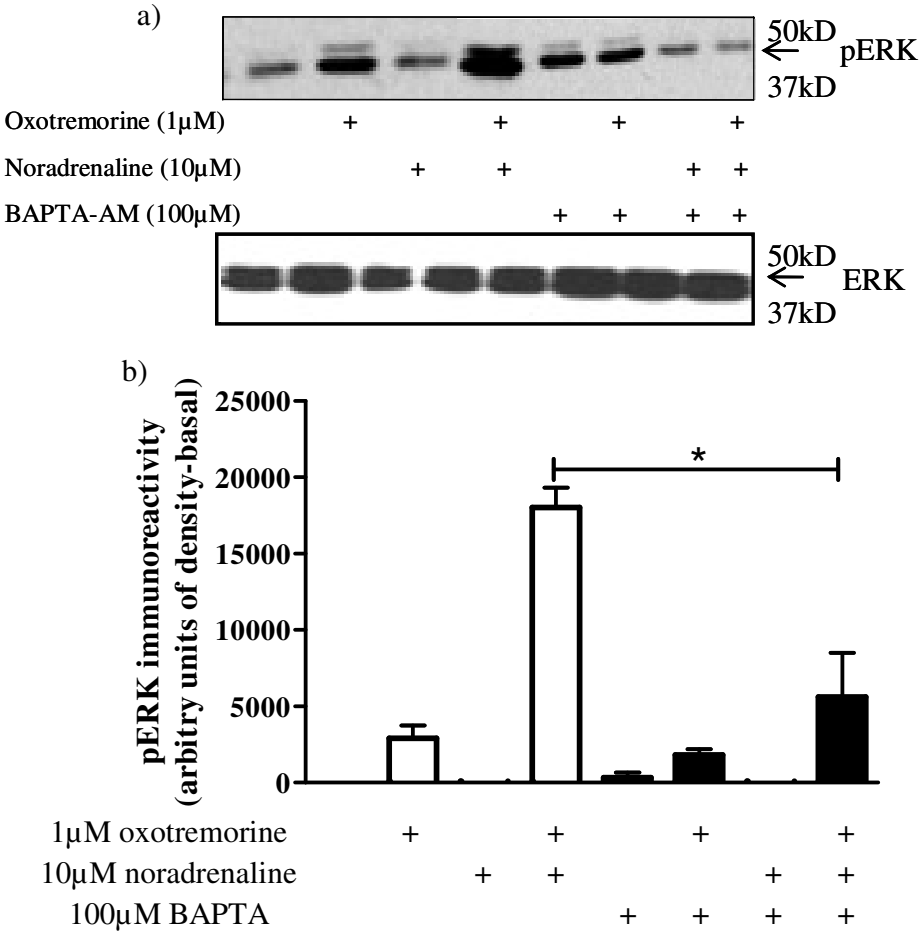


Figure 5.6 ERK phosphorylation is abolished by the Ca^{2+} chelator BAPTA-AM. Cells were cultured in a 24 well plate for 48h and either untreated (control) or pre-incubated with BAPTA-AM (100µM) for 30min before stimulation with agonists for 2min. Whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with pERK and total ERK antibodies a) and visualised with ECL+ and exposure to film. Optical densitometry was carried out on the blots and values expressed relative to the background b). Data are mean + s.e.mean, n=3. *$p<0.05$ one-way ANOVA (Bonferroni's multiple comparison test). For clarity not all the statistical significances are shown.

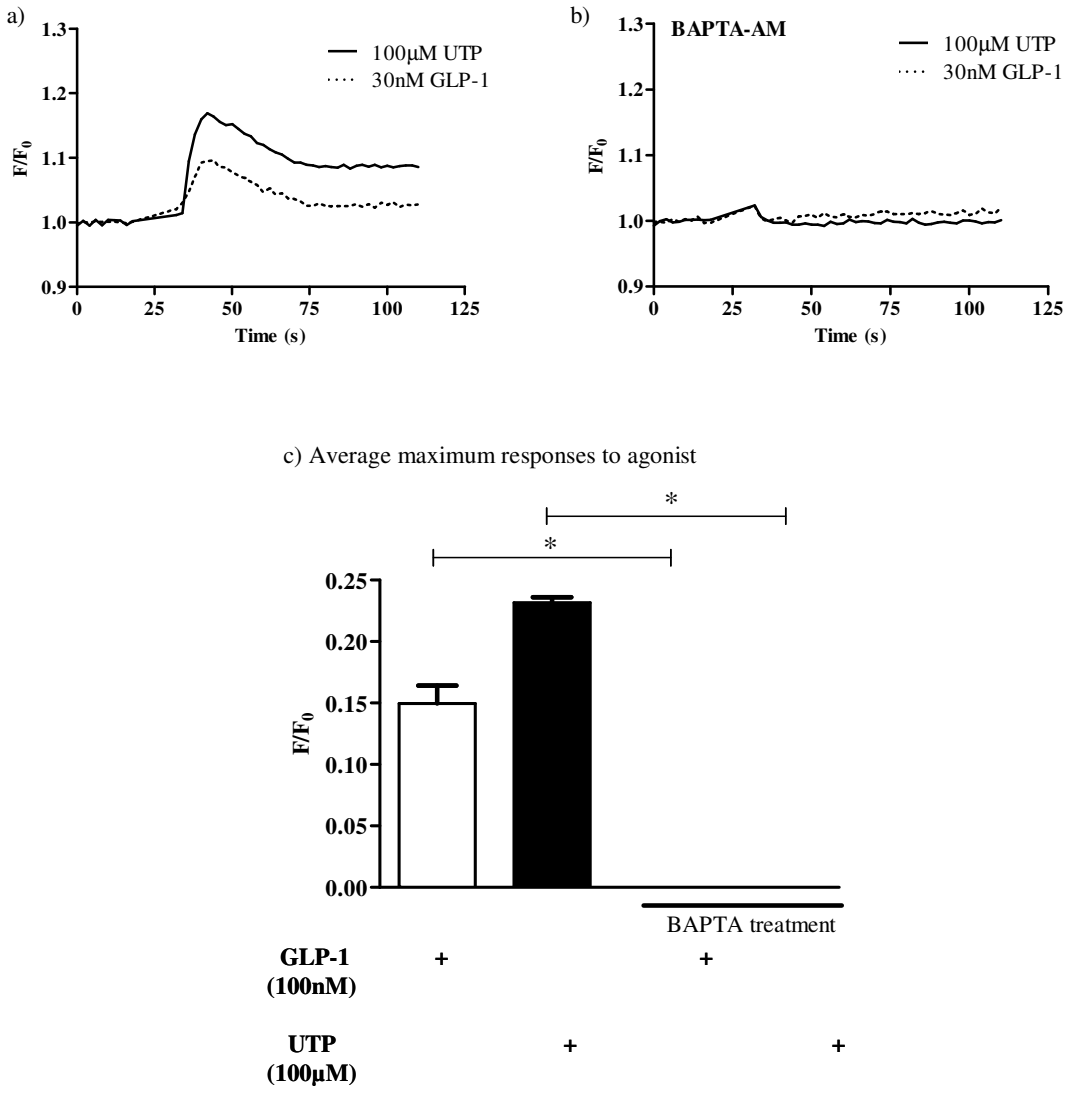


Figure 5.7 GLP-1- and UTP-mediated Ca^{2+} signalling is abolished by the Ca^{2+} chelator BAPTA-AM. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and stimulated with a maximal GLP-1 (300nM) or UTP (100µM) either in the absence a) or presence b) of BAPTA-AM (100µM, 30min). Maximal responses to GLP-1 and UTP in the absence and presence of BAPTA-AM are shown (c). Changes in fluorescence measured on the NOVOstar plate reader and calculated compared to the basal (F/F_0-1). Data are either representative of 3 individual experiments (a and b) or mean + s.e.mean, n=3 c). *$p<0.05$ Student's *t*-test

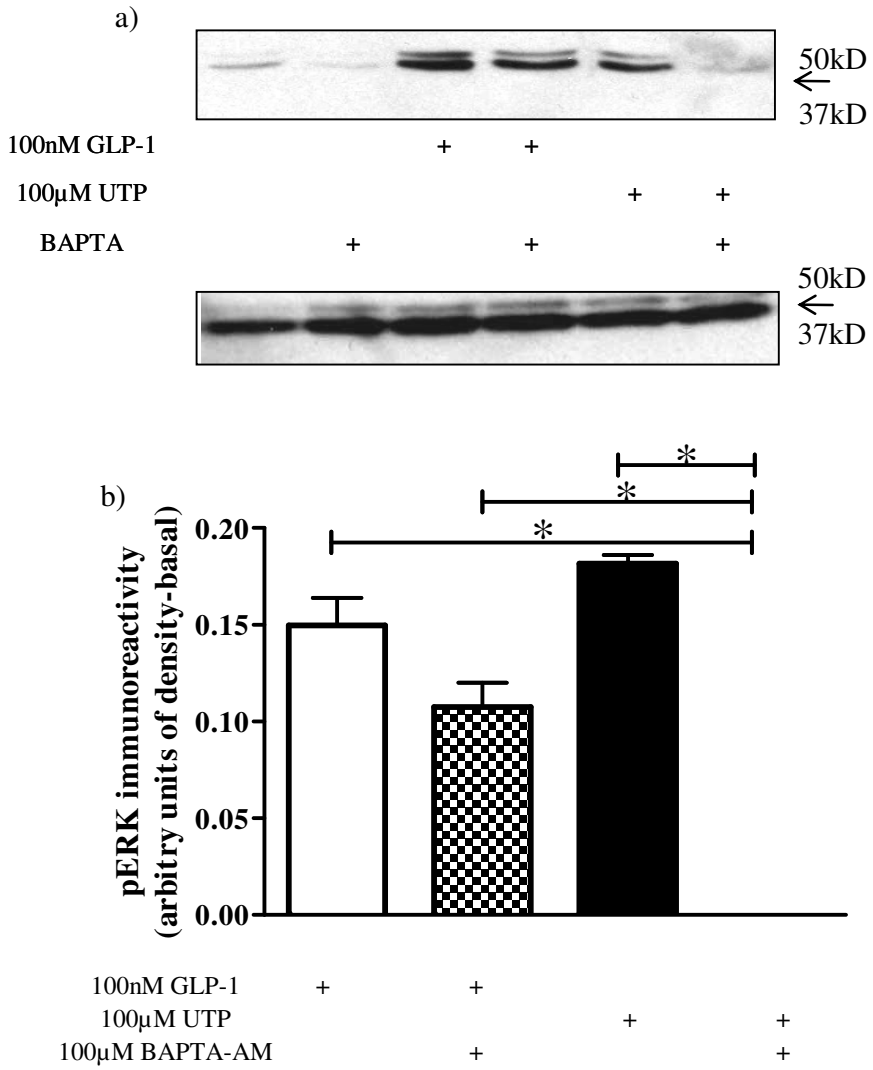


Figure 5.8 ERK phosphorylation mediated by UTP but not GLP-1 is abolished by the Ca^{2+} chelator BAPTA-AM. HEK 293 cells (stably transfected with the GLP-1 receptor) were cultured in a 24 well plate for 48h and either untreated (control) or pre-incubated with BAPTA-AM (100µM) for 30min before stimulation with agonists for 5min. Whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with either pERK or total ERK antibodies a) and visualised with ECL+ and exposure to film. Optical densitometry was carried out on the blots and values expressed relative to the background b). Data are mean + s.e.mean, n=3. *p<0.05 one-way ANOVA (Bonferroni's multiple comparison test).

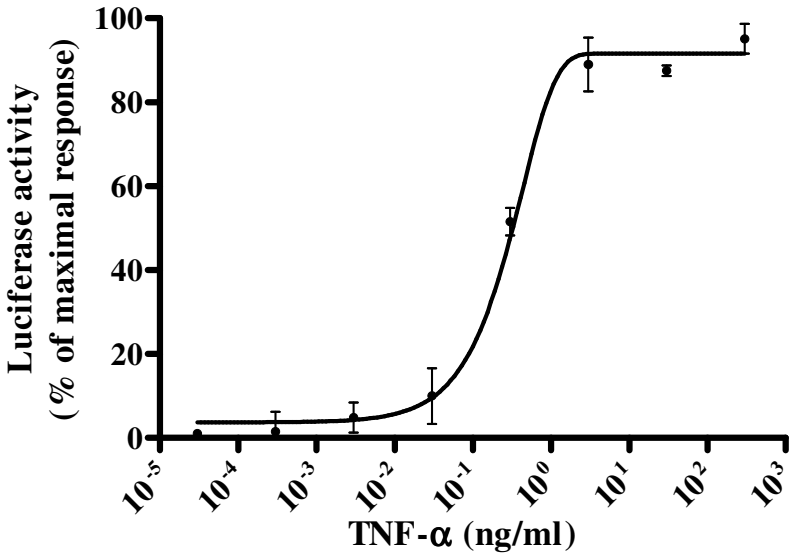


Figure 5.9. Activation of NF-κB by TNF-α. HEK 293 cells were transiently transfected with a luciferase reporter of NF-kB activity (NF-κB-luc) and 24h after transfection, cells were stimulated for 5.5h with TNF-α as described in *Materials and Methods*. Firefly luciferase activity was determined using luminometry and is calculated as a percentage of the maximum response. Data are mean ± s.e.mean, n=4.

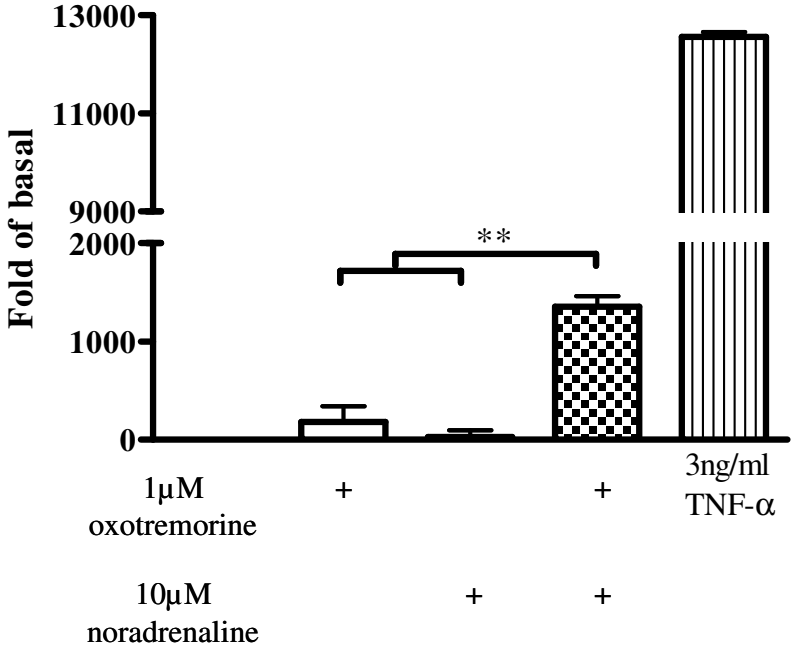


Figure 5.10. Activation of NF-kB by oxotremorine and noradrenaline. HEK 293 cells were transiently transfected with a luciferase reporter of NF-kB activity (NF-kB-luc) and 24h after transfection cells were stimulated for 5.5h with the indicated agonists as described in *Materials and Methods*. Firefly luciferase activity was determined using luminometry and is calculated as fold over basal-1. Data are mean+s.e.mean., n=5. **p<0.01.

5.4 Discussion

5.4.1 Summary of data

This thesis has focused on crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors leading to a synergistic increase in intracellular Ca^{2+} signalling. The aim of this chapter was to explore whether the crosstalk-mediated increase in Ca^{2+} signalling altered the activation of transcription factors downstream of Ca^{2+}.

Challenging the cells with methacholine or oxotremorine caused an increase in the phosphorylation of ERK. This is consistent with other studies demonstrating the activation of ERK by muscarinic receptor agonists (Budd *et al.,* 2001, Budd *et al.,* 2003, Gosens *et al.,* 2007). Treatment of the cells with noradrenaline alone did not cause an increase and actually caused a decrease in the phosphorylation of ERK, over the time course, suggesting activation of the β_2-adrenoceptors in these HEK 293 cells does not signal positively through ERK. Using the optimal agonist conditions for intracellular Ca^{2+} crosstalk (1μM oxotremorine and 10μM noradrenaline) a synergistic effect was observed in the phosphorylation of ERK 1/2. β_2-adrenoceptor activation leads to a decrease in ERK activation meaning the synergic increase in ERK, after co-stimulation with oxotremorine and noradrenaline, is even more impressive. Not only was a synergistic increase in phosphorylation of the protein observed but activation of ERK occurred more quickly with co-addition of oxotremorine (1μM) and noradrenaline (10μM) than with oxotremorine alone. The mechanism of this crosstalk is unknown. However, as discussed in Chapter 4, the results suggest cAMP may sensitise $Ins(1,4,5)P_3$ receptors at 'cAMP junctions'

increasing sensitivity of the receptors to Ins(1,4,5)P_3 and enhancing intracellular Ca^{2+} release. This increase in Ca^{2+} could augment Ras activation and therefore ERK activation.

Changes in the profile of ERK activation via crosstalk between GPCRs has also been demonstrated between $G\alpha_{q/11}$-coupled and $G\alpha_s$-coupled receptors in cardiac fibroblasts. Thus, when $G\alpha_{q/11}$-coupled α_1- and $G\alpha_s$-coupled β_2-adrenoceptors were concomitantly activated, ERK phosphorylation occurred and decreased rapidly. Activation of α_1-adrenoceptors alone produced a smaller and less rapid response with prolonged activation and stimulating only the β_2-adrenoceptors did not cause any phosphorylation of ERK (Cervantes *et al.,* 2010). This mechanism of crosstalk involved β_2-adrenoceptor-mediated recruitment of β-arrestin3 leading to the sequestration of ERK in the cytosol by direct binding between arrestin and ERK. The receptor/arrestin complex also caused phosphatase priming in the cytoplasm of the cells, leading to ERK dephosphorylation and rapid deactivation of the protein (Cervantes *et al.,* 2010). In this thesis the crosstalk mechanism in unknown but the evidence suggests that the synergistic ERK activation is caused by the synergistic increase in intracellular Ca^{2+}. Thus, when cells were treated with the cell permeable intracellular Ca^{2+} chelator BAPTA-AM, both intracellular Ca^{2+} signalling and ERK activation mediated via the crosstalk, were abolished. These different patterns of ERK activation are important and can regulate the fate of the cell. In PC12 pheochromocytoma cells the sustained but not transient activation of ERK caused differentiation of the cells into sympathetic-like neurons (Murphy and Blenis, 2006). Proteins such as c-Fos are also dependent on the kinetics of ERK signalling. The c-Fos protein is very unstable and accumulates only if ERK activation is sustained leading to phosphorylation of the c-terminal of the c-Fos protein (Murphy and Blenis, 2006).

In this study the $G\alpha_s$-coupled β_2-adrenoceptors did not activate ERK. Indeed, a decrease in basal ERK was observed over the time-course of incubation of the cells with noradrenaline. Although coupled to the cAMP β_2-adrenoceptors have been shown to elicit ERK activation in some clones of a cell line and inhibit ERK activation in other clones. In some cells β_2-adrenoceptors have been shown to inhibit ERK activation via PKA leading to phosphorylation and inhibition of c-Raf1 (Cook and McCormick, 1993, Crespo *et al.,* 1995). In the HEK 293 cells, used in the studies described in this thesis, the mechanism of ERK inhibition is unknown.

In HEK 293 and other cell types, activation of β_2-adrenoceptors has also been shown to activate ERK via a $G\alpha_s$-dependent pathway (Schmitt and Stork, 2000, Schmitt *et al.,* 2001). Different pathways have been implicated in this ERK activation with cAMP leading to activation of Epac and therefore Rap-1, whereas PKA activation can activate Rap-1 via Src (Wan and Huang, 1998). β_2-adrenoceptors have also been shown to phosphorylate ERK in HEK 293 cells by G-protein 'switching' and coupling to $G\alpha_i$ and increasing PLC activity by the $G\beta\gamma$-dimer (Daaka *et al.,* 1997, Luttrell *et al.,* 1999). Previous work in our laboratory demonstrated that crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors was dependent on cholera toxin-sensitive $G\alpha_s$-coupling. Therefore, the synergistic ERK activation demonstrated in this study is unlikely to be due to switching of the β_2-adrenoceptor G-protein coupling from $G\alpha_s$ to $G\alpha_i$.

Crosstalk between β_2-adrenceptors and muscarinic M_3 receptors also caused a synergistic response in another downstream effector. Treatment of HEK 293 cells transiently expressing the NF-kB luciferase construct with 1μM oxotremorine and 10μM

noradrenaline caused a synergistic increase in the NF-kB-mediated expression of luciferase suggesting an increase in the transcriptional activity of NF-kB. Previous studies have shown that intracellular Ca^{2+} can activate the transcription factor NF-kB via intermediate proteins including calmodulin kinases and calcineurin in a variety of cell types including skeletal muscle C2C12 cells, human T lymphocytes, human keratinocytes and mouse hippocampal neurons (Alzuherri and Chang, 2003, Howe *et al.,* 2002, Meffert *et al.,* 2003, Praskova *et al.,* 2002). Therefore, the increased intracellular Ca^{2+} signalling elicited by the crosstalk seen in this present study could directly lead to the increase in luciferase expression, indicating an increase in gene transcription. Intracellular Ca^{2+}-mediated increases in NF-kB have been demonstrated in the skin epithelial cell line NCTC2544 stably expressing the protease-activated receptor-2 (PAR2) (Macfarlane *et al.,* 2005). Treatment of the epithelial cell line with BAPTA-AM greatly reduced PMA- or trypsin-stimulated NF-kB reporter activity and NF-kB-DNA binding by reducing IKK activity. This indicated the NF-kB pathway was activated by intracellular Ca^{2+}.

Regulation of the NF-kB pathway by β_2-adrenoceptors has previously been demonstrated in HEK 293 cells (Gao, *et al.,* 2004). However, in that study, when β_2-adrenoceptors were activated with isoproterenol an inhibition of the NF-kB pathway was observed (Gao, *et al.,* 2004). The mechanism involved the interaction of β_2-adrenoceptors with IkBα (the inhibitor of NF-kB) via β-arrestin2, preventing phosphorylation and degradation of the IkBα and release of NF-kB (Gao, *et al.,* 2004). Although the mechanism for the increase in NF-kB in this thesis has not been investigated it is possible that a direct effect of the synergy in intracellular Ca^{2+} is the cause through binding to calmodulin and the activation of calcineurin (Alzuherri and Chang, 2003).

NF-kB is of particular interest because this transcription factor regulates inflammatory genes important in chronic inflammatory airway diseases such as asthma and COPD. The muscarinic M_3 receptor and β_2-adrenoceptor are co-expressed on airway smooth muscle regulating contraction and relaxation, respectively. NF-kB is essential for the pathogenesis of airway diseases (Barnes and Adcock, 1997, Barnes, 2006, Edwards *et al.,* 2009). Several studies have shown enhanced NF-kB in asthmatics including increased levels of NF-kB/p65 expression in peripheral blood mononuclear cells of asthmatic adults and children (Gagliardo *et al.,* 2003, La Grutta *et al.,* 2003). NF-kB has many downstream targets which are relevant in asthma and COPD. These include pro-inflammatory cytokines, chemokines, adhesion molecules and angiogenic factors (see Edwards *et al*., 2009 for general review). One enzyme of particular interest is cyclooxygenase-2 (cox-2) which catalyses the conversion of arachidonic acid into prostaglandins. These prostaglandins go on to become biologically active eicosanoids, which have inflammatory actions (Steer *et al.,* 2003). NF-kB is therefore a good target for therapeutic treatment of airway diseases but it is of the upmost importance not to inhibit beneficial pathways (Edwards *et al.,* 2009).

This chapter has demonstrated that crosstalk occurs at effector levels activated by intracellular Ca^{2+}. The synergistic effect demonstrated by ERK and NF-kB was most certainly linked to the synergistic increase in intracellular Ca^{2+} that was demonstrated in previous chapters, although the mechanism has not been directly examined for NF-kB. On chelation of the intracellular Ca^{2+}, ERK activation was abolished demonstrating a direct relationship between ERK activation and Ca^{2+}. These data indicate crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors could have physiological effects in airway

smooth muscle and in airway diseases leading to conditions such as enhanced inflammation.

Chapter 6: Does crosstalk occur in primary cells?

6.1 Overview of chapter

Crosstalk, between muscarinic M_3 receptors and β_2-adrenoceptors, resulting in a synergistic intracellular Ca^{2+} response has been observed in HEK 293 cells. Muscarinic M_3 receptors and β_2-adrenoceptors are co-expressed in primary tissue such as smooth muscle cells. This chapter explores whether crosstalk between these two receptor types occurs in primary rat tracheal smooth muscle (RTSM) cells and in an immortalised human bronchial smooth muscle (HBSM) cell line.

6.2 Introduction

Muscarinic M_3 receptors and β_2-adrenoceptors are co-expressed in smooth muscle tissue including urinary bladder, digestive tract and airway smooth muscle (Billington and Penn, 2002, Giglio *et al.,* 2007, Hool and Harvey, 1997, Proskocil and Fryer, 2005). In airway and other smooth muscle the stimulation of muscarinic M_3 receptors leads to contraction of smooth muscle via release of intracellular Ca^{2+} and activation of MLCKs (See Figure 6.1.1) (Billington *et al.*, 2003, Fryer *et al*., 1998, Tomkinson *et al.*, 1993, Shibata *et al*., 1998). The MLCKs activate MLCs by increasing its affinity for Ca^{2+}-calmodulin, allowing the formation of cross-bridges and contraction of the muscle (Billington *et al.*, 2003). Muscarinic receptors are expressed on the airway smooth muscle cells of many different species including human, mouse, guinea pig, bovine and rat (Preuss *et al*., 1999, Shibata *et al.,* 1998, Tomkinson *et al.,* 1993).

β_2-adrenoceptors have an opposing role to muscarinic M_3 receptors and cause relaxation of airway smooth muscle (Giembycz *et al*., 2006). They mediate this action through PKA which phosphorylates the MLCK, decreasing its affinity for ca^{2+}-calmodulin and reducing phosphorylation of MLC, leading to relaxation of the muscle (Figure 6.1.1.) (Billington *et al*., 2003). The co-expression of these two receptors enables the control of contraction and relaxation of airway smooth muscle and therefore receptors are useful targets for the therapeutic control of asthma and COPD (Kips and Pauwels, 2001, Peretto *et al.,* 2009, Walters *et al.,* 2007). Excessive contraction of airway smooth muscle via muscarinic M_3 receptors has long been known to cause some of the features of these diseases (Bellamy *et al*., 2007, Smith *et al.,* 2007). Crosstalk between the muscarinic M_3 receptors and β_2-adrenoceptors which leads to an increase in intracellular Ca^{2+} signalling and potentially contraction, seems counter-intuitive. Although it is unknown whether this crosstalk, enhancing intracellular Ca^{2+} signalling, occurs in airway smooth muscle a number of examples of crosstalk have been observed in smooth muscle.

The regulation of airway smooth muscle tone and crosstalk between the different receptors involved could be extremely important particularly when considering treatment of airway diseases. In human bronchial smooth muscle (HBSM) cells, 24h treatment with salmeterol and isoprenaline (selective and non-selective β_2-adrenoceptor agonists respectively) augmented histamine- and bradykinin-mediated InsPx production. The β_2-adrenoceptor agonists mediated this increase in InsPx production by elevating the mRNA levels of histamine and bradykinin receptors, therefore causing an increase in receptor expression (Smith *et al*., 2007). In bovine airway smooth muscle, activation of β_2-adrenoceptors by fenoterol has also been shown to upregulate histamine H_1 receptors (Mak *et al*., 2000).

These examples of crosstalk are probably dependent on protein synthesis and although very different from the crosstalk studied in this thesis, they demonstrate that crosstalk occurs between GPCRs in smooth muscle.

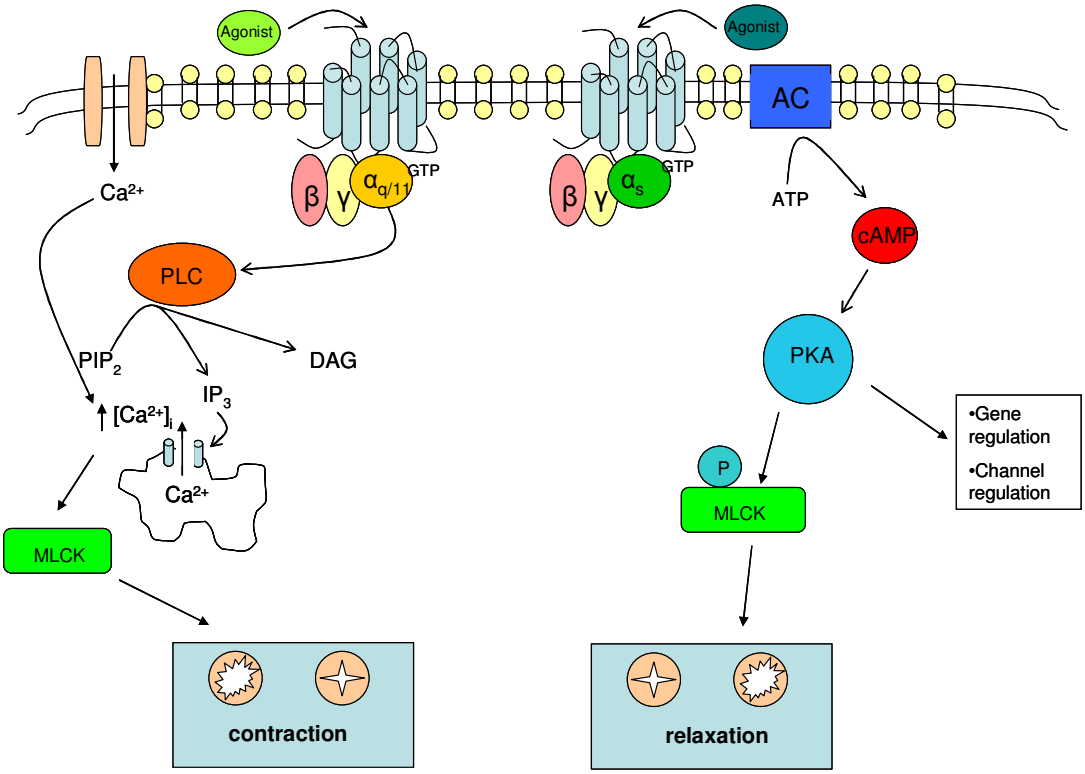


Figure 6.1.1 Contraction and relaxation of airway smooth muscle. $G\alpha_{q/11}$-coupled muscarinic M_3 receptors are activated by endogenous acetylcholine. This causes an increase and/or activation of downstream signalling causing contraction of the muscle. Circulating catecholamines activate $G\alpha_s$-coupled β_2-adrenoceptors leading to the phosphorylation and therefore deactivation of proteins activated by the muscarinic M_3 receptor. This leads to relaxation of the airway smooth muscle (Adapted from Billington *et al.* 2002).

Inhibitory crosstalk between muscarinic receptors and β-adrenoceptors, has been demonstrated in bovine tracheal smooth muscle. When activating the muscarinic receptors with carbachol or directly activating PKC there was a significant decrease in maximum binding capacity of β-adrenoceptors, on stimulation with isoproterenol. These results suggested that PKC activation via muscarinic stimulation may lead to a reduction in the number of functioning β-adrenoceptors and may explain the reduction of β-adrenoceptors in asthmatic airways (Grandordy *et al*., 1994). Crosstalk leading to sensitisation of pathways has also been demonstrated in HASM cells over a much longer time frame (Pascual *et al.,* 2001). Thus, when the cytokine IL-1β or TNF-α were added in the presence or absence of EGF (for 16h), two distinct signalling pathways were activated. This led to the desensitisation of PGE_2 receptors and β_2-adrencoceptors via PKA activation but also the sensitisation of adenylate cyclase leading to an increase in cAMP (Pascual *et al.,* 2001).

Based on the findings in previous chapters and evidence in the literature, this chapter aims to determine if crosstalk occurs between muscarinic receptors and β_2-adrenoceptors in isolated rat tracheal smooth muscle (RTSM) cells and an immortalised human bronchial smooth muscle (HBSM) cell line. The expression of muscarinic M_3 receptors and β_2-adrenoceptors was explored in both cell types along with crosstalk between β_2-adrenoceptors and other $G\alpha_{q/11}$-coupled receptors. Furthermore, mechanisms to upregulate muscarinic receptor expression in the primary rat tracheal smooth muscle cells were also examined.

6.3 Results

6.3.1 Staining of α-actin in tracheal smooth muscle cells

Staining the rat tracheal smooth muscle cells with an α-actin antibody demonstrated that on the day of isolation and also on each day of *in vitro* culture, actin (found in the myofilaments of muscle) was detected, indicating the presence of smooth muscle cells. On the day of isolation the coverslips were only approximately 10% covered with cells, however all these cells stained for actin (Figure 6.1). When culturing the cells for 1 to 6 days, the number of α-actin-stained cells increased indicating an increase in the number of airway smooth muscle cells. On day 7 and 8, smooth muscle cells were still visible with actin clearly present within the cell. However, their shape was becoming less elongated and pockets of fat were forming within the cells (Figure 6.1).

6.3.2 Challenge of rat tracheal smooth muscle cells with methacholine caused little or no intracellular Ca^{2+} response

Addition of methacholine (100μM) to rat tracheal smooth muscle cells on either the day of isolation or after 1 day of culture caused no intracellular Ca^{2+} response. Ionomycin (1μM) addition 570s after methacholine caused a Ca^{2+} response demonstrating the adequacy of fluo-4 AM loading. When cells had been cultured for two days, adding methacholine caused 12±6% (n=3 coverslips from different preparations, each containing 10 cells in the field of view) of cells to respond. The number of cells responding to methacholine after 3 to 4 days of culture was 28±3 and 46±6% respectively. On days 5 to 7 of culture all of the cells responded to methacholine (Figure 6.2). On this basis culturing the cells for 5 days was chosen for further experiments.

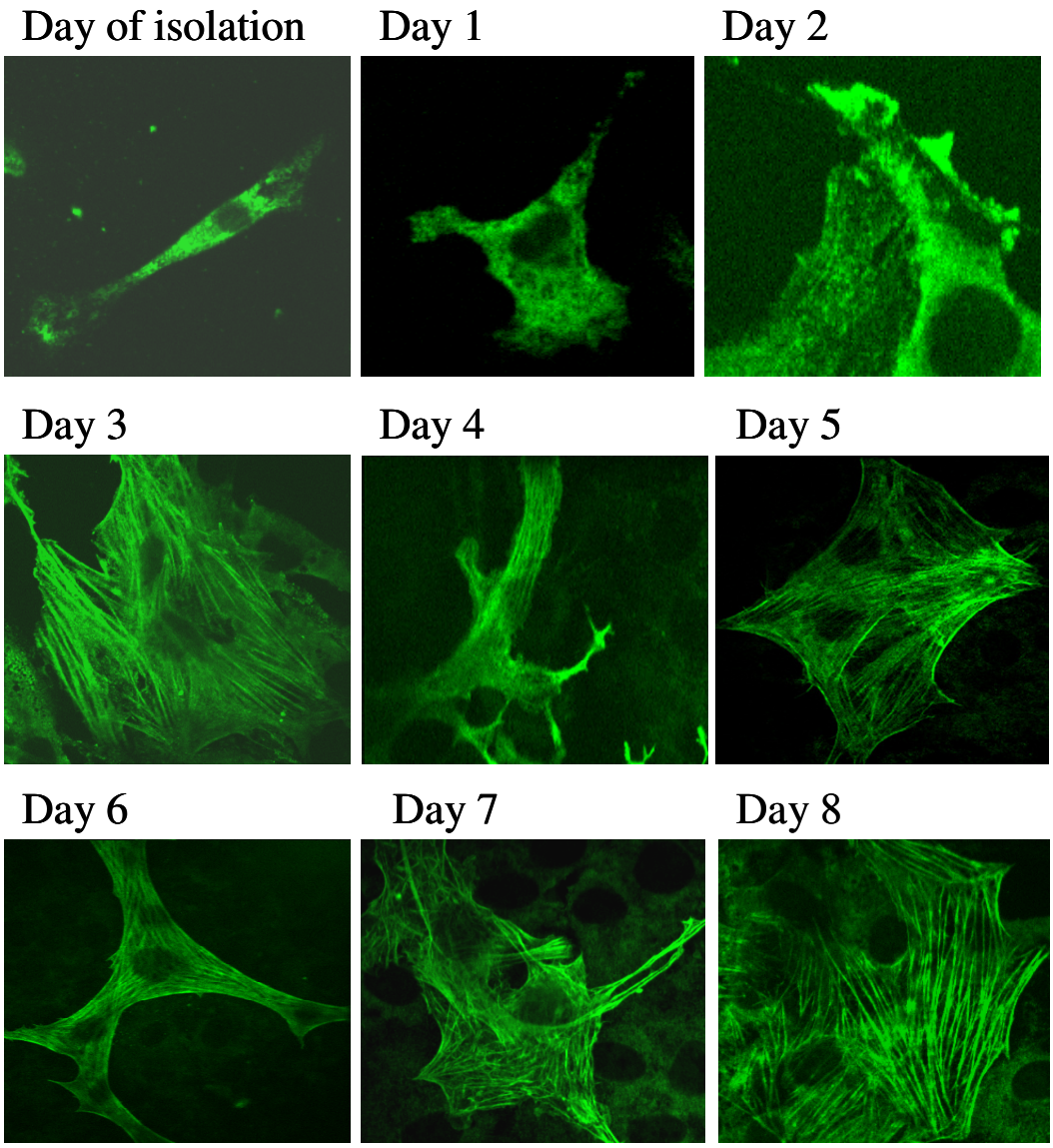


Figure 6.1. ICC confocal images of α-actin in rat tracheal smooth muscle cells at isolation (as soon as adherent) and following *in vitro* culture. Rat tracheal smooth muscle cells were isolated and cultured from one up to eight days. On each day of culture, cells were stained with an α-actin monoclonal antibody overnight and incubated with an anti-mouse IgG FITC secondary antibody. The cells were examined on a confocal microscope as described in *Materials and Methods*. The Figure demonstrates the classical elongated cell shape of smooth muscle cells and the actin filaments can clearly be seen on each of the nine images.

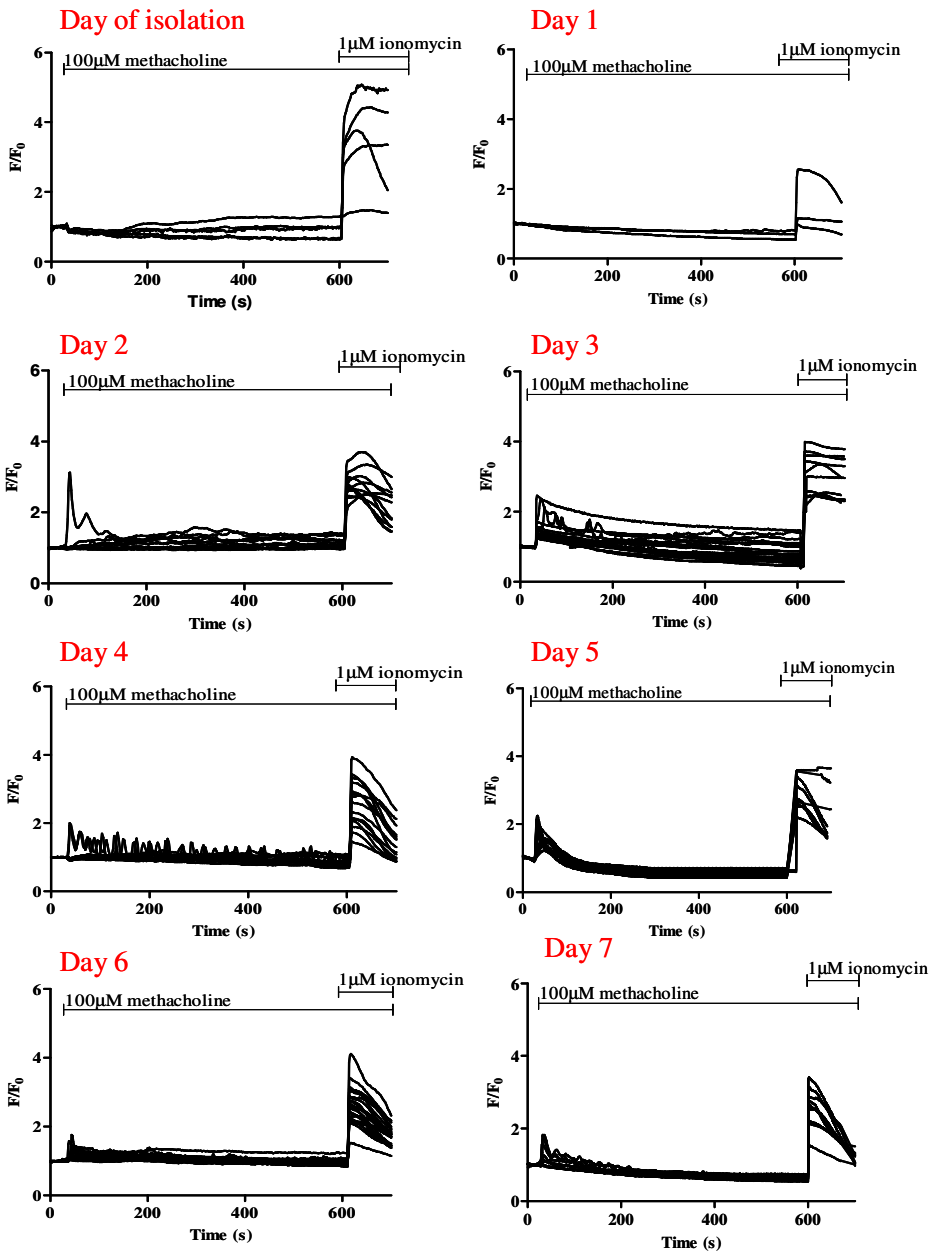


Figure 6.2. Single cell imaging of Ca^{2+} responses to 100µM methacholine and 1µM ionomycin in rat tracheal smooth muscle cells at isolation and following *in vitro* culture. Cells were stimulated with 100µM methacholine at 30s and susequently challenged with 1µM ionomycin at 600s. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 15 cells in the field of view. Data are representative of n=3 coverslips, from 3 different preparations.

6.3.3 Pre-treatment of cells with the nicotinic receptor antagonist hexamethonium chloride abolished methacholine-mediated Ca^{2+} responses.

When methacholine (100μM) was added to cells after 5 days in culture, all cells responded with some Ca^{2+} oscillations (Figure 6.3a). However when the experiments were carried out in presence of the nicotinic acetylcholine receptor antagonist, hexamethonium chloride (1μM), the methacholine-mediated Ca^{2+} response was abolished (Figure 6.3b).

6.3.4 Noradrenaline-mediated intracellular Ca^{2+} responses in rat tracheal smooth muscle cells

Addition of noradrenaline (10μM) to rat tracheal smooth muscle cells after 5 days in culture caused an intracellular Ca^{2+} response (Figure 6.4a). When the cells were incubated with phentolamine (10μM), an α-adrenoceptor antagonist, the noradrenaline-mediated response was abolished (Figure 6.4b).

6.3.5 Would crosstalk occur between muscarinic receptors and β_2-adrenoceptors in the presence of hexamethonium chloride and phentolamine?

Treating the cells with methacholine (100μM) in the presence of hexamethonium chloride (1μM) and phentolamine did not elicit an intracellular Ca^{2+} response. Addition of noradrenaline at 360s into the experiment was also unable to elicit an intracellular Ca^{2+} response (Figure 6.5).

6.3.6 Does serum withdrawal reveal muscarinic receptor-mediated Ca^{2+} signalling?

Although experiments failed to demonstrate crosstalk at the level of Ca^{2+} signalling between methacholine and noradrenaline, it was notable that methacholine alone did not evoke a Ca^{2+} response. This could suggest a lack of $G\alpha_{q/11}$-coupled muscarinic receptors, which is consistent with loss of muscarinic M_3 on culture of smooth muscle cells (Boselli *et al.,* 2002, Gosens *et al.,* 2003). Some evidence suggests that serum-withdrawal may re-establish muscarinic receptor-mediated Ca^{2+} signalling (Gosens *et al.,* 2003, Singer *et al.,* 2002). Thus, serum-withdrawal from the cells was carried out in an attempt to upregulate muscarinic receptor expression. Following serum-withdrawal for 24 hours all cells gave a Ca^{2+} response on addition of methacholine (Figure 6.6a). However, these responses were abolished after the serum-starved cells were treated with hexamethonium chloride (Figure 6.6b).

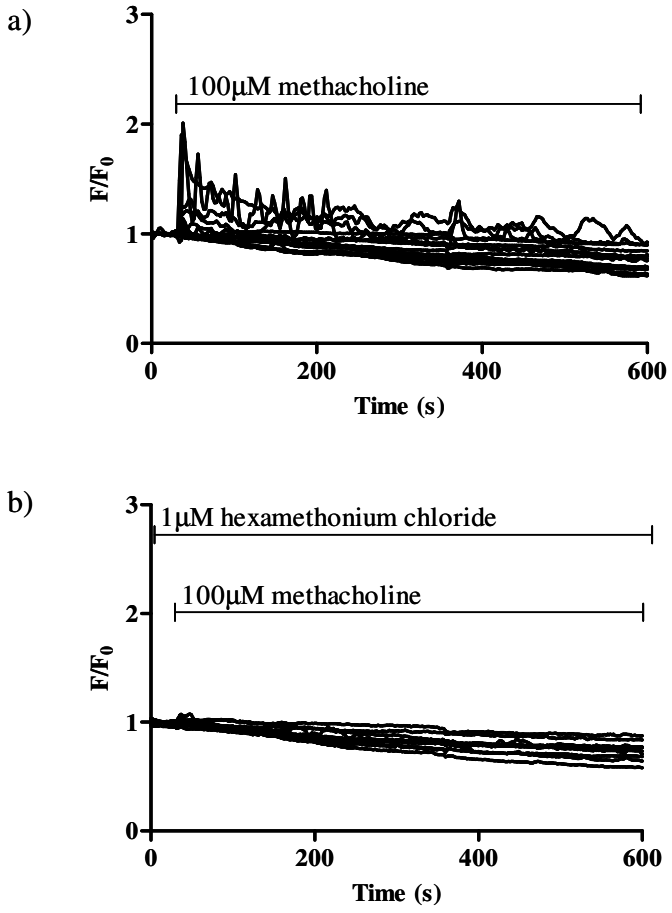


Figure 6.3. Single cell imaging of Ca^{2+} responses to 100µM methacholine in the absence and presence of 1µM hexamethonium chloride, a nicotinic receptor antagonist. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and challenged with 100µM methacholine at 30s in the continued absence (a) or presence (b) of 1µM hexamethonium chloride. Experiments were performed on cells after 5 days in culture. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 15 cells in the field of view. Data are representative of n=3 coverslips.

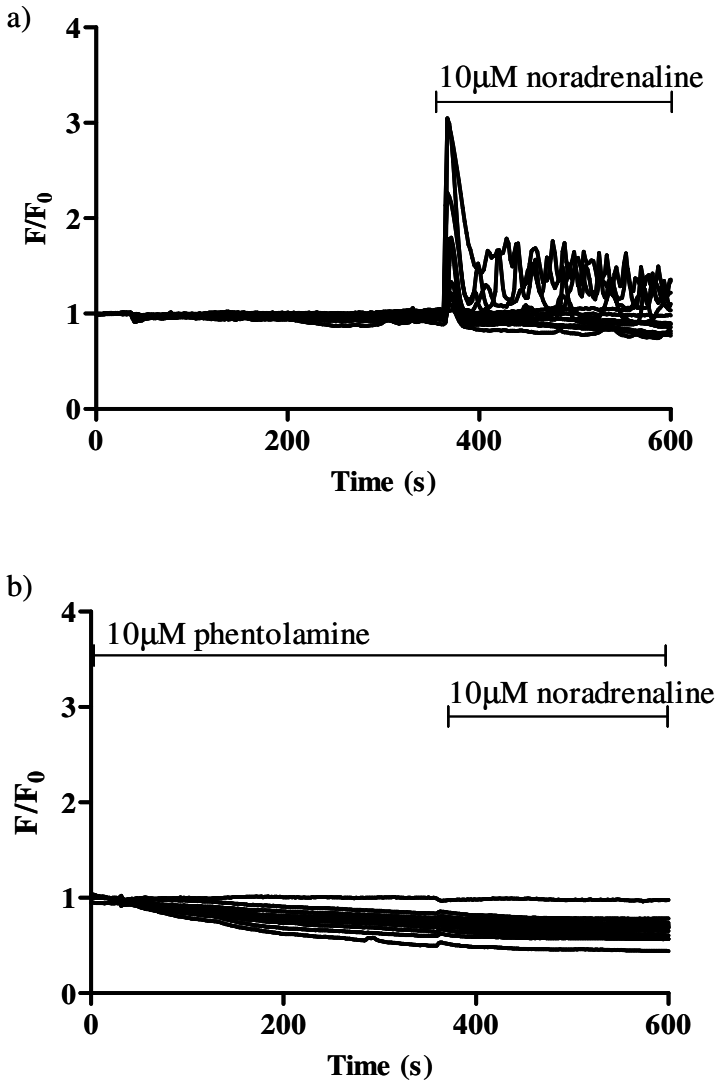


Figure 6.4. Single cell imaging of Ca^{2+} responses to 10μM noradrenaline in the absence and presence of 10μM phentolamine, an α-adrenoceptor antagonist in rat tracheal smooth muscle cells after 5 days in culture. Cells were loaded with fluo-4 AM as specified in *Materials and Methods,* in the absence or presence of phentolamine (10μM) then imaged by confocal microscopy. Buffer was added to the cells at 30s and 10μM noradrenaline at 360s in the continued absence (a) or presence (b) of phentolamine. Changes in cytosolic fluorescence were calculated compared to basal (F/F_0) and are shown for approximately 15 cells in the field of view. Data are representative of n=3 coverslips.

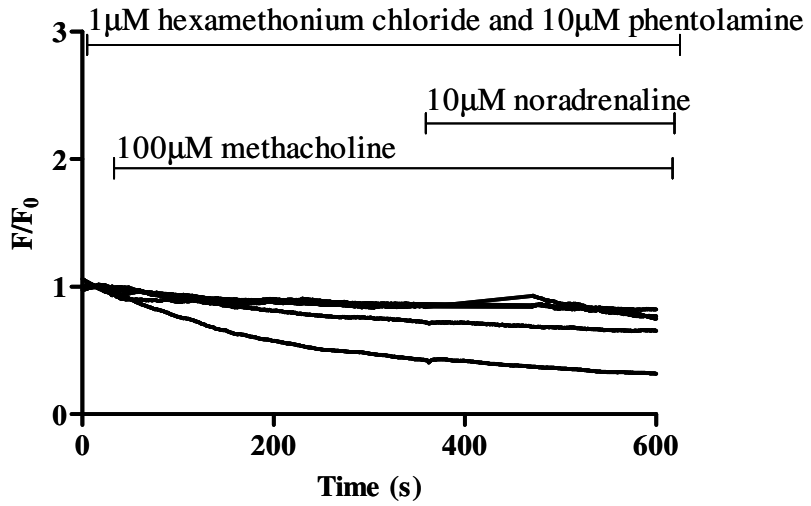


Figure 6.5. Single cell imaging of Ca^{2+} responses to 100μM methacholine and 10μM noadrenaline in the presence of 1μM hexamethonium and 10μM phentolamine in rat tracheal smooth muscle cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and challenged with 100μM methacholine at 30s and 10μM noradrenaline in the continued presence of 1μM hexamethonium chloride and 10μM noradrenaline. Experiments were performed on cells after 5 days in culture. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 15 cells in the field of view. Data are representative of n=3 coverslips.

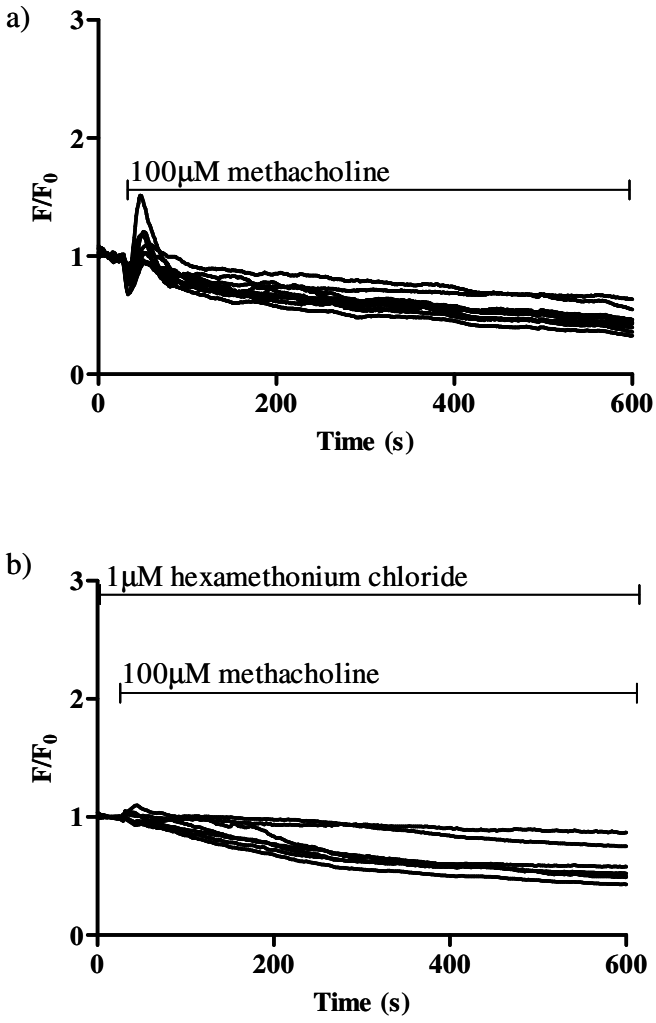


Figure 6.6. Single cell imaging of Ca^{2+} responses to 100µM methacholine without or with 1µM hexamethonium chloride pre-incubation, after 24h serum withdrawal in rat tracheal smooth muscle cells. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and challenged with 100µM methacholine at 30s in the continued absence (a) or presence (b) of 1µM hexamethonium chloride. Experiments were performed on cells after 5 days in culture with serum withdrawal for the last 24h. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 15 cells in the field of view. Data are representative of n=3 coverslips.

6.3.7 Examination of crosstalk between an alternative $G\alpha_{q/11}$-coupled receptor and the β_2-adrenoceptor

Attempts to upregulate muscarinic receptor-mediated Ca^{2+} signalling were not successful and therefore experiments were carried out using two other agonists, UTP and bradykinin. These are known to activate other $G\alpha_{q/11}$-coupled receptors i.e. P_2Y receptors and the bradykinin receptors both of which are expressed on RTSM cells (Jacobson and Boevnaems, 2010, Newton *et al.,* 2002).

Addition of UTP (10-0.1μM) caused an intracellular Ca^{2+} response from all of the cells (Figure 6.7a-c). In the presence of phentolamine (10μM), to block the α-adrenoceptors, the magnitude of the UTP-mediated intracellular Ca^{2+} responses were unaffected. In the presence of UTP (10-0.1μM) and phentolamine, noradrenaline did not mediate an intracellular Ca^{2+} response (Figure 6.8a-c).

Addition of bradykinin (10-0.1μM), an agonist for $G\alpha_{q/11}$-coupled bradykinin receptors, resulted in Ca^{2+} responses in all cells at all concentrations tested (Figure 6.9a-c). In the presence of bradykinin (1μM and 0.1μM) and the α-adrenoceptor antagonist, phentolamine, noradrenaline was unable to mediate a Ca^{2+} response (Figure 6.10a and b).

6.3.8 Is the muscarinic M_3 receptor expressed at the surface of cultured rat tracheal smooth muscle cells?

The experiments to date had failed to show a muscarinic receptor-mediated response in the RTSM cells. One potential explanation was that the receptor was not expressed at the cell

surface. To examine receptor expression, ICC was performed on RTSM and HEK 293 cells (both untransfected and transfected with muscarinic M_3 receptors) using an antibody for the mouse muscarinic M_3 receptor (Figure 6.11). There is sequence similarity between the rat and mouse muscarinic M_3 receptor in the region against which the antibody was generated, suggesting that the mouse muscarinic M_3 antibody would detect the rat muscarinic M_3 receptor. Using the mouse muscarinic M_3 antibody, ICC of untransfected HEK 293 cells or HEK 293 cells transiently transfected with the human muscarinic M_3 receptor did not reveal any staining (Figure 6.11b and c). However, ICC of HEK 293 cells transiently transfected with the rat muscarinic M_3 receptor demonstrated staining consistent with detection of the rat muscarinic M_3 receptor with the mouse muscarinic M_3 antibody. This also suggested the receptor was localised at the plasma membrane (Figure 6.11d). Indeed, an overlay of the staining patterns of the mouse muscarinic M_3 receptor antibody and F-actin antibody indicated that the rat muscarinic M_3 receptors were present at the cell surface. However, no staining was observed using the mouse muscarinic M_3 receptor for ICC of RTSM cells suggesting an absence of muscarinic M_3 receptor expression or expression of the receptor below that of the sensitivity of the technique (Figure 6.11a). In contrast, F-actin within the cells was detected by the phalloidin antibody demonstrating the presence of cells on the coverslip.

6.3.9 RT-PCR of muscarinic M_3 receptor

Attempts to demonstrate crosstalk in RTSM cells had been unsuccessful. Further, ICC had suggested an absence of receptor expression. As an alternative strategy to investigate the expression of muscarinic M_3 receptors in these cells RT-PCR was carried out on RNA

extracted from RTSM, HEK 293 and HBSM cells to examine whether muscarinic M_3 receptor mRNA was present and also in the cell lines used in the crosstalk experiments as positive controls (Figure 6.12). mRNA was reverse transcribed and part of the muscarinic M_3 receptor sequence amplified using primers designed to give products of 461bp and 376bp in size for the rat and human muscarinic M_3 receptors respectively. Primers complementary to the human M_3 sequence were used for amplification of sequences from the 379bp, present in the third intracellular loop. Sequences for the primers were the same as those used previously (Section 2.3.2.2) (Iwanaga *et al,.* 2009 (rat muscarinic M_3 receptor primers), Frucht *et al.,* 1999 (human muscarinic M_3 receptor primers)). When the RT-PCR products were separated by agarose gel electrophoresis bands were clearly seen at the expected sizes, except in lane 2, which represented the 5 day culture of RTSM cells (Figure 6.12). The absence of a band indicates a lack of muscarinic M_3 mRNA suggesting the receptor may not be expressed.

6.3.10 RT-PCR of the β_2-adrenoceptor

RT-PCR of the β_2-adrenoceptor was also carried out on RNA extracted from RTSM cells, HEK 293 cells and the human HBSM cell line (Figure 6.13). This cell line was used because it was more physiologically relevant than HEK 293 cells and expressed muscarinic M_3 receptors. Therefore these cells may have allowed further exploration of crosstalk not possible in the RTSM cells because of the loss of the muscarinic M_3 expression. mRNA was reverse transcribed and part of the β_2-adrenoceptor sequence amplified using primers designed to give a 559bp and 401bp product, the expected size of the specific coding sequences of the rat and human β_2-adrenoceptor respectively (Figure 6.13 lane 1). Sequences for the primers were the same as those used previously (Section 2.3.2.2) (Fujii et

al. 1998 (human β_2-adrenoceptor primers), Tanaka et al. 2002 (rat β_2-adrenoceptor primers)).

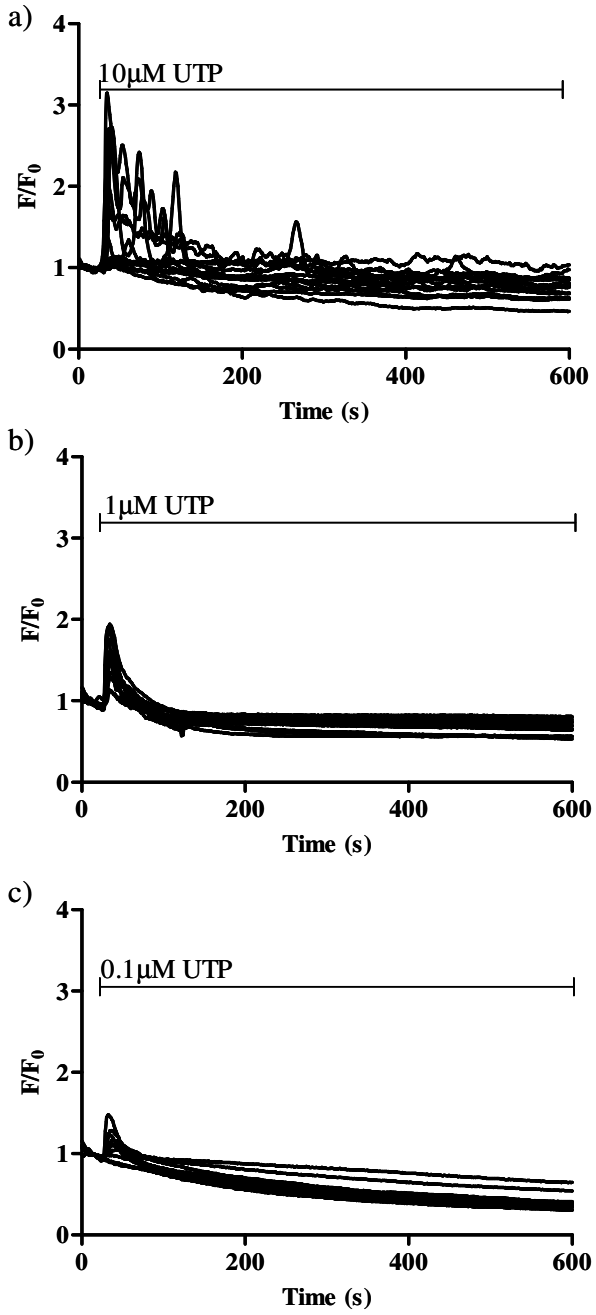


Figure 6.7. Single cell imaging of Ca^{2+} responses to UTP in rat tracheal smooth muscle cells after 5 days in culture. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and imaged by confocal microscopy. Cells were challenged with 10μM (a), 1μM (b), or 0.1μM (c) UTP at 30s. Changes in cytosolic fluorescence were compared to the basal (F/F_0) and shown for approximately 13 cells in the field of view. Data are representative of n=3 coverslips.

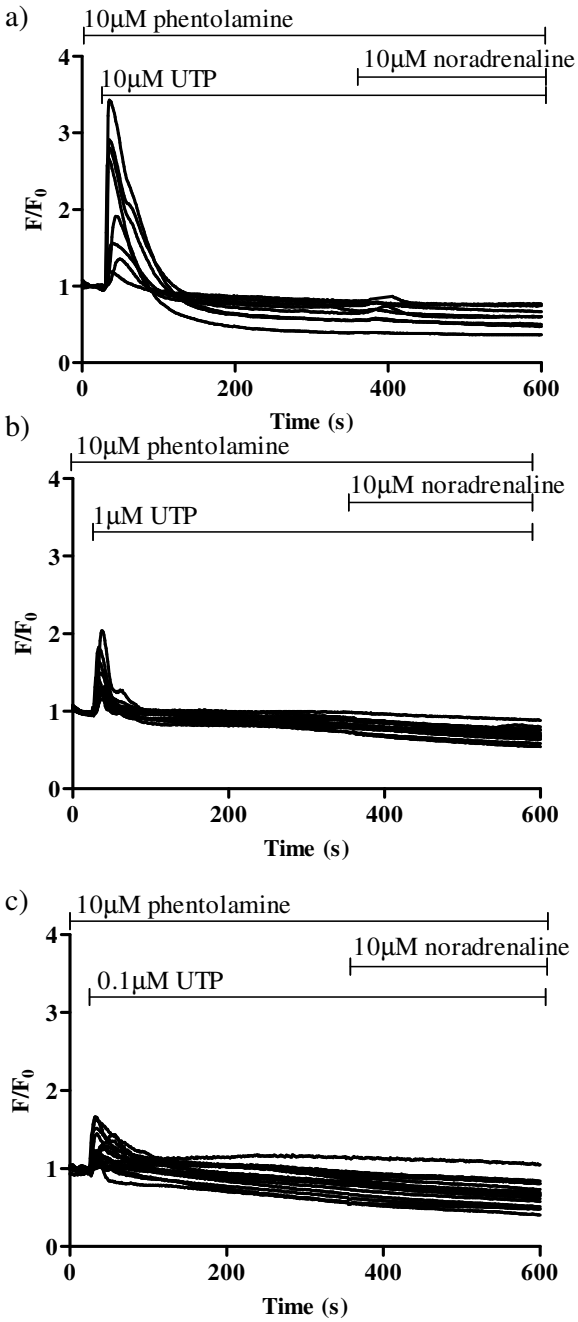


Figure 6.8. Single cell imaging of Ca^{2+} responses to UTP and noradrenaline in the presence of the α-adrenoceptor antagonist phentolamine in rat tracheal smooth muscle cells after 5 days in culture. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and incubated with phentolamine (10μM) and imaged by confocal microscopy. Cells were challenged with 10μM (a) 1μM (b) or 0.1μM (c) UTP at 30s followed by 10μM noradrenaline at 360s. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 13 cells in the field of view. Data are representative of n=3 coverslips.

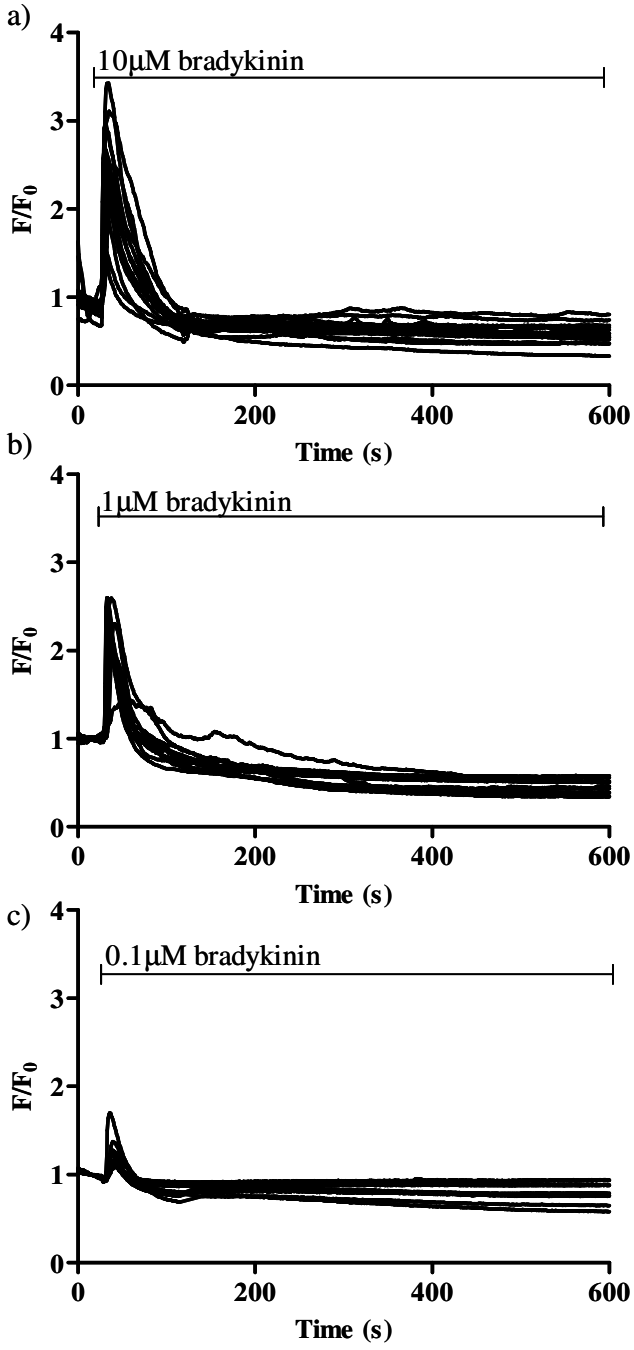


Figure 6.9. Single cell imaging of Ca^{2+} responses to bradykinin in rat tracheal smooth muscle cells after 5 days in culture. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and imaged by confocal microscopy. Cells were challenged with 10µM (a) 1µM (b) or 0.1µM (c) bradykinin at 30s. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 13 cells in the field of view. Data are representative of n=3 coverslips.

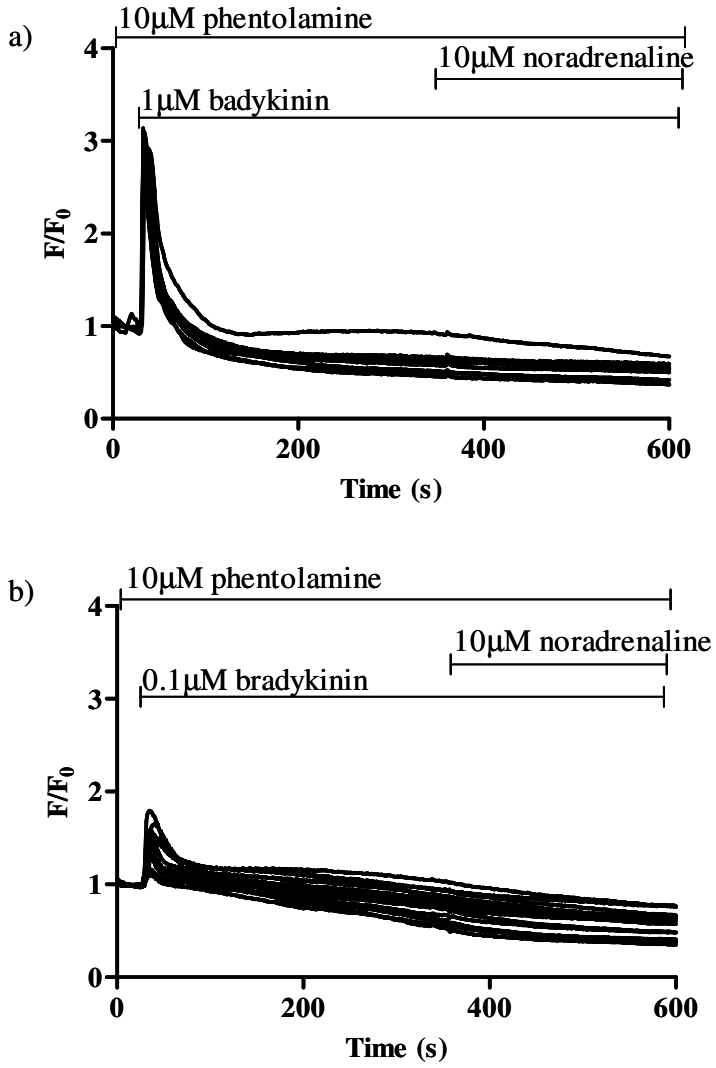


Figure 6.10. Single cell imaging of Ca^{2+} responses to bradykinin and noradrenaline in the presence of the α-adrenoceptor antagonist phentolamine in rat tracheal smooth muscle cells after 5 days of growth. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and incubated with 10μM phentolamine for 10 minutes before imaging by confocal microscopy. Cells were challenged with 1μM (a) or 0.1μM (b) bradykinin at 30s followed by 10μM noradrenaline at 360s. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 13 cells in the field of view. Data are representative of n=3 coverslips.

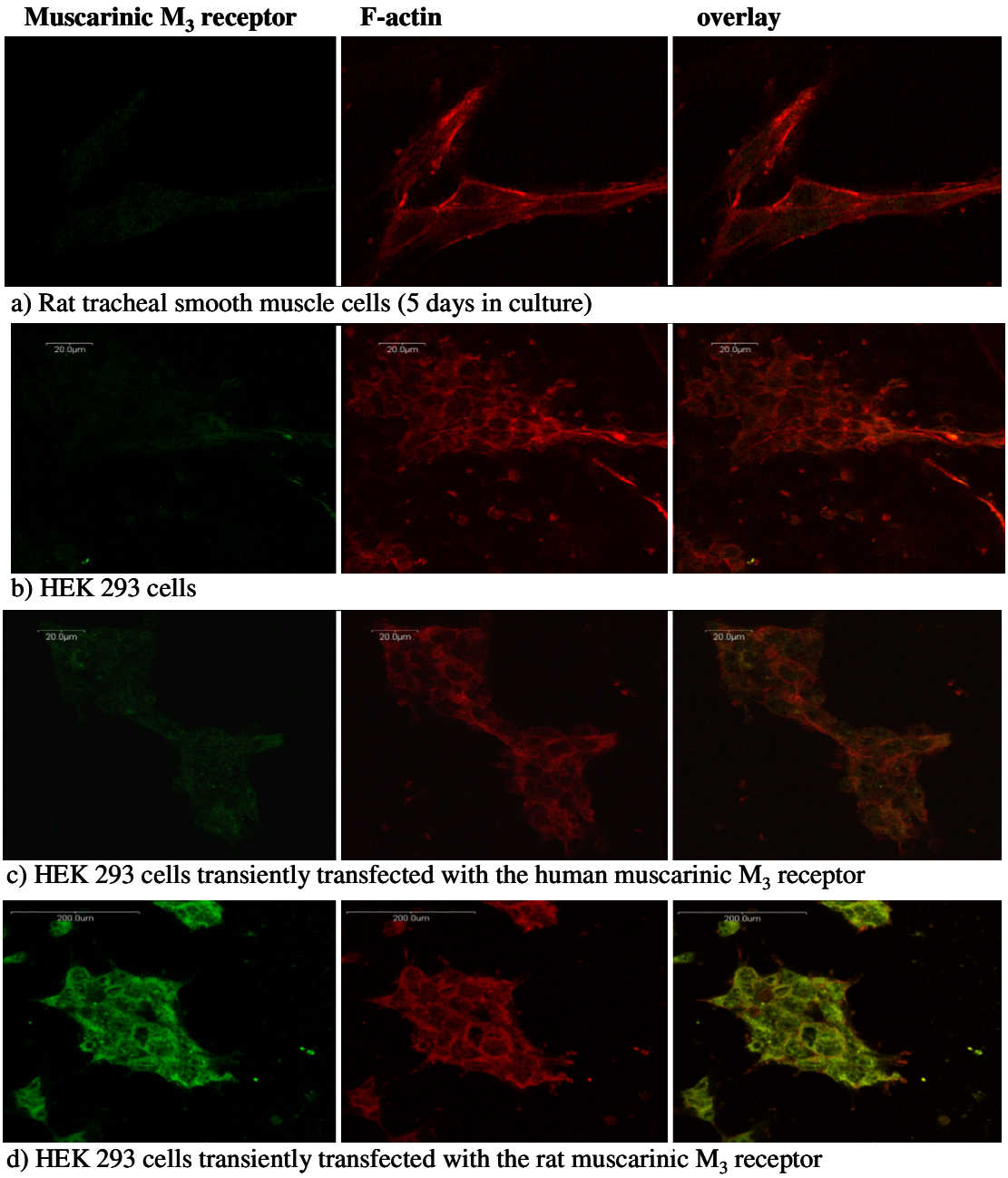
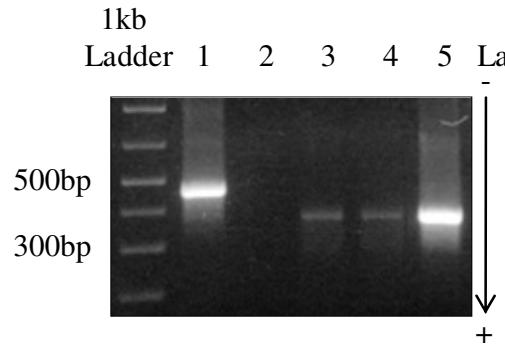
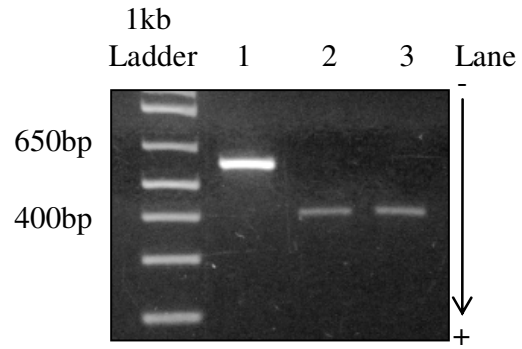


Figure 6.11. ICC confocal images of muscarinic M_3 receptor expression and F-actin staining. Muscarinic M_3 receptor expression was detected using a mouse muscarinic M_3 receptor antibody and secondary anti-mouse IgG FITC antibody, produced in goat. F-actin was detected using a FITC-phalloidin conjugate antibody and the cells were examined on a confocal microscope. The green cells demonstrate those expressing muscarinic M_3 receptors. The F-actin demonstrates cells are present on the coverslips and the overlay shows the cells that express the muscarinic M_3 receptor do so on their surface.



Lane	Source of DNA	Expected size of product
1	Rat muscarinic M_3 receptor DNA control plasmid	461bp
2	Rat tracheal smooth muscle cells	461bp
3	HEK 293 cells	376bp
4	Human bronchial smooth muscle cell line	376bp
5	Human muscarinic M_3 receptor DNA control plasmid	376bp

Figure 6.12. RT-PCR analysis of the muscarinic M_3 receptor. RNA was extracted from the cells and cell lines shown in the table. RT-PCR was performed as described in *Materials and Methods* (35 cycles, 58ºC). The figure represents gel electrophoresis of the PCR products.



Lane	Source of DNA	Expected size of product
1	Rat tracheal smooth muscle cells	559bp
2	HEK 293 cells	401bp
3	Human bronchial smooth muscle cell line	401bp

Figure 6.13. RT-PCR analysis of the β_2-adrenoceptor. RNA was extracted from the cells and cell lines shown in the table. RT-PCR was performed as described in *Materials and Methods* (35 cycles, 55ºC). The figure represents gel electrophoresis of the PCR products.

6.3.11 Does crosstalk occur in the human bronchial smooth muscle cell line?

Staining the HBSM cells with an F-actin antibody, found in the myofilaments of muscle, indicated the presence of smooth muscle cells (Figure 6.14). Addition of methacholine (100µM) caused an intracellular Ca^{2+} response from all the cells (Figure 6.15a) and the same response was observed in the presence of the nicotinic receptor blocker, hexamethonium chloride (Figure 6.15b). However, addition of noradrenaline 330s after methacholine did not cause a second Ca^{2+} response (Figure 6.15c). Addition of noradrenaline in the presence of either 1µM methacholine, 100µM or 1µM oxotremorine also did not evoke a Ca^{2+} response, therefore providing no evidence of crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors (Figures 6.16a to c). Reducing the time between addition of the muscarinic receptor agonist (100µM or 1µM methacholine) and noradrenaline to 30s did not result in a Ca^{2+} response to noradrenaline (Figure 6.17a and b).

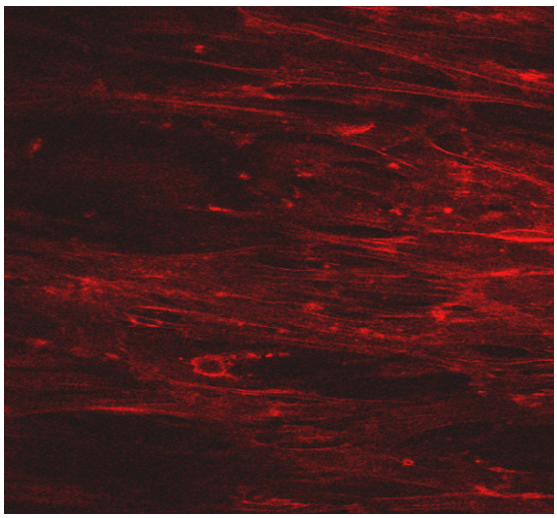


Figure 6.14. ICC confocal images of F-actin staining in a human bronchial smooth muscle cell line 2 days after plating. F-actin was detected using a FITC-phalloidin conjugated antibody and the cells were examined by confocal microscopy as described in *Materials and Methods*.

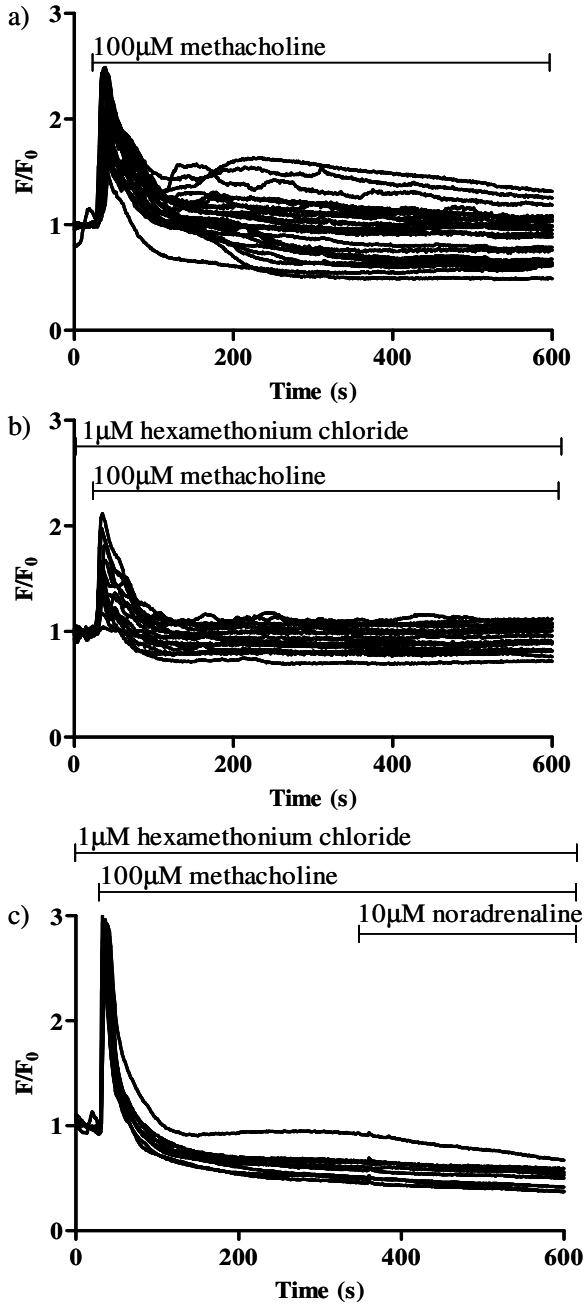


Figure 6.15. Single cell imaging of Ca^{2+} responses during addition of methacholine and noradrenaline in the absence and presence of 1μM hexamethonium chloride in a human airway smooth muscle cell line. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and if needed incubated with hexamethonium chloride for 15 minutes before imaging by confocal microscopy. Cells were challenged with 100μM methacholine at 30s (a and b) or 100μM methacholine at 30s followed by 10μM noradrenaline at 360s (c). Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 13 cells in the field of view. Data are representative of n=3 coverslips.

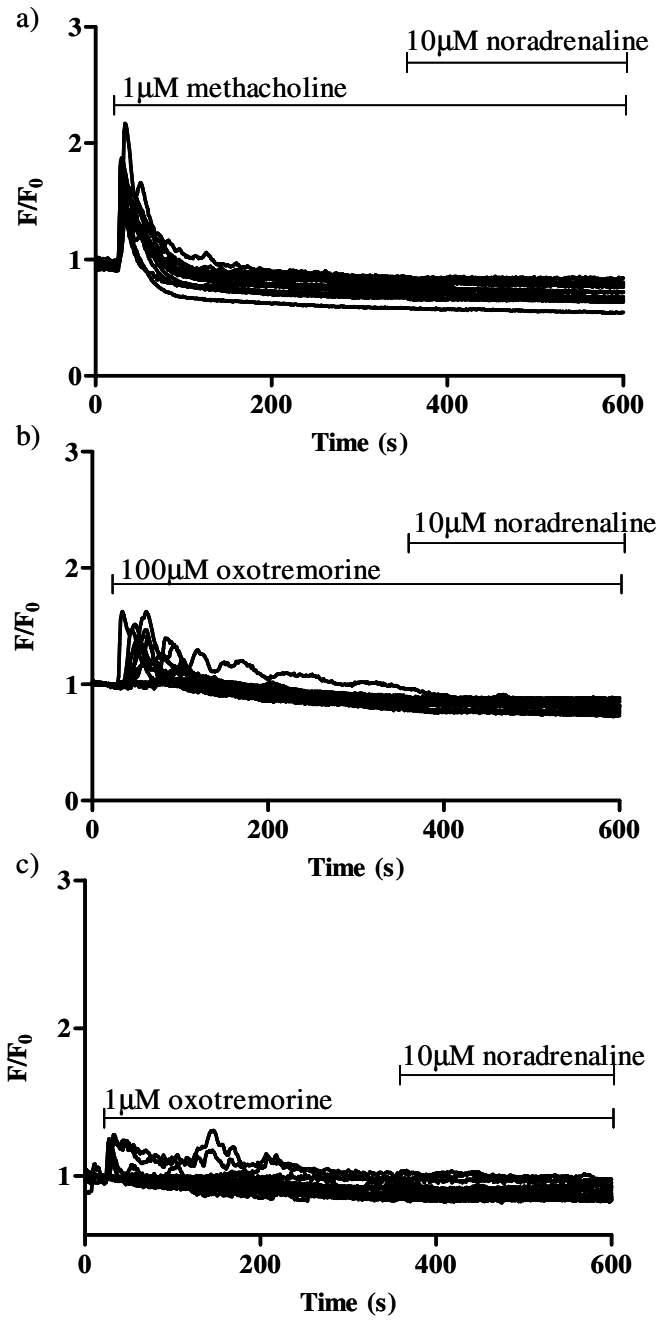


Figure 6.16. Single cell imaging of Ca^{2+} responses addition of noradrenaline in the presence of methacholine or oxotremorine in a human airway smooth muscle cell line. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and imaged by confocal microscopy. Cells were challenged with 1μM methacholine at 30s (a), 100μM (b) or 1μM (c) oxotremorine at 30s followed by 10μM noradrenaline at 360s. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 13 cells in the field of view. Data are representative of n=3 coverslips.

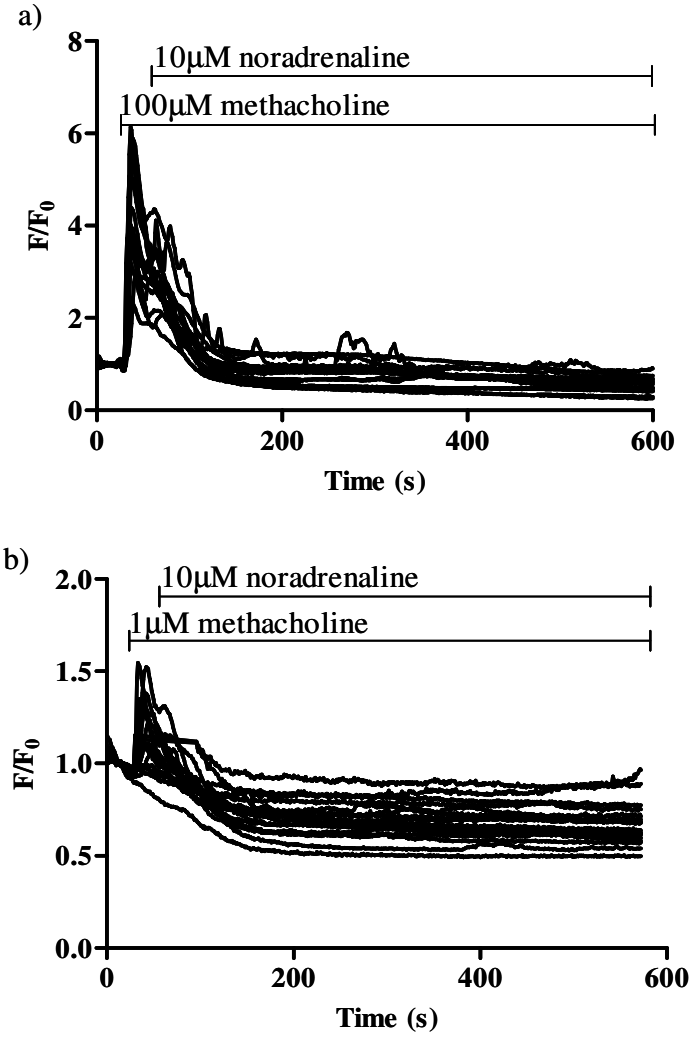


Figure 6.17. Single cell imaging of $[Ca^{2+}]_i$ during addition of noradrenaline in the presence of methacholine in a human airway smooth muscle cell line. Cells were loaded with fluo-4 AM as specified in *Materials and Methods* and imaged by confocal microscopy. Cells were challenged with 100μM (a) or 1μM (b) methacholine followed by 10μM noradrenaline at 60s. Changes in cytosolic fluorescence were calculated compared to the basal (F/F_0) and are shown for approximately 13 cells in the field of view. Data are representative of n=3 coverslips.

6.4 Discussion

6.4.1 Summary of data

The aim of this chapter was to determine whether crosstalk could occur in primary cells, which are clearly both physiologically, pathologically and therapeutically of more relevance to understanding potential crosstalk in airway smooth muscle than HEK 293 cells.

The data in this chapter demonstrates that RTSM cells were viable in culture and could initiate an intracellular Ca^{2+} signal on agonist stimulation following isolation. However, muscarinic receptor-mediated intracellular Ca^{2+} signalling could not be established either at the time of isolation or after culture and no crosstalk was detected. Attempts to demonstrate crosstalk between β_2-adrenoceptors and other $G\alpha_{q/11}$-coupled receptors in both the RTSM and HBSM cells were also unsuccessful although RT-PCR on mRNA extracted from these two types of cells demonstrated the presence of β_2-adrenoceptor mRNA, suggesting that the β_2-adrenoceptor is expressed by these cells.

Although RTSM cells responded to methacholine, treatment with the nicotinic receptor antagonist hexamethonium chloride abolished methacholine-mediated intracellular Ca^{2+} signalling suggesting that the Ca^{2+} response was a result of nicotinic receptor activation (Beker *et al.,* 2003, Oomori *et al.,* 1998). Nicotinic receptors are ligand gated ion channels, permeable to Na^+, which can lead to membrane depolarisation and activation of VGCC. Nicotinic receptors also show Ca^{2+} permeability, particularly when composed of specific subunits, and activation could, therefore, directly elevate $[Ca^{2+}]_i$. It is also possible that

Ca^{2+} signalling through release from intracellular stores could occur due to CICR (Dajas-Bailador and Wonnacott, 2004, Sharma and Vijayaraghavan, 2001).

As previously shown in this study, crosstalk between muscarinic receptors and β_2-adrenoceptors, of the type demonstrated in HEK 293 cells, can only occur in the presence of activated muscarinic receptors. Therefore, any down-regulation of muscarinic receptors could reduce or abolish crosstalk with β_2-adrenoceptors. Indeed, down-regulation of muscarinic M_3 receptors was confirmed by RT-PCR showing that the mRNA for the receptor was no longer expressed in RTSM cells once isolated and cultured. This loss of muscarinic receptor-mediated signalling is a problem in isolated smooth muscle cells (Boselli *et al.,* 2002, Hedin *et al.,* 1990) and manipulations have been performed in an attempt to regain muscarinic receptor expression using either complete serum-withdrawal or a low concentration of serum. For example, culturing canine tracheal cells in medium containing 1% FBS, rather than the normal 10%, caused the expression of muscarinic receptors to be greatly increased (Yang *et al.*, 1993). Furthermore, studies in canine tracheal smooth muscle showed that a prolonged (7 day) complete serum withdrawal resulted in the re-appearance of muscarinic M_3 expression (Halayko *et al.,* 1999). After prolonged serum-withdrawal, the up-regulated muscarinic M_3 receptors had the ability once again to signal through Ca^{2+} (Mitchell *et al.,* 2000). As experiments here demonstrated a lack of muscarinic M_3 receptor expression in the RTSM cells, serum-withdrawal was used in an attempt to up-regulate muscarinic M_3 receptor expression. However, the culture of RTSM cells in serum-free medium was unable to upregulate muscarinic M_3 receptors. This lack of effect of serum-withdrawal on muscarinic receptor expression is not uncommon. For example, swine tracheal smooth muscle cells had a low expression of muscarinic

receptors after isolation but this was unaffected by culture in media containing low (1%) serum (Hsieh *et al.,* 2002). The RT-PCR performed on RTSM cells before serum-withdrawal showed no mRNA for the muscarinic M_3 receptors suggesting cell surface expression of the receptor would be highly unlikely. This was supported by the ICC experiments that failed to demonstrate cell surface expression.

An added complication to the study of muscarinic and β_2-adrenoceptor crosstalk in RTSM cells, is that on addition of noradrenaline, even in the absence of muscarinic agonists, an intracellular Ca^{2+} response was elicited. Both α- and β-adrenoceptors are expressed on airway smooth muscle with α_1-adrenoceptors being $G\alpha_{q/11}$-coupled and therefore having the potential to elicit a Ca^{2+} response to noradrenaline (Koto *et al.,* 1996, Noveral *et al.,* 1994, Roux *et al.,* 1998). When the cells were treated with phentolamine, a non-selective α-adrenoceptor antagonist, the intracellular Ca^{2+} signals were abolished. Addition of noradrenaline to cells, in the presence of a muscarinic agonist and pre-incubated with phentolamine, was still unable to elicit an intracellular Ca^{2+} response.

As the RTSM cells did not express muscarinic receptors, attempts were made to identify other $G\alpha_{q/11}$-coupled receptors to explore the possibility of crosstalk with β_2-adrenoceptors. Bradykinin is an agonist for the $G\alpha_{q/11}$-coupled bradykinin B1 and 2 receptors, both of which are expressed on human airway smooth cells and upregulated during chronic inflammation, an effect that can be seen in airway diseases such as asthma (Newton *et al.,* 2002). Stimulation with bradykinin causes bronchoconstriction in asthmatic patients but reportedly has no effect in normal individuals (Barnes, 1992). The RTSM cells were also stimulated with noradrenaline in the presence of UTP, an agonist for the $G\alpha_{q/11}$-coupled

$P2Y_2$ receptor in an attempt to mediate crosstalk (Jacobson and Boevnaems, 2010). In isolated RTSM cells UTP increases intracellular Ca^{2+} via P2Y receptors (Michoud *et al.,* 1997). When stimulated with either UTP or bradykinin, the RTSM cells responded with an increase in $[Ca^{2+}]_i$ but noradrenaline could not elicit a Ca^{2+} response in the presence of either of these agonists. These $G\alpha_{q/11}$-coupled receptors may have been unable to sensitise the mechanism for crosstalk that has been demonstrated in HEK 293 cells and potentially occurs between muscarinic M_3 receptors and β_2-adrenoceptors. Being unable to elicit crosstalk in the RTSM cells, experiments were carried out in an immortalised HBSM cell line as they have been reported to express muscarinic M_3 receptors (Gosens *et al.,* 2006). RT-PCR was performed on the cells and confirmed the presence of mRNA for both muscarinic M_3 receptors and β_2-adrenoceptors suggesting they may indeed be present. Cell surface expression of the muscarinic receptors was confirmed by the methacholine-mediated intracellular Ca^{2+} response from the cells, even in the presence of the nicotinic acetylcholine receptor antagonist hexamethonium chloride. However, this did not confirm the receptors were M_3 as both M_1 and M_5 muscarinic receptors also couple to $G\alpha_{q/11}$ and therefore signal through elevation of intracellular Ca^{2+}. Although these cells could produce a muscarinic-mediated intracellular Ca^{2+} response, addition of noradrenaline in the presence of muscarinic receptor activation did not evoke a Ca^{2+} response. These cells expressed the mRNA for β_2-adrenoceptors but it is possible that receptors are not present at the cell surface and therefore unable to mediate responses to noradrenaline.

Different mechanisms of crosstalk between receptors have been demonstrated in cell lines and primary tissues allowing them to elicit either a synergistic or additional intracellular Ca^{2+} response (see Werry *et al.,* 2003 for review). In rabbit ciliary body epithelial cells,

crosstalk between $G\alpha_{q/11}$- and $G\alpha_s$-coupled receptors has been demonstrated with simultaneous activation of the $G\alpha_{q/11}$-coupled muscarinic receptors and $G\alpha_{i/o}$-coupled α_2-adrenoceptors causing a synergistic increase in $Ins(1,4,5)P_3$ and intracellular Ca^{2+} (Cilluffo *et al*., 2000). The mechanism involved a synergistic effect of both the βγ-dimer, released following activation of α_2-adrenoceptors and the α-subunits released following activation of muscarinic receptors. The presence of both βγ-dimers and $G\alpha_{q/11}$ enhanced PLCβ activity and thereby increased the intracellular Ca^{2+} response (Cilluffo *et al*., 2000). Crosstalk via the mechanism of enhanced PLC activity has been demonstrated in other studies (Chan *et al.,* 2000, Werry *et al.,* 2002, Yoon *et al.,* 1999). Sensitisation of $Ins(1,4,5)P_3$ receptors via PKA-mediated phosphorylation is another mechanism of crosstalk. In mouse acinar cells, PKA phosphorylation of $Ins(1,4,5)P_3$ type 2 receptors potentiated Ca^{2+} signalling elicited by carbachol activation of muscarinic receptors (Bruce *et al.,* 2002). Other mechanisms that synergistically increase intracellular Ca^{2+} also exist including second messengers accessing different Ca^{2+} stores and increasing the availability of intracellular Ca^{2+}. The Ca^{2+}-releasing agent nicotinic acid-adenine dinucleotide phosphate (NAADP) has been shown to enhance local muscarinic receptor-stimulated Ca^{2+} responses into global signals in mouse pancreatic acinar cells (Cancela *et al.,* 1999, Cancela *et al.,* 2002). The exact mechanism is unknown. However, it is likely to involve a local interaction between $Ins(1,4,5)P_3$ receptors and NAADP. NAADP releases Ca^{2+} from thapsigargin-insensitive stores due to elevations in cAMP therefore representing a potential mechanism of crosstalk between $G\alpha_s$ and $G\alpha_{q/11}$-coupled receptors. Increasing the availability of Ca^{2+} for signalling has also been described as a mechanism for crosstalk involving Ca^{2+} synergy. Crosstalk between the PLC and AC pathways has been demonstrated in guinea pig hepatocytes. In the presence of angiotensin II, isoprenaline was able to cause a synergistic Ca^{2+} release. The exact mechanism is

unclear but could involve either store shifting of intracellular Ca^{2+} or enhanced activation of SERCA. This increased SERCA would allow increased store loading allowing more Ca^{2+} for release, on agonist stimulation of the pathway (Burgess *et at.,* 1991).

From the conclusions in this chapter it is difficult to determine whether crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors, causing an increase in intracellular Ca^{2+} signalling, is physiologically relevant or a quirk that is demonstrated in the HEK 293 cell line. Crosstalk between these two receptors has previously been shown but all of these studies have been in cells lines (Budd *et al.,* 1999, Kurian *et al.,* 2009, Liu and Simon, 1996). These experiments in RTSM cells were only carried out in cells under 'normal' conditions from models that did not possess an asthmatic phenotype. Perhaps in sensitised tissue the crosstalk may have been expressed. For example, in rabbit TSM cells sensitised with serum from atopic asthmatic patients, β_2-adrenoceptor-mediated relaxation was significantly attenuated in the presence of stimulated muscarinic M_2 receptors (Hakonarson *et al.,* 1995). In rabbit TSM cells treated with non-atopic human serum this synergy was not detected. Although this is an example of negative crosstalk it demonstrates how signalling can be different between asthmatic and non-asthmatic tissue. Another example of crosstalk regulating the affect of β_2-adrenoceptor bronchodilation has been shown in murine airway smooth muscle (McGraw *et al.,* 2003). Here, activation of the $G\alpha_{q/11}$-coupled prostanoid-EP_1 receptor with prostaglandin E_2 (PGE_2), which is abundant in asthmatic airways, caused the β_2-adrenoceptor to uncouple from it G-protein therefore preventing signalling of the receptor and reducing bronchodilation. This crosstalk is likely to contribute to the reduction in response to β_2-adrenoceptor agonists that occur in patients with severe asthma when endogenous PGE_2 is elevated. Positive crosstalk between $G\alpha_s$- and $G\alpha_{q/11}$-coupled receptors

leading to an increase in signalling has been demonstrated in cells, such as mast cells present during the allergic reactions seen in asthma (Ryzhov *et al.,* 2006). A_{2B} adenosine receptors are able to couple to both $G\alpha_s$ and $G\alpha_{q/11}$ pathways and are activated by adenosine, which is found in elevated concentrations in bronchoalveolar lavage fluid in asthma (Driver *et al.,* 1993). A_{2B} adenosine receptors stimulate IL-4 production via the $G\alpha_{q/11}$ pathway but this activation is potentiated when the $G\alpha_s$ pathway is also activated. The mechanism involves phosphorylation of NFAT by PKA increasing the protein's activation and therefore leading to a synergistic increase in IL-4 production.

With muscarinic M_3 receptors and β_2-adrenoceptors being co-expressed in airway smooth muscle, studying and understanding the crosstalk between these two receptors may be important for the effective treatment of asthma and COPD. A recent study demonstrated how crosstalk between these two receptors could depend on the genotype of the receptor and cause the efficacy of COPD treatment to differ in patients (Umeda *et al.,* 2008). The study showed that patients with the homologous Arg16 β_2-adrenoceptor genotype exhibited greater sensitivity to the muscarinic M_3 antagonist than those expressing heterologous β_2-adrenoceptors. Although several studies have examined the mechanism of crosstalk between these two receptors, the reason for the different β_2-adrenoceptor genotype affecting the muscarinic response is unknown (Budd *et al.,* 1999, Kurian *et al.,* 2009, Liu and Simon, 1996, McGraw *et al.,* 2003). Further study in airway smooth muscle is needed to fully understand the signalling pathways and allow effective treatment of airway diseases.

Chapter 7: General discussion

This thesis investigated crosstalk between GPCRs which couple to $G\alpha_{q/11}$ and $G\alpha_s$-proteins leading to a synergistic increase in intracellular Ca^{2+}. In particular this study has focussed on crosstalk between the $G\alpha_{q/11}$-coupled muscarinic M_3 receptor and the $G\alpha_s$-coupled β_2-adrenoceptor. These receptors are of interest as they are often co-expressed, for example, on smooth muscle tissue where they control muscle tone (Billington and Penn, 2002) (see section 1.6.1 and 6.1). Muscarinic M_3 receptors cause contraction of smooth muscle when activated by their endogenous ligand acetylcholine. Acetylcholine is released from parasympathetic nerves and regulates many fundamental actions in the periphery and central nervous system (Proctor, 2006). These receptors are therefore potential targets in asthma and COPD treatment, where antagonists may relieve and bronchoconstrictor drive. Asthma is a chronic inflammatory disease of the airways that leads to increased contraction of the smooth muscle and narrowing of the airway, preventing normal breathing (Bousquet *et al.,* 2000). In COPD, like in asthma, the airways become narrowed however, this bronchoconstriction is difficult to reverse and the disease often becomes progressively worse with time.

Currently the main focus of asthma treatment is the use of bronchodilators, which cause relaxation of the airway smooth muscle via the β_2-adrenoceptors. Additionally, steroids are used to treat the inflammation associated with more severe asthma (Hawkins *et al.,* 2003). Acute asthma attacks are treated with short-acting inhaled β_2-adrenoceptor agonists such as salbutamol whereas long-acting β_2-adrenoceptor agonists salmeterol and formoterol can be

prescribed as a preventative treatment (Bousquet *et al.,* 2000, Lipworth, 2007). Increased intracellular Ca^{2+} signalling could potentially cause increased bronchoconstriction, one of the symptoms of asthma. The crosstalk studied in this thesis leads to an increase in intracellular Ca^{2+} and therefore could be an important factor in treating diseases such as asthma and COPD where muscarinic M_3 receptors and β_2-adrenoceptors are targets for treatment.

Crosstalk leading to a synergistic intracellular Ca^{2+} response was most prevalent at sub-maximal concentrations of the full muscarinic agonist and also when a partial muscarinic agonist was used to prime the cells. Partial muscarinic agonists were used to explore their ability to participate in crosstalk as they will elicit only a small degree of the pharmacological response of a full agonist, even if all the receptors are occupied (Lape *et al.,* 2008). The partial agonist of the muscarinic receptor also allowed exploration of intracellular Ca^{2+} oscillations which, under the conditions tested, were most robust and consistent when cells were stimulated with an EC_{50} concentration of the partial agonist. Partial agonists can be useful in therapeutic treatments and are considered to cause less of the key adverse effects that can be associated with the use of full agonists including dependency, tolerance and withdrawal (Zhu, 2005). A number of partial agonists of the β_2-adrenoceptor are widely used as bronchodilators. The rate of phosphorylation of the β_2-adrenoceptor is greatly reduced following activation of the receptors with the partial agonists salbutamol and salmeterol when compared to adrenaline. Therefore this reduces the desensitization and potential down-regulation of the receptor, allowing greater bronchodilation (January, *et al.,* 1998). Although the partial muscarinic agonist oxotremorine was unable to elicit a full intracellular Ca^{2+} response, partial activation of

downstream effectors of Ca^{2+} still occurred and oxotremorine was more effective at eliciting crosstalk then methacholine.

The patterns of intracellular Ca^{2+} signalling associated with crosstalk were initially examined to determine how crosstalk affected the magnitude and frequency of signalling. Addition of the β_2-adrenoceptor agonist, noradrenaline, alone to the cells did not elicit a Ca^{2+} response, as would be expected for a $G\alpha_s$-coupled receptor coupling to adenylate cyclase to increase cAMP. However, in the presence of sub-maximal concentrations of methacholine and maximal and sub-maximal concentrations of oxotremorine, the β_2-adrenoceptor elicited an intracellular Ca^{2+} response. Other studies have demonstrated the ability of $G\alpha_s$-coupled receptors to elicit intracellular Ca^{2+} responses in the presence of an activated $G\alpha_{q/11}$-coupled receptor but not between muscarinic M_3 receptors and β_2-adrenoceptors (Cervantes *et al.,* 2010, Ostrom *et al.,* 2003, Tovey *et al.,* 2003, Werry *et al.,* 2002). Noradrenaline was only able to elicit an intracellular Ca^{2+} response in the presence of a muscarinic agonist and this Ca^{2+} response was independent of extracellular Ca^{2+} but dependent on a thapsigargin-sensitive store. This increase in Ca^{2+} signalling synergistically increased ERK activation, potentially enhancing gene transcription. Nuclear translocation of ERK causes the activation of cellular processes including proliferation, differentiation and growth (Cervantes *et al.,* 2010). This is of particular interest as airway smooth muscle proliferation is an important step in remodelling of the airway wall in asthma and has been suggested as a potential target for the treatment of the disease (Stewart *et al.,* 2004). ERK activation via second messengers, including intracellular Ca^{2+}, regulates targets in the cytosol but also translocates to the nucleus. Once in the nucleus, ERK phosphorylates transcription factors to regulate gene transcription. AP-1 proteins, mostly those that belong

to the Jun group, have been shown to regulate the expression and function of cell cycle regulators such as cyclin D1 (Ravenhall *et al.,* 2000, Shaulian and Karin, 2001). C-Jun is able to positively regulate cell proliferation by repressing tumour suppressor gene expression and function, and inducing cyclin D1 transcription (Shaulian and Karin, 2001). In fibroblasts, sustained activation of ERK for several hours causes the cell to enter S phase whereas transient activity does not (Murphy *et al.,* 2002). The c-Fos is a molecular sensor of ERK and contains an ERK DEF domain (the ERK docking site). Inhibition of this domain prevents c-Fos-mediated activity (Murphy *et al.,* 2002). Activation of muscarinic M_3 receptors induces immediate early gene expression encoding nuclear proteins in T- and B-lymphocytes and astrocytoma cells (Fujii and Kawashima, 2000a, Fujii and Kawashima, 2000b, Trejo *et al.,* 1991). Members of this early gene family include c-Fos and c-Jun, both proto-oncogenes that form a dimeric complex (AP-1), which acts as a transcription factor (Trejo *et al.,* 1991). Along with ERK DEF domains, c-Fos contains regulatory elements for phorbol esters and Ca^{2+} allowing both PKC and Ca^{2+} to regulate induction of the gene (Trejo *et al.,* 1991). A change in intracellular Ca^{2+} signalling could therefore alter regulation of c-Fos. In human B- and T-lymphocyte cell lines muscarinic M_3 receptors elicit Ca^{2+} oscillations which have been shown to upregulate c-Fos gene expression (Fuji and Kawashima, 2000a, Fuji and Kawashima, 2000b). An increase in ERK translocation leading to increased cell proliferation could, therefore, be an important factor in the remodelling of asthmatic airways. In HEK 293 cells, crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors leads to synergistic increases in both $[Ca^{2+}]_i$ and pERK. Although, for reasons discussed within this thesis, this crosstalk was not demonstrated in RTSM cells, there is still potential that such a mechanism of enhanced signalling could

occur within a patho-physiological setting leading to increased proliferation of the airway in asthmatic patients.

PKA and Epac were investigated in this thesis to determine if either of these effectors were involved in the mechanism of crosstalk, as previous work in the laboratory had established a role for cAMP (Kurian *et al.,* 2009). Several studies of crosstalk between $G\alpha_s$- and $G\alpha_{q/11}$-coupled receptors have demonstrated a role for both proteins (Brown *et al.,* 2004, Bruce *et al.,* 2002, den Dekker *et al.,* 2002, Kang *et al.,* 2001, Kang *et al.,* 2003, Tanimura *et al.,* 1999). For crosstalk between $G\alpha_i$- and $G\alpha_{q/11}$-coupled receptors there is much evidence demonstrating an increase in PLC activity and some clear evidence that an important mechanism for this may be $G\alpha_{q/11}$ and $\beta\gamma$ synergy on PLC activation. Studies have shown crosstalk between $G\alpha_i$- and $G\alpha_{q/11}$-coupled receptors in which this was specifically mediated by PLCβ3. The synergy involves a two-state allosteric mechanism (between active and inactive states) with the synergistic state enhancing the active state of PLCβ3, when both $G\alpha_{q/11}$ and $\beta\gamma$ are bound. The synergism requires a low level of basal activity in the absence of activation and only becomes significant when the activity level rises about 0.1% of the maximal response. This synergy is unique to PLCβ3 and does not occur with PLCβ1, 2 or PLCβ4 (Philip *et al.,* 2010). This mechanism is most likely highlighted in RAW264.7 cells, where synergy between the $G\alpha_i$-coupled C5a receptor and $G\alpha_{q/11}$-coupled $P2Y_6$ receptor is mediated by enhanced PLCβ3 activity and does not involve either PLCβ2 or PLCβ4 (Roach *et al.,* 2008). Previous work in the laboratory has demonstrated no increase in [^{3}H]-InsPx accumulation suggesting enhanced PLC activity is not the mechanism involved in crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors. Whilst the lack of [^{3}H]-InsPx accumulation alone does not absolutely rule out this as the

mechanism, other studies of crosstalk have observed increases in [^{3}H]-InsPx accumulation, indicating the sensitivity of the technique to demonstrate enhanced PLC activity underlying crosstalk (Selbie *et al.,* 1995, Werry *et al.,* 2002, Werry *et al.,* 2003). Therefore other proteins/effectors must be involved. Downstream effector molecules of the β_2-adrenoceptor are PKA and Epac, so potentially either of these could have a role. However, over-expressing Epac1, which is endogenously expressed in HEK 293 cells (Section 4.4), or inhibiting PKA did not affect the intracellular Ca^{2+} signals elicited by crosstalk suggesting that these proteins are not involved (Section 4.4). A possible mechanism for this crosstalk is the direct sensitisation of Ins(1,4,5)P_3 receptors by cAMP. This has previously been shown in HEK 293 cells (Tovey *et al.,* 2008). In that study, cAMP was demonstrated to sensitise Ins(1,4,5)P_3 type 2 receptors via intracellular 'cAMP junctions' where cAMP passes directly from AC isoform 6 to Ins(1,4,5)P_3 type 2 receptors, increasing sensitivity of the receptor to Ins(1,4,5)P_3. The study suggested that cAMP binds to a low affinity site on the Ins(1,4,5)P_3 receptor. The affinity of cAMP for this binding site is ~600 times lower than Epac and ~20,000 times lower than PKA but its location is unknown (Tovey *et al.,* 2008). The crosstalk in this thesis has been shown to be independent of Epac1 and PKA. However, previous work in the laboratory has demonstrated the requirement for AC activation and an increase in cAMP, for the crosstalk to occur, therefore this mechanism could fit (Kurian, 2006). Another possibility is that G$\beta\gamma$-dimers released from the β_2-adrenoceptor sensitise the Ins(1,4,5)P_3 receptors. However, this sensitisation alone is not enough to release intracellular Ca^{2+} as noradrenaline alone is unable to mediate a Ca^{2+} response, although in the presence of an activated G$\alpha_{q/11}$-coupled receptor, Ins(1,4,5)P_3 levels may be increased sufficiently to activate the sensitised receptors and thereby increase Ca^{2+} release.

Addition of noradrenaline to single cells demonstrated a change in the pattern of intracellular Ca^{2+} oscillations in the presence of muscarinic agonists. After stimulation with noradrenaline in the presence of a muscarinic agonist, an increase in the number of cells signalling and the frequency and magnitude of signal was observed. The relevance of this change in oscillation Ca^{2+} pattern is unknown. However, Ca^{2+} oscillations regulate gene transcription (Dolmetsch *et al.,* 1998, Hu *et al.,* 1999, Tomida *et al.,* 2003) (Sections 3.4 and 5.4). In the presence of a submaximal concentration of oxotremorine, noradrenaline significantly increased the activity of a luciferase reporter construct containing NF-kB, a transcription factor involved in the expression of many pro-inflammatory genes. This potentially demonstrates a regulatory role, via crosstalk, for controlling the transcription of inflammatory cytokines, chemokines and other mediators that contain a kB site within their promoter region and which may be important in diseases such as asthma and COPD. Oscillatory Ca^{2+} signalling has been shown, both in this thesis and in previous studies, to increase the activity of transcription factors by indirectly phosphorylating (in the case of NF-kB) or dephosphorylating (in the case of NFAT) proteins, leading to translocation of the transcription factor into the nucleus. The transcription factors NF-AT, NF-kB and Oct/OAP can all be differentially affected by different frequencies of Ca^{2+} oscillations (Dolmetsch *et al.,* 1997, Dolmetsch *et al.,* 1998, Hu *et al.,* 1999, Li *et al*, 1998, Tomida *et al.,* 2003). For example, in T-lymphocytes, rapid oscillations activate NF-AT, NF-kB and Oct/OAP, whereas less frequent oscillations only activate NF-kB (Dolmetsch *et al.,* 1998). Asthma sufferers are often prescribed glucocorticosteriods (GCs) which have an inhibitory effect on inflammation by targeting transcription factors including NF-kB (Barnes and Karin, 1997, Barnes, 2006). GCs diffuse into cells and activate their receptors (GRs)

causing an antagonistic effect on NF-kB (Almawi and Melemedjin, 2002, Barnes, 2006). Activated receptors translocate into the nucleus of the cells and can modulate transcription effects by directly associating with various sequences in the DNA including the GC response element. GC activation of GRs increases the synthesis of IkB in some cells by binding to positive glucocorticoid response elements (GREs), resulting in increased gene transcription of IkB, increasing the inhibition of NF-kB activation (Barnes, 2006). Some patients do not respond to GC treatment and this could be caused by impaired binding at the GRE site, with tolerance being more common in COPD sufferers (Adcock and Lane, 2003). IkB kinase-2, which phosphorylates IkB allowing NF-kB activation, has been investigated as a selective therapeutic treatment for airway diseases. This would inhibit NF-kB-mediated gene expression preventing processes such as proliferation, which can induce thickening of the airway (Catley *et al.,* 2006). Treating asthma with a combination of GCs and β_2-adrenoceptor agonists is more effective than using either one alone (Durham 1999).

Polymorphisms of the β_2-adrenoceptor can be a potential issue when treating asthma with agonists of this receptor (Liggett, 1997, Zhang *et al.,* 2007). Single nucleotide polymorphisms of the β_2-adrenoceptor most commonly occur at positions 46 and 79 with the substitution of glycine for arginine at codon 16 and glutamic acid for glutamine at codon 27 (Leineweber and Brodde, 2004, Tattersfield and Hall, 2004). The β_2-adrenoceptor is not a gene that is suspected to be involved in the susceptibility of asthma, however, the variants in the alleles may have an effect on the phenotype that develops, effectiveness of medication, airway hyperresponsiveness and the severity of the asthma (Holloway *et al.,* 2000, Taylor *et al.,* 2000, Taylor and Kennedy 2001). When treating asthma, studies have shown different outcomes after regular use of β_2-agonists (Dennis *et al.,* 2000, Drazen *et*

al., 1996, Sears *et al.,* 1990, Taylor *et al.,* 1998). Polymorphisms at codon 16 and 27 cause down-regulation of the receptor in response to isoprenaline, therefore reducing the number of receptors that contribute to bronchodilation and limiting recovery from asthma (Tattersfield and Hall 2004, Turki *et al.,* 1995). It is unclear whether crosstalk between β_2-adrenoceptors and muscarinic M_3 receptors occurs or only occurs in some patients or under certain conditions. β_2-adrenoceptor function can also be impaired in asthma sufferers by the inhibiting effect of muscarinic M_2 receptors, preventing relaxation of the airway. This demonstrates a second reason why treating asthma with muscarinic antagonists is effective (Emala *et al.,* 1997). Animal models of asthma have shown an increase in acetylcholine release via presynaptic muscarinic M_2 receptors leading to an increase in muscarinic M_3 receptor activation and increased hyperresponsiveness (Coulson and Fryer, 2003, Fryer and Jacoby, 1998).

Increasing or altering Ca^{2+} responses in the tissue allow control over numerous physiological processes including contraction. Muscarinic M_3 receptors control contraction of smooth muscle whereas activation of β_2-adrenoceptors leads to relaxation, hence the use of β_2-adrenoceptor agonists to treat asthma. Therefore crosstalk between these two receptors leading to an augmentation in Ca^{2+} signalling would indicate an increase in contraction, which is unexpected and counters the recognised role of the β_2-adrenoceptor. However, crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors could play a role in the longer term effects of asthma rather than the acute regulation of airway contractility. For example, Ca^{2+} signalling via crosstalk following treatment of airway disease with β_2-adrenoceptor agonists could contribute to remodelling of the airway by increased proliferation and the development of scar tissue (Lipworth, 2007). Long-acting

β_2-adrenoceptor agonists have been shown to increase severe and life-threatening exacerbations and asthma related deaths (Salpeter *et al.,* 2006). In a study of 26,000 asthma patients over a 6 month period there was a 2-fold increase in life-threatening asthma exacerbations and a 4-fold increase in death in patients treated with salmeterol compared to placebo (Salpeter *et al.,* 2006). Exacerbations result from asthma attacks in which the bronchial tubes suddenly become constricted. This increased contraction could be the consequence of increased Ca^{2+} signalling on activation of β_2-adrenoceptors, which could occur in the presence of activated muscarinic M_3 receptors, by acetylcholine (Coulson and Fryer, 2003).

In order to study the crosstalk in a more physiologically relevant tissue, rat tracheal smooth muscle cells were isolated. Unfortunately the HEK 293 crosstalk experiments were not repeatable in the smooth muscle cells because the muscarinic M_3 expression was lost and could not be regained.

7.1 Future directions

There are several future directions for this project. The exact mechanism involved in the crosstalk has not been discovered. This study has demonstrated that Epac1 and PKA are not involved and an alternative mechanism has been suggested but not definitively proven. Epac1 was over-expressed in cells and this had no effect on intracellular Ca^{2+} signalling. However, to ensure Epac is not involved in the mechanism, siRNA for Epac1 and 2 could be used to knockdown expression. Both Epac1 and 2 are expressed in HEK 293 cells although Epac1 has higher expression at least in some clones (Keiper *et al.,* 2004).

Crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors was shown to significantly increase the activity of a luciferase reporter construct containing NF-kB. NF-kB is a positive regulator of enzymes such as cyclooxygenase-2 (cox-2) a key enzyme in the production of prostaglandins, which are central mediators in inflammatory diseases such as asthma (Chun and Surh, 2002, D'Acquisto *et al.,* 1997, Kojima *et al.,* 2000, Kosaka *et al.,* 1994, Lai and Rogers, 2010, Tsatsanis *et al.,* 2006). Cox-2 contains a TATA box and two putative NF-kB binding sites. The increase in NF-kB activation could lead to an increase in cox-2 expression (D'Acquisto *et al.,* 1997). It would therefore be interesting to examine the impact of crosstalk on cox-2 expression by immunoblotting or PCR.

Finally experiments carried out on rat tracheal smooth muscle cells isolated after 5 days were not successful in maintaining muscarinic expression or eliciting a muscarinic receptor-mediated response. Further attempts could be made to maintain/restore muscarinic M_3 expression. Alternatively, airway smooth muscle cells could be transfected to express muscarinic receptors at physiological levels (around 48fmol/mg protein in trachea, the endogenous level in the HEK 293 cells used in this thesis was 40fmol/mg protein) to investigate the possibility of crosstalk in primary cells (Emala *et al.,* 1995). These transfections can be difficult with poor transfection efficacy rates. However, being able to examine single-cell signalling allows for the study of transfected cells, even at low transfection efficiency, providing the transfected plasmid encodes a protein containing a fluorescent tag. Additionally, previous studies have successfully transfected primary airway smooth muscle cells (Dalby *et al.,* 2004, Gresch *et al.,* 2004, Kang *et al.,* 2009, Maurisse *et al.,* 2010, Meng *et al.,* 2004).

7.2 Summary and conclusions

This study has demonstrated that full and partial muscarinic agonists facilitate crosstalk i.e. enable β_2-adrenoceptors to signal through Ca^{2+}. This crosstalk is more prevalent at lower concentrations of full muscarinic agonists and with a partial agonist of the muscarinic receptor. Importantly this crosstalk has been shown not only to increase intracellular Ca^{2+} signalling but to alter the oscillatory pattern of Ca^{2+} signalling and was dependent on a thapsigargin-sensitive intracellular store.

The mechanism of the crosstalk is still unclear. However, evidence suggests that PKA and Epac are not involved and that the mechanism could possibly require cAMP sensitisation of $Ins(1,4,5)P_3$ receptors via a low affinity site, as recently described (Tovey *et al.,* 2008). The enhanced Ca^{2+} signalling mediated by this crosstalk had clear effects on downstream processes including ERK and NF-kB. Therefore there is a possibility of crosstalk affecting cellular functions including proliferation and increasing the production of inflammatory mediators in diseases such as asthma and COPD. The crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors could not be established in primary cultures of RTSM cells due primarily to the loss of the muscarinic M_3 expression.

Although it is yet to be established, the possibility exists that such crosstalk between muscarinic M_3 receptors and β_2-adrenoceptors exists in pathophysiological circumstances in cells co-expressing these receptors. This could potentially complicate understanding of

disease processes and highlight the importance of therapies targeting more than one receptor type.

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