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Interaction of differentially-coupled GPCRs resulting in enhanced intracellular Ca^{2+} signalling

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The superfamily of G-protein-coupled receptors (GPCRs) represents a major group of cellular receptors that convert extracellular signals from hormones, neurotransmitters and pharmacological ligands into cellular responses. These receptors instigate intracellular signalling by activating heterotrimeric GTP-binding proteins (G-proteins), which subsequently regulate effectors including ion channels and enzymes involved in second messenger production. Examples of the activation of one type of GPCR influencing the activity of a different type of GPCR are becoming increasingly frequent. Such interactions have far-reaching implications, potentially having significance in diverse patho-physiological circumstances.

Many chemokines signal *via* GPCRs, including interleukin-8 and GROα acting through the $G\alpha_i$-coupled CXC chemokine receptor 2 (CXCR2). This receptor stimulates pertussis-toxin sensitive Ca^{2+} responses (i.e. *via* $G\alpha_i$) in many native cell types, but not when expressed recombinantly in an immortalized cell line. This study shows that stimulation of endogenous $G\alpha_q$-coupled P2Y nucleotide receptors in CXCR2-transfected human embryonic kidney (HEK) cells allows a subsequent robust Ca^{2+} response to stimulation of CXCR2. This interaction required continued nucleotide receptor activation and was dependent on the activation of $G\alpha_i$ G-proteins. Extracellular Ca^{2+} was not required, indicating release of additional Ca^{2+} from internal stores following CXCR2 stimulation. However, the extent of potentiation induced by P2Y2 receptors was markedly different to that induced by P2Y1 receptors, a difference attributable to the rapid and full desensitization of P2Y1 receptors. Co-stimulation of CXCR2 and P2Y2 receptors also potentiated phosphoinositide generation, suggesting an involvement of phospholipase C (PLC) and $InsP_3$ in the crosstalk. A similar crosstalk phenomenon occurs between $G\alpha_q$-coupled M3 muscarinic- and $G\alpha_s$-coupled β2 adrenoceptors. However, the muscarinic M3 receptor-β2-adrenoceptor interaction is not observed at the phosphoinositide level, indicating two divergent mechanisms, although it was shown that protein kinase A activation was not required. The involvement of the PLCβ/$InsP_3$ signalling pathway was supported by the inhibitory effects of the putative $InsP_3$ receptor blocker, 2-aminoethoxy-diphenylborane. That additional Ca^{2+} stores could be accessed simply by increasing $InsP_3$ production was confirmed by the demonstration of quantal Ca^{2+} release. The possibility that potentiation was due to co-operativity between $G\alpha_q$ and Gβγ acting on PLCβ was supported by the demonstration that inhibition of $G\alpha_q$ by regulator of G-protein signalling 2 (RGS2), or the sequestration of Gβγ by $G\alpha_t$, markedly attenuated the response to CXCR2 stimulation.

In addition, the physiological relevance of these studies was examined in a variety of primary cells expressing both P2Y receptors and CXCR2 endogenously. Similar crosstalk was not observed in any of these cell types, and the potential reasons for this apparent discrepancy between the recombinant system and the native system is discussed. However, this work represents a significant advance in the understanding of a potential mechanism of interaction by which GPCRs can modulate the intracellular Ca^{2+} signalling of another receptor.

Contents

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Publications

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Full Papers

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Werry, T.D., Wilkinson, G.F., Willars, G.B. Title to be decided. Communication of data showing non-involvement of PKA in muscarinic M3 receptor-β2 adrenoceptor crosstalk. **(In preparation)**

Werry T.D, Wilkinson, G.F., Willars, G.B. GPCR crosstalk: potential mechanisms of enhanced intracellular Ca^{2+} signalling. **(Review article, in preparation)**

Chapter 1 – Main introduction: Overview of GPCR signalling, P2Y nucleotide receptors, chemokine receptors and GPCR crosstalk

Section 1.1: G-protein-coupled receptors

The ability to sense the extracellular environment and react to it is fundamental to the growth, development and function of all cells and, as such, there is a critical role to be played by the factors that mediate the conversion of extracellular signals into intracellular responses. This role is played by transmembrane receptors, whose extracellular domains interact with hormones, neurotransmitters and growth factors causing a change in the cytosolic domain of the receptor, which transduces the signal into an intracellular response. However, the staggering array of extracellular stimuli demands that cells are flexible in their range of responses to these factors, and can respond accordingly to different specific circumstances. This flexibility is reflected in the evolution of a number of super-families of receptors that modulate the activity of a highly diverse group of 'effector' species (mainly enzymes and ion channels). One of the largest of these families is the family of G-protein-coupled receptors (GPCRs; also known as seven transmembrane domain (7TMD) receptors)). GPCRs are the target molecules for a large number of endogenous ligands such as hormones and neurotransmitters, and are currently the most commonly targeted molecules for therapeutic intervention with pharmacological ligands. A knowledge of GPCR functioning, not only of their linear transduction cascades but also their increasingly recognised ability to interact with one another at various points if these cascades, is therefore of vital importance. This project aims to investigate a crosstalk between two such GPCRs, the CXC chemokine

receptor 2 (CXCR2), and the nucleotide receptor, P2Y2, characterising this interaction in terms of its effect on intracellular signalling by the second messenger calcium ion (Ca^{2+}), and exploring the molecular mechanisms that are involved in it.

Section 1.1.1: Structure and function

GPCRs are excellently reviewed by Pierce *et al*, (2002). They are a group of proteins in the order of 40-120kDa in size that are expressed at the plasma membrane to communicate extracellular signals to various intracellular machineries. The tertiary structure of these proteins is of seven hydrophobic α-helices joined by short hydrophilic loops (hence 'seven trans-membrane domain' receptors). These receptors also contain an extracellular N-terminal domain of variable length that often has some role in determining the ligand binding properties of the receptor, and a cytoplasmic C-terminal tail that has important roles in G-protein coupling and receptor trafficking (the modulation of receptor activity and presence at the cell surface). It is *via* this coupling to heterotrimeric G-proteins (guanosine triphosphate (GTP)-binding proteins; see below) that a wide array of hormones, neurotransmitters and therapeutic ligands link to intracellular effector mechanisms including adenylyl cyclase and phospholipase C (PLC), and to a range of divergent and convergent signalling pathways utilising various ion channels, kinases, phosphatases and transcription factors.

Heterotrimeric G-proteins consist of three distinct subunits - α, β and γ. There are currently 21 identified genes for Gα subunits, 5 for Gβ and 11 for Gγ, opening up the possibility of over 1000 potential variations on the basic trimeric structure. For a long time, specificity of signalling to a transduction pathway was considered to be solely a function of the Gα subunit, of which there are four main families - $G\alpha_{q/11}$, $G\alpha_i$, $G\alpha_s$ and $G\alpha_{12/13}$[§]. $G\alpha_i$ and $G\alpha_s$

[§] The general scheme used in this thesis to refer to G-proteins is as follows: "Gq" will denote a heterotrimeric G-protein containing α,β and γ subunits, the "q" deriving from the identity of the α subunit. "$G\alpha_q$" will be used when referring specifically to actions or properties of a G-protein α subunit in isolation from the Gβγ subunit.

subunits inhibit and stimulate, respectively, the activity of adenylyl cyclase to regulate the production of the cyclic nucleotide, cyclic adenosine monophosphate (cAMP) from ATP. $G\alpha_q$ promotes the breakdown of the membrane phospholipid, phosphatidylinositol-(4,5)-bisphosphate ($PtdIns(4,5)P_2$) by PLC enzymes into inositol trisphosphate ($InsP_3$) and diacylglycerol (DAG) and thus stimulates increases in both intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and protein kinase C (PKC) activity. In addition to these three major varieties, other types of $G\alpha$ subunits exist which bear some functional homology to the main $G\alpha$ groups. $G\alpha_{14}$, α_{15} and α_{16} subunits bear significant similarity to $G\alpha_{q/11}$ (indeed these subunits are classified within the $G\alpha_q$ family), mediating their actions mainly through PLC stimulation and Ca^{2+} release/PKC activation. The $G\alpha_i$ family contains all $G\alpha_i$ members and also $G\alpha_o$, $G\alpha_z$ and $G\alpha_t$ subunits, all of which inhibit adenylyl cyclase and have some activity at plasma membrane ion channels (mainly Ca^{2+} and K^+ channels). An additional family contains $G\alpha_{12}$ and α_{13}, which are thought to regulate guanine nucleotide exchange factors (GEFs), resulting in activation of the small GTPase protein, Rho, and in effects on the cytoskeleton. Na^+/K^+ exchangers have also been shown to be activated by $G\alpha_{12}$ and $G\alpha_{13}$ (Voyno-Yasenetskaya *et al*, 1994). In addition, alternative effectors exist for most G-protein subunits, including Src tyrosine kinases, phosphodiesterase enzymes and ion channels (summarised in Pierce *et al*, 2002. G-protein classes and effector specificity are also reviewed by Gilman, 1987; Clapham & Neer, 1993; and Neer, 1994 and 1995, and references can be found herein).

The mode of G-protein activation is, with some exceptions (e.g. the protease-activated receptor), identical for all GPCRs (See Fig. 1.1. Basic G-protein organisation and activation are also excellently reviewed by Neer, 1995). Agonist binding to the external surface of the receptor causes a conformational change in the secondary/tertiary structure of the receptor, of which little is known other than the disruption of an ionic interaction between two of the transmembrane helices that occurs in β2 adrenoceptors (Ballesteros *et al*, 2001).

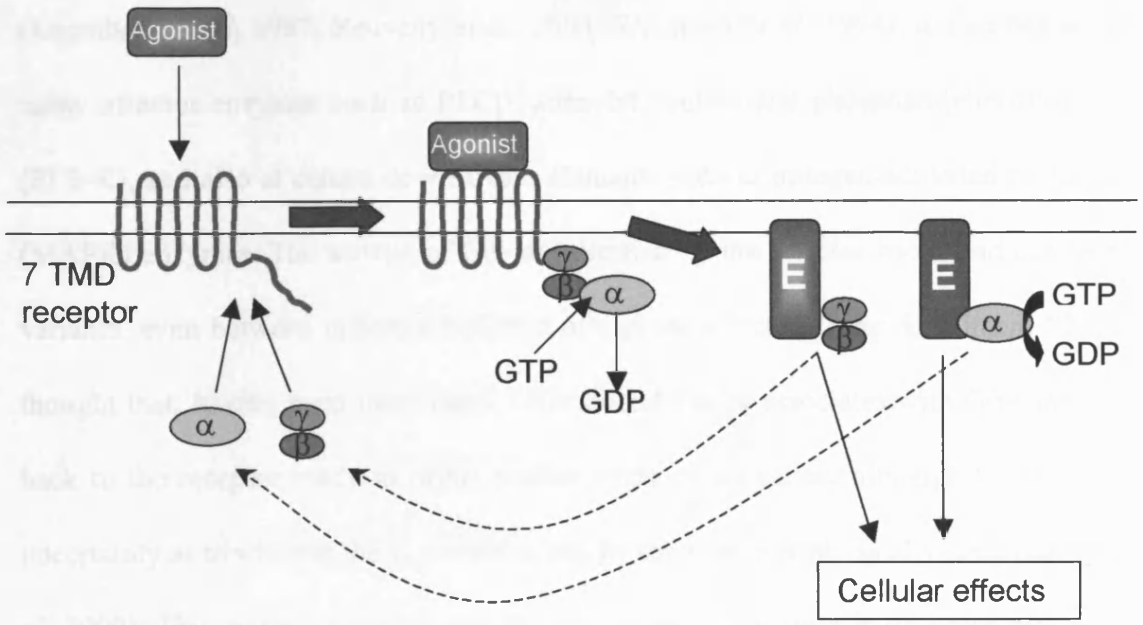


Figure 1.1: Simplified diagram of the G-protein-linked transduction cycle. Agonist stimulation of a 7-TMD GPCR causes GTP-GDP exchange on the Gα subunit of a heterotrimeric G-protein. The subsequent dissociation of Gα and Gβγ from the receptor allows modulation of the effector enzymes (E). GTP hydrolysis causes the G-protein to revert back to its heterotrimeric state, in a competent form to re-associate with the receptor.

Furthermore, agonist binding causes the transient formation of a high affinity complex between agonist, receptor and G-protein. The agonist:receptor complex acts as a GEF for the heterotrimeric G-protein, decreasing its affinity for GDP, thus facilitating GDP (inactive state):GTP (active state) exchange. Once bound to GTP, the α subunit dissociates from both the receptor and Gβγ, and moves within the membrane to its specific effector. Here it interacts, causing either an up- or down-regulation of enzyme activity or the modulation of ion flow through plasma membrane ion channels. This interaction with effector can occur for several seconds before the α subunit hydrolyses the terminal phosphate of GTP (by means of intrinsic GTPase activity: see below), forming GDP and thereby inactivating the G protein. In addition, contrary to initial hypotheses, Gβγ is not simply a localisation factor (Logothetis *et al*, 1987; Clapham & Neer, 1993). Initially discovered to activate potassium channels

(Logothetis *et al*, 1987; Reuveny *et al*, 1994; Wickman *et al*, 1994), it also has activity at many effector enzymes such as PLCβ, adenylyl cyclase and phosphatidylinositol 3-kinase (PI 3-K), and also at certain downstream elements such as mitogen-activated protein kinase (MAPK) enzymes. The actions of Gβγ are dictated by the effector itself, and can be highly variable, even between different isoforms of a given effector (Tang & Gilman, 1991). It is thought that, having been inactivated, GDP-bound Gα re-associates with Gβγ and recycles back to the receptor ready to begin another cycle of activation, although there is growing uncertainty as to whether the G-protein α and βγ subunits ever physically dissociate (Klein *et al*, 2000). This system is perpetuated for as long as the receptor is occupied by agonist, or until the receptor becomes desensitised to the agonist (see below). This mechanism serves to amplify the agonist signal many times as a single active receptor can activate several G-protein molecules that can in turn cause prolonged (i.e. seconds) regulation of their respective effectors. This prolonged activity leads to significant production (or inhibition of production) of second messenger.

However, the signal must also shut off at some point to retain the fine control that GPCRs exert over cellular events. There are a number of points in the signal transduction pathway that this can be achieved, including deactivation of the receptor, G-protein or effector. The rate of deactivation of G-protein signalling is highly variable, and has clear significance in determining the duration and magnitude of the signal generated by it. Variation in the rate of GTP hydrolysis is usually the central factor. *In vitro* analysis has shown that GTP hydrolysis occurs with a half-life of several seconds for most G-proteins (Brandt & Ross, 1985; Breitwieser & Szabo, 1988). In some cases, this correlates well with the physiological rate of signal termination *in vivo* (Cassel *et al*, 1979). However, *in vivo* kinetics are often rather different to those *in vitro*, with visual G-protein signal termination a particularly striking example. These signals have a half-life of a matter of milliseconds *in vivo* (Vuong & Chabre,

1991), suggesting that cellular constituents that are lost in G-protein purification are crucial in determining the rate of signal deactivation. Among these constituents are the members of a group of GTPase activating proteins (GAPs). The regulator of G-protein signalling (RGS) proteins are perhaps the most well-known GAPs (Berman and Gilman, 1998; Ross and Wilkie, 2000), but some effector molecules (e.g. PLC) also exhibit GAP activity (Berstein *et al*, 1992; Paulssen *et al*, 1996; Chidiac and Ross, 1999). Prolongation of GPCR/G-protein/effector signalling duration represents a simple method of potentiating signal magnitude or intensity, and the potential relevance of this to the positive interactions seen in this study are discussed further in Chapter 4.

Certain disease states arise from disturbances in the balance of the G-protein system. Pertussis toxin (PTX) (whooping cough) causes ADP-ribosylation of $G\alpha_i$ which disrupts its linkage to adenylyl cyclase and thus uncouples adenylyl cyclase from inhibitory control. The symptomatology of whooping cough can be entirely attributed to over-production of cAMP. Cholera toxin (CTX) also causes ADP-ribosylation, this time of $G\alpha_s$, resulting in the abolition of its GTPase activity and thus the persistent activation of the α subunit and over-activity of adenylyl cyclase.

Adenylyl cyclase activity causes the production of cyclic AMP (cAMP). This messenger activates cAMP-dependent protein kinase (PKA), a serine-threonine kinase that mediates many downstream effects of cAMP. These effects vary depending on cell type but can be a direct consequence of PKA-mediated phosphorylation (e.g. receptor desensitisation, enzyme activation etc) or by a more indirect method *via* the action of PKA-phosphorylated proteins such as the cAMP response element binding (CREB) protein that binds to the CRE site on certain genes, initiating transcription at the command of cAMP signals. Both main factors in this signalling pathway are under control from neutralising enzymes: cAMP by cAMP-

specific phosphodiesterases, and PKA by phosphatases acting in a negative feedback loop following activation by PKA.

$G\alpha_q$ couples positively to members of the phospholipase Cβ (PLCβ) family, and by doing so stimulates the hydrolysis of the membrane phospholipids known as the inositol phospholipids (namely PtdIns(4,5)P_2) into $InsP_3$ and DAG. The PLCβ family is one of four sub-families of PLC enzymes - β, γ, δ and ε. The PLCβ sub-family has four members (PLCβ1-4), and these each have isotypic differences. The general conserved structure of the PLCβ isozymes is of two catalytic domains, X and Y, while the remainder of the molecule is fairly non-homologous between isoforms. However, all PLCβ isozymes have a pleckstrin homology (PH) domain (that serves as a docking interface mediating membrane association by binding to PtdIns(4,5)P_2), an EF hand motif, and an elongated C-terminal tail that contains a C2 domain, within which is the $G\alpha_q$-binding motif (see Fig. 1.2). It is *via* this motif that $G\alpha_q$ stimulates PLCβ activity, but there are also sites within the enzyme that bind and respond to Gβγ subunits, again causing PLCβ activation. However, the responsiveness to both of these activators varies between isozymes. PLCβ1 is more responsive to $G\alpha_q$ stimulation than PLCβ2, while the opposite is the case for Gβγ stimulation. PLCβ3 has intermediate sensitivity to both $G\alpha_q$ and Gβγ. The rank order of PLCβ stimulation by $G\alpha_q$ is PLCβ1≥PLCβ3>PLCβ2, and for stimulation by Gβγ is PLCβ2>PLCβ3>PLCβ1. PLCβ4 is not stimulated by Gβγ (Lee *et al*, 1994). Measurement of PLCβ4 activation by either $G\alpha_q$ or Gβγ is difficult given the inhibition of basal activity in PLCβ4 by ribonucleotides, including GTPγS, which is used extensively in studies of PLCβ activity (Lee *et al*, 1994). There also exist certain phosphorylation sites on PLCβ enzymes that may be targeted during effector desensitisation by, for example, PKC (see below). The regulation of phospholipase C enzymes is excellently reviewed by Rhee (2001), and will also be discussed

Figure 1.2: Common structural features of PLCβ (after Singer *et al*, 1997). Amalgamation of various pieces of evidence showing some characteristics of PLCβ enzymes. This diagram does NOT represent any single PLC isoform. The features shown are likely to be conserved among PLCβ enzymes, but the positions will be subject to slight variations. The green box indicates a PKC phosphorylation site (Ryu *et al*, 1990). Orange boxes show the Gβγ binding domains of PLCβ2 (Wu *et al*, 1993a; Sankaran *et al*, 1998; Kuang *et al*, 1996b) and PLCβ3 (Barr *et al*, 2000)). The X and Y domains form the PLCβ catalytic domain. The Y segment also contains a Gβγ binding site(s) that may contribute directly to enzyme activation. The grey box (PH) is the pleckstrin homology domain. The $G\alpha_q$ binding site within a C2 domain in the C-terminus is depicted by the yellow box (Wu *et al*, 1992).

later (Chapter 4) when considering the potential of $G\alpha_q$ and Gβγ to act co-operatively at PLCβ.

One of the products of PLCβ-mediated hydrolysis of PtdIns(4,5)P_2, DAG, causes direct activation of some members of the PKC family of serine-threonine kinases, and release of arachidonic acid (by further cleavage events). PKC isoforms (of which there are currently more than ten known) are responsible for the phosphorylation and control of a plethora of signalling molecules, including GPCRs, effector enzymes and ion channels/transporters (reviewed by Liu and Heckman, 1998). Its mode of activation varies between isotypes, but usually involves DAG. It also requires phosphatidylserine (PS) fatty acids in the plasma membrane, which act as membrane localisation co-factors (Bell & Burns, 1991; Orr & Newton, 1992). The interaction of PKC with phosphatidylserine can be dependent on Ca^{2+}, which can act as a bridge between PKC and PS, although novel isoforms of PKC do not require this type of interaction. The classical mode of activation of PKC involves

translocation to the plasma membrane mediated by association with DAG and (in Ca^{2+}-dependent isoforms) with Ca^{2+}, which increases the affinity of PKC for PS. Once at the membrane, PKC associates with PS, and penetrates the membrane. The presence of Ca^{2+} facilitates this process by increasing the affinity of PKC for negatively-charged lipids. This chain of events serves to activate PKC, and the catalytic site becomes accessible for substrate binding. PKC mediates the phosphorylation of a multitude of proteins on serine and threonine residues to up- or down-regulate their activity. The structure, function and regulation of PKC are covered in detail by Newton (1995).

$InsP_3$ acts on $InsP_3$ receptors in the membrane of the endoplasmic reticulum. These receptors contain an intrinsic Ca^{2+} channel, and $InsP_3$ activation causes rapid and considerable efflux of stored Ca^{2+} into the cytosol. This initiates various other events including the opening of plasma membrane Ca^{2+} channels and the activation of PKC, resulting in capacitative Ca^{2+} entry and protein phosphorylation, respectively. Ca^{2+} can also act as an agonist in its own right, either by activating ryanodine receptors (RyR) present on the endoplasmic reticulum (ER) membrane and liberating further Ca^{2+} stores by so-called Ca^{2+}-induced calcium release (CICR), or by facilitating further Ca^{2+} release *via* $InsP_3$ receptors (although at higher concentrations is inhibitory to $InsP_3$ receptor channel opening). However, Ca^{2+}, while performing certain functions directly, performs many of its actions following the formation of a complex with calmodulin, the ubiquitous 'Ca^{2+}-receptor' protein. The predominant group of Ca^{2+}-calmodulin (CaM)-dependent effectors are the CaM kinases, which include myosin light chain kinase (MLCK), particularly important in muscle contraction, and the multi-functional CaM kinase II.

It is an area of intense interest exactly how Ca^{2+} can mediate such a diverse array of cellular functions given its simplicity. Ca^{2+} is responsible for processes such as neurotransmission,

muscle contraction, cell proliferation, secretion and gene expression. Such plasticity is achieved by modulating the magnitude, frequency and spatio-temporal characteristics of $[Ca^{2+}]_i$ variations, and each cell type is equipped with a unique 'toolkit' that decodes the changes in $[Ca^{2+}]_i$, allowing the cell to respond appropriately (reviewed by Bootman *et al*, 2001). This toolkit includes Ca^{2+} sensing equipment such as calmodulin, which has the sensitivity to respond oppositely at different concentrations and spatio-temporal patterns of $[Ca^{2+}]_i$ elevation (DeMaria *et al*, 2001). The Ca^{2+} signal itself is also a source of variation, with many grades of response ranging from small, highly localised events such as 'puffs' and 'sparks' (Bootman, 1996) to globalised Ca^{2+} waves. These small sub-cellular Ca^{2+} events, which indicate Ca^{2+} current either through plasma membrane Ca^{2+} channels or through intracellular release sites such as the $InsP_3$ receptor, create microdomains of high $[Ca^{2+}]$, and it is this spatial resolution that allows differentiation of responses within a single cell. For instance, the higher sensitivity of certain adenylyl cyclase isoforms to Ca^{2+} entry through store-operated Ca^{2+} channels (SOCCs) rather than from intracellular release suggests that adenylyl cyclase and SOCCs are in close apposition (Fagan *et al*, 2000). Similarly, mitochondria compensate for the low affinity of their Ca^{2+} uptake systems by positioning themselves next to sites of high $[Ca^{2+}]$ e.g. the mouths of Ca^{2+} channels (Rizzutto *et al*, 1998; Csordas *et al*, 1999). It is clear, then, that minute changes in $[Ca^{2+}]_i$ can propagate profound changes in the final outcome of the signal, and that any change in either the magnitude or the spatio-temporal pattern of the signal may radically alter the response observed following cell stimulation with agonist.

It is therefore evident that there is some considerable diversity in receptor-mediated responses to GPCRs. This study investigates a phenomenon whereby the activation of one such second messenger system, namely Ca^{2+} release from intracellular stores, can be influenced by the presence or absence of other simultaneous inputs into the cell.

Predominant in this work are the $G\alpha_i$-coupled chemokine receptor, CXCR2, and $G\alpha_q$-coupled P2Y nucleotide receptors, although some attention is also focused on a further example of interaction between $G\alpha_q$-coupled muscarinic M3 receptors and $G\alpha_s$-coupled β2 adrenoceptors. The crux of this study concerns the ability of certain receptor combinations to cause potentiation of the magnitude and potency of Ca^{2+} release in response to receptor stimulation, and aims to begin to elucidate the mechanisms underlying such interactions in the hope that it may provide some further understanding of the integration of pathways of intracellular Ca^{2+} signalling.

Section 1.1.2: Modulation of GPCR activity

In keeping with most other cellular functions, GPCR activation and signalling must be under strict control. To afford some protection against excessive receptor activity, there exists a system of GPCR regulation that tightly controls the cell surface presence and sensitivity of these receptors. This system involves the diminution of receptor responsiveness to agonist over time ('desensitisation') and the withdrawal of receptors from the cell surface ('internalisation'). In addition, modulation of gene expression leads to down-regulation of *de novo* receptor synthesis and therefore to a reduction in the population of these receptors expressed at the cell surface. These events are balanced against the re-activation and re-presentation of receptors at the cell surface by a number of intracellular processes ('resensitisation'). (These events have been reviewed previously by Ferguson (2001), and by Bünemann & Hosey (1999), and extensive references are available therein).

Desensitisation is generally a phosphorylation-dependent process (although examples of phosphorylation-independent desensitisation have been demonstrated (Dhami *et al*, 2002)), and this modification is performed mainly by either second messenger-dependent protein kinases (PKA, PKC) or by the family of GPCR kinases (GRKs; reviewed by Premont *et al*,

1995; and Stoffel *et al*, 1997). Both mediate the phosphorylation of GPCR molecules leading to the uncoupling of a receptor from its G-protein and subsequently to an inability to activate G-protein-dependent signalling events. A fundamental difference between these two groups of kinases is the ability to mediate a process termed heterologous desensitisation (i.e. the desensitisation of GPCRs other than the one which initially stimulated the kinase). GRKs are only able to phosphorylate activated receptors, and have a degree of specificity for the Gβγ subunits with which they combine (Daaka *et al*, 1997a), and thus only contribute to homologous desensitisation. In contrast, PKA and PKC are more indiscriminate in their phosphorylation targets, including non-activated receptors and certain downstream targets of GPCRs such as phospholipase C and adenylyl cyclase, and requiring only a suitable consensus sequence and a serine or threonine residue to catalyse phosphorylation. In the cases where phosphorylation is inhibitory to normal function, this effectively constitutes heterologous desensitisation. Kinase activation occurs in response to a variety of factors: PKA and PKC are activated by GPCR second messengers (cAMP and Ca^{2+}/diacylglycerol, respectively), while GRKs respond positively to such diverse factors as polycations, PKC, PtdIns(4,5)P_2 and Gβγ subunits (see Pitcher *et al*, 1998 for extensive review and references). In addition to PKA, PKC and GRK-mediated phosphorylation, other kinases such as tyrosine kinases (Pak *et al*, 1999) and casein kinase 1α (Tobin *et al*, 1997; Budd *et al*, 2000) are also able to modulate GPCR signalling activity through phosphorylation.

A further group of molecules central to GPCR desensitisation is the family of arrestins, a group of 'arresting agents' that contribute both to GPCR uncoupling from G-protein, and to endocytosis of these phosphorylated GPCRs. Initially cloned in 1985, visual arrestin typifies this class of proteins (Pfister al, 1985), and three other variants (cone arrestin, and two β-arrestins) bearing significant homology to this molecule have since been discovered. The general paradigm governing β-arrestin action is that these molecules, following GPCR

activation, bind preferentially (but not exclusively) to agonist-occupied, GRK-phosphorylated GPCRs, and in doing so physically uncouple the GPCR from its G-protein and target the GPCR for internalisation by endocytosis. It is unclear whether GPCRs recruit β-arrestin actively by signal generation or whether the process is a simple passive diffusion of arrestin molecules resulting in chance association with activated GPCRs. Endocytosis is achieved by targeting the receptor to a special membrane region enriched in clathrin, a process that is regulated by membrane phosphoinositide lipids, particularly inositol hexakisphosphate ($InsP_6$) (Gaidarov & Keen, 1999; Gaidarov *et al*, 1999), although the mechanism by which this regulation occurs is unknown. It is from these 'clathrin-coated pits' that the receptor is sequestered into a membrane invagination ('endosome'). Contrary to initial hypotheses that internalisation played a role in receptor desensitisation by withdrawing receptors from the cell surface, popular opinion now states that internalisation plays a critical role in receptor *re*-sensitisation. This hypothesis was strongly supported by the demonstration that interruption of internalisation (either by pharmacological or molecular methods) severely affected receptor resensitisation, without affecting either receptor-G-protein coupling or desensitisation (Pippig *et al*, 1995; Garland *et al*, 1996; Hasbi *et al*, 2000). Early endosomes are sorted into either degradative lysosomes, or into late endosomes in which GPCRs are resensitised by the activity of phosphatases (Sibley *et al*, 1986; Pitcher *et al*, 1995) and recycled back to the cell surface in an active state. This sorting process determines how rapidly a receptor recovers from desensitisation, as does the rate of receptor manufacture by *de novo* protein synthesis. This balance between receptor desensitisation, internalisation and recycling/replacement is of critical importance in determining the kinetics/duration of receptor activity and the effectiveness of agonists at these receptors over time.

Effector enzymes themselves are also targets for desensitising inputs. Phospholipase C is known to be phosphorylated (and inactivated) by PKC (Nishizuka, 1995; Ali *et al*, 1997; Strassheim *et al*, 1998), as is adenylyl cyclase (Teitelbaum & Berl, 1994). The ability of the second messenger-dependent protein kinases to induce heterologous desensitisation is due in large part to their ability to desensitise effector enzymes and, by doing so, interrupt the signalling of every receptor that uses that effector. Desensitisation of PLCβ can be achieved in a number of ways. There is some evidence that responsiveness of PLCβ to $G\alpha_q$-mediated stimulation may be lost due to extreme C-terminal modification of the PLCβ enzyme by calpain (Lee *et al*, 1993; Banno *et al*, 1994), which cleaves the $G\alpha_q$-binding site contained within the C-terminal, rendering the enzyme insensitive to this activator. However, the modification need not be so extreme. GPCR-activated PKC is known to feedback on PLCβ, limiting its ability to hydrolyse PtdIns(4,5)P_2 and thus constituting a protective mechanism against over-activity. This type of feedback control may be one explanation for the observation that certain $G\alpha_q$-coupled receptors are unable to stimulate phosphoinositide turnover in cells pre-stimulated at an alternative $G\alpha_q$-coupled GPCR (Megson *et al*, 1995) (although there are alternative explanations such as Ca^{2+} store emptying or PLCβ substrate depletion).

It is clear that the interplay between GPCR signalling pathways mentioned above contributes to the enormous complexity of receptor desensitisation (e.g. heterologous desensitisation), but interactions of other sorts can also cause positive effects on receptor sensitivity and signalling. The work detailed here involves a study of two such interactions between two pairs of receptors, in each of which one receptor couples to intracellular calcium elevation, the other to either inhibition or stimulation of adenylyl cyclase. Co-stimulation of cells with agonists at these receptors results in the enhancement of calcium signalling. The subsequent

section looks at previously published observations of similar interactions between GPCRs, and begins to explore the potential mechanisms underlying these demonstrations of crosstalk.

Section 1.1.3: Crosstalk interactions[§]

Heterologous interactions between GPCRs can result in both loss of function (desensitization) and gain or enhancement of function (here referred to as "crosstalk") and it is this latter aspect that this study is concerned with. Crosstalk between GPCRs has been demonstrated by the measurement of a number of different functional outputs but many studies have focused on those associated with phosphoinositide and Ca^{2+} signalling. Although not exhaustive, Table 1.1 highlights some examples of such crosstalk. While based predominantly on enhanced Ca^{2+} signalling, several of these studies have reported enhanced signalling upstream of Ca^{2+} but from which it could be reasonably predicted that Ca^{2+} release would also be enhanced. Despite growing evidence that such crosstalk may be widespread in model cell systems and primary cultures there is little direct evidence of the mechanisms involved. This study is intended to characterise two such phenomena and extend the investigation to attempt to unravel the mechanism(s) that permit such interactions.

Most research into Ca^{2+} crosstalk has been performed in recombinant expression systems, but there is also accumulating evidence for parallels in primary cells. Potentiated Ca^{2+} signalling has been shown in a wide variety of cells derived from both the central nervous system and systemic tissues, suggesting that the phenomena observed in immortalised cells, often with over-expressed recombinant receptors, reflects a more physiological phenomenon.

[§] 'Crosstalk' has been used occasionally elsewhere to describe negative interactions between combinations of receptors, but is more generally used, as within this study, to imply convergence that leads to a positive outcome (that is, enhanced receptor function). Further, the term 'crosstalk' has not been qualified as either 'potentiating crosstalk' or 'gain-of-function crosstalk' as this implies knowledge of the underlying mechanism. For example, in interactions involving $G\alpha_q$- and $G\alpha_i$-coupled GPCRs it is unclear as to which receptor is the donor and which receptor is the target of the crosstalk. Thus, the interaction could be 'potentiating' (i.e. the $G\alpha_i$-coupled GPCR enhancing the existing $G\alpha_q$-mediated signal) or a 'gain-of-function' (i.e. the $G\alpha_q$-coupled GPCR promoting the coupling of the $G\alpha_i$-coupled GPCR to a previously inaccessible pathway).

Table 1.1: **Interactions between GPCRs leading to modulation of Ca^{2+} and/or phosphoinositide signalling**

Gi-coupled receptor	Other receptors	Gq-coupled receptor	Consequence	Refs
Adenosine A1		α1 adrenoceptor	↑Ca^{2+} mobilisation	Delumeau *et al*, 1991b
		Bradykinin	↑IP_3 production/↑Ca^{2+} mobilisation	Gerwins *et al* 1992b
			↑PKC activation	
		CCK	↑IPx accumulation	Gerwins *et al*,1995
				Dickenson and Hill, 1996, 1998b
		H1	Various	
		mGluR	↑Ca^{2+} mobilisation	Dickenson *et al*, 1993, Dickenson and Hill, 1994
		Muscarinics	↑PLC activity	Ogata *et al* (1994), Toms *et al* (1999)
			↑Ca^{2+} mobilisation	Biden *et al* (1993)
		P2Y2	↑IPx accumulation etc	Farahbakhsh *et al* (1997)
		P2Y (unspecified)	↑Ca^{2+} mobilisation	Dickenson *et al*, 1993, 1998b; Megson *et al* 1995
		Thyrotropin receptor	↑PLC activity	Jimenez *et al*, 1999, Gerwins *et al*, 1992a
				Tomura *et al*, 1997
Opioid receptors		Bombesin R	↑IPx accumulation	Chan *et al*, 2000
		Bradykinin receptor(s)	↑IP_3 production/↑Ca^{2+} mobilisation	Okajima *et al*, 1992, 1993, Tomura *et al*, 1992
		Muscarinics	↑IPx accumulation/↑Ca^{2+} mobilisation	Connor *et al*, 1996, Chan *et al*, 2000, Yeo *et al*, 2001
			Enhanced mAChR-generated EPSPs	Kearns *et al*, 2001
		P2Y2	↑IP_3 production	Chan *et al*, 2000, Okajima *et al*, 1993
Somatostatin receptors		α1 adrenoceptors	↑Ca^{2+} influx	Delumeau *et al*, 1992
		Bradykinin receptor(s)	↑Ca^{2+} mobilisation	Okajima *et al*, 1992
		PTX-insensitive muscarinics	↑Ca^{2+} mobilisation	Connor *et al*, 1997b, Xia *et al*,1997b
α2 adrenoceptor	5-HT receptor (unspecified)		↑Ca^{2+} mobilisation	Shah *et al*, 1999
		α1 adrenoceptors	↑IPx accumulation	Wilson *et al*,1991
		Bradykinin receptor(s)	↑Ca^{2+} mobilisation	Okajima *et al*,1992
		PTX-insensitive muscarinics	↑Ca^{2+} mobilisation	Farahbakhsh *et al*,1997, 1994
NPY-Y1/2		Muscarinic receptors	↑Ca^{2+} mobilisation	Connor *et al*, 1997b
	PTX-sensitive P2Y	Oxytocin receptor	↑Ca^{2+} mobilisation	Nakano *et al*, 2001

Table 1.1 **(cont): Interactions between GPCRs leading to modulation of Ca^{2+} and/or phosphoinositide signalling**				
Gi-coupled receptor	Other receptors	Gq-coupled receptor	Consequence	Refs
	β2 adrenoceptor	Histamine P2Y2 ATII receptor α1 adrenoceptor	↓Ca^{2+} mobilisation to Hist ↑Ca^{2+} mobilisation ↑Ca^{2+} mobilisation ↑Ca^{2+} mobilisation	Dickenson *et al,* 1993 Werry *et al,* 2000, 2002), Jimenez *et al,* 1999 Burgess *et al,* 1991 Delumeau *et al* 1991a
	PTH	Muscarinic M3 P2Y1	↑Ca^{2+} mobilisation ↑Ca^{2+} mobilisation	Short and Taylor,2000 Buckley *et al,*2001
$5HT_{1B}$		CCK P2Y2	↑IPx accumulation ↑Ca^{2+} mobilisation	Dickenson and Hill,1996 Dickenson and Hill,1998a
Muscarinic M2/M4		Bradykinin	↑Ca^{2+} mobilisation	Okajima *et al,*1992
CXCR2		P2Y2	↑Ca^{2+} mobilisation	Werry *et al,* 2000, 2002
LTB_4		P2Y2	↑Neutrophil degranulation	Kannan *et al,*2001
CCR4		P2Y (unspecified)	↑Ca^{2+} mobilisation	Rosethorne *et al,*2002

Examples of GPCR crosstalk in the CNS include the enhancement of muscarinic receptor-induced excitatory post-synaptic potentials (EPSPs) by μ-opioid receptor agonists in hippocampal neurons (Kearns *et al*, 2001) and the augmentation of GABA release in rat periaqueductal gray neurons co-stimulated with 5-HT_{1A} serotonergic and μ-opioid receptor agonists (Kishimoto *et al*, 2001). $GABA_B$ receptors have been shown to enhance both metabotropic glutamate receptor (mGluR)-mediated Ca^{2+} signalling and, consequently, excitatory transmission in cerebellar synapses (Hirono *et al*, 2001). In the periphery, the range of effects of receptor interactions is somewhat broader. Activation of ($G\alpha_s$-coupled) parathyroid hormone receptors causes potentiated $[Ca^{2+}]_i$ responses to activation of $G\alpha_q$-coupled P2Y1 nucleotide receptors in rat osteoblasts (Buckley *et al*, 2001). In addition, interleukin-1β (IL-1β) causes potentiation of phosphoinositide hydrolysis and Ca^{2+} signalling downstream of the bradykinin B2 receptor in canine tracheal smooth muscle cells (Yang *et al*, 2001), and serotonin participates in synergistic interactions with various agonists in the control of vascular smooth muscle cell proliferation (Watanabe *et al*, 2001a and b). Furthermore, 5-HT- and noradrenaline-mediated vasoconstriction is potentiated by co-stimulation with neuropeptide Y (Andriansitohaina *et al*, 1988).

Although the G-protein coupling specificities have not been detailed individually for the above examples, it is sufficient to state that, as a group, these examples encompass most of the main possible combinations of differentially-coupled GPCRs – e.g. Gi-Gq, Gq-Gs, Gi-Gi etc. It is perhaps surprising that positive crosstalk should occur in a cellular paradigm, since many recognised interactions between GPCRs involve receptor cross-desensitisation downstream of kinase activation, or in some instances desensitisation at a later point in the transduction pathway such as effector inactivation or Ca^{2+} store depletion. It is important, then, to consider why such interactions do take place physiologically. It is likely that the complexity of Ca^{2+} signal generation and translation (i.e. the conversion of Ca^{2+} signals into a strikingly diverse range of cellular responses) (Bootman *et al*, 2001) is favourable to a

situation where small variation in the magnitude and spatio-temporal pattern of a Ca^{2+} response may have disproportionately large effects on the final outcome of the signal. Therefore, an interaction that improves the coupling of a receptor to a Ca^{2+} signal, maximises that signal, or converts it into one with a rather different pattern of release in terms of spatio-temporal characteristics or oscillation frequency, may also be expected to determine the final outcome of receptor stimulation in these circumstances.

Therefore, while the physiological role of Ca^{2+} crosstalk is not well understood, it could be of great significance amongst the many other regulatory features occurring in cellular signalling pathways. In addition to the spatio-temporal considerations of a Ca^{2+} crosstalk interaction, there are also further potential reasons for a cell to mediate these types of phenomena. The introduction of Ca^{2+} signalling capability into a GPCR following pre-stimulation is somewhat different to the modulation of an existing Ca^{2+} signal (see above), and may have rather different roles. For example, the requirement for joint stimuli could act as a coincidence detector, or may serve as a safeguard against inappropriate signalling given the potentially damaging effects of excessive $[Ca^{2+}]_i$ elevation. The repertoire of conditions under which Ca^{2+} crosstalk occurs has not been fully explored, but the diversity of situations (different cell types and receptor combinations) suggests a fundamental role for such interactions.

The significance of crosstalk is therefore of clear interest. However, it is evident from the literature that there is unlikely to be a single mechanism underlying Ca^{2+} crosstalk. This study explores several models describing potentiation of Ca^{2+} signalling and assesses their relevance to the examples of crosstalk alluded to here. These paradigms will be discussed in depth in later sections of this thesis. One of the receptor combinations investigated here is the CXCR2 chemokine receptor-P2Y nucleotide receptor axis. The following sections describe some distinguishing features of each of these receptors and their family members.

Section 1.2: Nucleotide receptors

Section 1.2.1: Classification and pharmacology

Work on purinergic transmission systems has been well established for many years, but for a significant period went without recognition by a scientific community sceptical of the ability of an essentially intracellular molecule to transmit signals extracellularly. It was not until 1972, and the work of Burnstock in moulding previous independent work into a convincing argument for purinergic signalling, that the role of nucleotides in cell-cell signalling was fully appreciated (Burnstock, 1972). The widespread acceptance of this theory led to the explosion of research into the field of purinergic pharmacology and the discovery of a complex system of receptors modulating the effects of these molecules.

Current classification groups purinergic nucleotide receptors into three main families: the P2X ligand-gated ion channel family, and the $G\alpha_i$-coupled P1 and $G\alpha_q$-coupled P2Y classes. In addition, a fourth potential class, P2T, is in the process of being defined molecularly (for a full review of purinergic pharmacology, see Williams & Jarvis, 2000). The P1 class is sensitive to the unphosphorylated ATP parent molecule, adenosine, and analogues of it, while ATP (and analogues) are the active molecules at the seven P2X receptor channels identified to date. However, the pharmacology of the P2Y nucleotide receptor family members is rather more complicated, involving di- and tri-phosphate forms of both adenine and uridine. There are several members of this P2Y receptor family, which can be discriminated pharmacologically only by assessing agonist orders of potency given the scarcity of any potent, selective antagonists at any of the receptor subtypes. The order of potency for all seven of the currently recognised P2Y receptor subtypes has been established by several different laboratories in different cell lines, but the work of Nicholas *et al* (1996b) went a long way towards bringing together much past work. It also refined previous findings to eliminate such factors as sample contamination found in commercial preparations of nucleotide agonists (see also Fagura *et al* 1998, Leon *et al*, 1997). Table 1.2 summarises the

Table 1.2: Summary of the pharmacological characteristics of the P2Y nucleotide receptor family.			
Subtype	Preferred agonist	Agonist order of potency	Cloned from:
P2Y1	Adenine-specific, diphosphate-preferring	2MeSADP>2MeSATP>ADP>ATP UTP/UDP inactive	Chick (Webb *et al*, 1993) Turkey (Filtz *et al*, 1994) Human (Schachter *et al*, 1996) (Janssens *et al*, 1996)
P2Y2	Nucleotide non-specific, triphosphate preferring	UTP=ATP ADP/UDP inactive	Mouse (Lustig *et al*, 1993) Human (Parr *et al*, 1994)
P2Y4	UTP selective (50-fold over ATP)	UTP>>ATP ADP/UDP inactive	Human (Nguyen *et al*, 1995) (Communi *et al*, 1995) Rat (Webb *et al*, 1998)
P2Y6	Uridine selective, diphosphate preferring	UDP>>UTP>ADP ATP inactive	Rat (Chang *et al*, 1995) Human (Communi *et al*, 1996)
P2Y11	Adenine-specific, no phosphorylation preference	dATP>ATP>ADPβS>2MeSATP>>>ADP	Human (Communi *et al*, 1997)
P2Y12	Adenine-specific, diphosphate-preferring Putative "ADP receptor"	2MeSADP>ADP=ADPβS	Human orphan (Zhang *et al*, 2001) (Hollopeter *et al*, 2001)
P2Y13	Adenine-specific, diphosphate-preferring Putative "ADP receptor"	2MeSADP>ADPβS>ADP>ATP	Human orphan and mouse (Zhang *et al*, 2002; Communi *et al*, 2001)

Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; UTP, uridine tripohosphate; UDP, uridine diphosphate; 2MeSATP, 2-methyl-thio-adenosine triphosphate; 2MeSADP, 2-methyl-thio-adenosine diphosphate; dATP, deoxyadenosine triphosphate; ADPβS, β-thio-adenosine diphosphate.

basic pharmacological properties of the five main members of the P2Y family in terms of agonist selectivity.

P2Y11 is one of the more recently discovered subtypes, cloned by Communi *et al* in 1997. Exhibiting 33% sequence homology with P2Y1, P2Y11 has a somewhat different agonist profile: dATP>ATP>ADPβS>2MeSATP>>>ADP (i.e lacking the diphosphate-preferring characteristic of P2Y1) (Communi *et al*, 1997; Communi *et al*, 1999). The most potent agonist at this receptor, an AstraZeneca compound AR-C67085 (Communi *et al*, 1999), was initially developed as an antagonist at the platelet-expressed P2T receptor (Humphries *et al*, 1995).

The most recently discovered P2Y subtypes are P2Y12 and P2Y13. These are putative "ADP receptors", being selective for adenine diphosphates above other nucleotide agonists. Both were cloned from orphan receptor studies (Zhang *et al*, 2001; Hollopeter *et al*, 2001; Communi *et al*, 2001; Zhang *et al*, 2002). The two receptors have high homology to one another, and very similar orders of agonist potency.

Agonist specificities at the P2Y1 receptor have come under much scrutiny. Initial results using the putative P2Y1 receptor agonist, ATP, suggested that this ligand did indeed activate this receptor. However, subsequent studies using HPLC-purified ATP showed that it had no agonist activity at this receptor and was in fact a neutral antagonist (Fagura *et al*, 1998; Leon *et al*, 1997). It was concluded that commercial preparations of ATP contained significant amounts of ADP contamination (up to 2%) and were therefore giving rise to an apparent potency of ATP that lay around two orders of magnitude to the right of that for ADP. Further study of P2Y1 turned the ATP agonist/antagonist issue on its head again. Palmer *et al* (1998) found that ATP appeared to be a low efficacy agonist at P2Y1 and concluded that its activity depended on the extent of receptor reserve. By reducing the receptor reserve using a 24hr

desensitisation period with ADPβS, ATP agonist efficacy was abolished, while the effects of a stronger agonist, ADP, were decreased in potency (and slightly in magnitude) but not abolished. The conclusions drawn from this were that both ATP and ADP are both agonists at P2Y1 receptors but that ADP was more efficacious, ATP being only a weak partial agonist. Given the uncertainty surrounding the true action of ATP, the work on P2Y1 receptors described herein utilised the relatively P2Y1-selective agonist, 2-methylthio-ADP (2MeSADP) when investigating differential effects of P2Y receptor subtypes.

The study of nucleotide receptor agonists and receptors is complicated by release of endogenous nucleotides from the receptor host cell and the activities of enzymes such as ectonucleotidases and nucleoside diphosphokinases expressed extrafacially on certain cell lines, all of which will alter the composition of a supposedly pure agonist solution. Methods employed to combat this include the use of apyrase (ATP diphosphohydrolase; Curdova *et al*, 1982), which is not ideal because, in addition to hydrolysing ATP, it also degrades many other types of nucleoside polyphosphates, including those added exogenously for pharmacological study. A more selective alternative is the use of hexokinase in the presence of glucose. This enzyme catalyses the transfer of the γ-phosphate group of any triphosphate to glucose, forming nucleotide diphosphates and glucose-6-phosphate (Lazarowski *et al*, 1997). However, it should be noted that this technique also has drawbacks in that nucleotide diphosphates also have agonist activity at P2Y receptors, in some cases more so than their triphosphate counterparts, and thus may limit the usefulness of this procedure.

Although the effects of adenine nucleotides were considered the most important given the prevalence of ATP release in many physiological situations, UTP has come to be recognised as also being of significance. This concept was borne out of the discovery of uridine-specific P2Y receptors, which are much less sensitive to ATP activation. Further research redefined the properties of P2Y2, P2Y4 and P2Y6 (Nicholas *et al*, 1996a). ATP is now believed to be

equipotent with UTP at P2Y2, while acting as a full (albeit weak) agonist at P2Y4. P2Y6 is almost completely selective for uridine-nucleotides (see Table 1.2).

Antagonists at P2Y receptors that exhibit any selectivity or potency have been hard to come by, hindering rapid progress in defining the pharmacological properties of these receptors (Burnstock & Williams, 2000). In addition, some antagonists also have ectonucleotidase-inhibiting activity that only serves to potentiate the actions of ATP. Nevertheless, high throughput screening has uncovered a P2Y1-selective antagonist, MRS2216. This remains the only antagonist with any appreciable selectivity among P2Y receptors. Weak, non-selective, but still widely used antagonists of the P2Y receptor family also exist, including Reactive Blue-2, suramin and PPADS (pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid). It is this group of antagonists that exhibit ectonucleotidase-inhibiting activity.

Section 1.2.2: Effector coupling

The general stereotype of a P2Y receptor is that it couples to the release of Ca^{2+} from intracellular stores in a PTX-insensitive manner (i.e. independent of any $G\alpha_i$-mediated effects). The classical route of this pathway is that the α subunit of $G_{q/11}$ activates PLCβ, leading to the hydrolysis of PtdIns(4,5)P_2 and the production of $InsP_3$ and DAG. This causes $InsP_3$-mediated release of Ca^{2+} from intracellular stores leading to the Ca^{2+}-dependent effects mediated by P2Y receptors (see below). However, in addition to stimulating PLCβ, some P2Y receptors have alternative signalling properties, notably a P2Y2 receptor-mediated activation of the sphingosine-1-phosphate Ca^{2+} release pathway that acts to amplify the P2Y2 Ca^{2+} signal in certain cell types (Alemany *et al*, 2000). In HL-60 neutrophil precursor cells, P2Y2 also couples to a G-protein that is proposed to be $G\alpha_{16}$ (Baltensperger and Porzig, 1997). The limited tissue distribution of $G\alpha_{16}$ means that this type of coupling is unlikely to have wider significance to P2Y2 signalling in non-hematopoietic cells, but such promiscuity of coupling should be considered to be beneficial in terms of crosstalk potential.

P2Y11 is known to additionally couple to G_s type G-proteins (Communi *et al*, 1997; Communi *et al*, 1999; Qi *et al*, 2001), and P2Y2 is thought to couple to $G_{i/o}$ proteins (Erb *et al*, 2001), as are the recently discovered P2Y12 and P2Y13 putative "ADP receptors" (Zhang *et al*, 2001; Hollopeter *et al*, 2001; Zhang *et al*, 2002). P2Y2 receptors have also been reported to have effects on basal cAMP levels (Post *et al*, 1998; Hopfner *et al*, 2001; Suh *et al*, 2001), but these seem to be rather cell-type specific.

The functional consequences of this coupling are unclear. Ca^{2+} signalling obviously occurs downstream of $G\alpha_{q/11}$ G-proteins, and accounts for most of the actions of nucleotide receptors. However, other actions such as activation of the MAPK cascade (Boarder *et al*, 1995) or sphingosine-1-phosphate generation (Alemany *et al*, 2000) have not been attributed to a specific G-protein, although there is some suggestion that the MAPK effects may occur downstream of the $G\alpha_{q/11}$-mediated Ca^{2+} elevation (Gao *et al*, 1999). This, however, probably requires other non-$G\alpha_{q/11}$-activated accessory inputs such as PI 3-K and Src family tyrosine kinases (Sellers *et al*, 2001). PI 3-K activation downstream of P2Y2 is certainly mediated by $G\alpha_i$ proteins and stimulates protein kinase B (PKB/Akt) activity (Huwiler *et al*, 2002).

This apparent dual coupling of P2Y receptors to markedly different types of G-protein may be of relevance to this study in terms of crosstalk. For instance, if the balance between $G\alpha_q$- and $G\alpha_i$-coupling in P2Y2 receptors could be shifted such that these receptors tended to be selective for $G\alpha_q$, this may potentiate Ca^{2+} signalling downstream of these receptors. While GPCRs have not been shown to be able to influence the coupling specificity of another GPCR, it is an essential property of the target receptor to be able to couple to multiple G-proteins such that a shift in coupling specificity is at all feasible.

P2Y receptors appear to be able to discriminate between PLCβ isoforms. For instance, P2Y2 receptors in CHO cells activate only PLCβ3, whereas muscarinic m3 receptors in the same cells appear to activate both PLCβ1 and PLCβ3 (Strassheim & Williams, 2000). In support of this is evidence in rat neonatal cardiomyocytes showing that P2Y2 nucleotide receptors and α1-adrenergic receptors couple differently to PLCβ1 and PLCβ3 (Arthur *et al*, 2001). It is not clear why this selectivity of effector coupling should occur, but it is possible that the PLCβ expression profile of a cell may have a bearing on the type or strength of signal it can generate in response to P2Y receptor activation. Furthermore, if a P2Y receptor could be induced to couple more efficaciously to a particular isoform of PLCβ, this may serve to potentiate Ca^{2+} signalling.

Nucleotide receptors appear, therefore, to be good candidates to participate in crosstalk at the level of Ca^{2+} signalling. There is a distinct flexibility in their coupling specificities, both in relation to other P2Y receptor subtypes and to the tissue in which they are expressed. This fluidity of coupling is the property that makes these receptors a good target for modulation by an accessory GPCR, underlying the decision to study these receptors for potential crosstalk interactions.

Section 1.2.3: Response desensitisation

As described above (Section 1.1.2), functional responses to GPCRs can desensitise at several levels, commonly at the receptor, effector, and second messenger levels. Little is known about the exact mechanism(s) underlying P2Y receptor desensitisation or internalisation. It is now known that P2Y2 receptors are desensitised in a PKC-dependent manner (Otero *et al*, 2000), although agonist-induced desensitisation is to some degree dependent on kinases other than PKC (Otero *et al*, 2000). The PKC-mediated component of desensitisation involves phosphorylation of the C-terminal tail (Otero *et al*, 2000; Garrad *et al*, 1998). Likewise, P2Y1 receptor desensitisation also seems to involve a PKC isoform, proposed to

be PKCβI (Chen & Lin, 1999). In contrast, PKC appears to have no role in P2Y4 or P2Y6 receptor inactivation or internalisation (Brinson & Harden, 2001). Indeed, P2Y6 has remarkable resistance to any desensitisation mechanism (Robaye *et al*, 1997). Even less is known about the internalisation of P2Y receptors. Desensitisation and sequestration appear to be discrete events (Garrad *et al*, 1998). P2Y2 receptors have been shown to internalise fairly rapidly (Sromek & Harden, 1998). An N-terminally hemagglutinin (HA)-tagged P2Y2 receptor expressed in 1321N1 cells was shown to internalise to a significant extent within 15mins: agonist-driven loss of cell surface immunoreactivity (25% loss at 15mins, 60% loss at 1hr) paralleled increases in cytosolic HA-immunoreactivity. Inositol phosphate production was also desensitised, over a quicker time scale and to a greater extent than loss of surface expression. This is consistent with a receptor desensitisation event preceding internalisation, although could also be due to a down-regulation of effector activity. It is not known by what mechanism P2Y receptors are internalised, although the failure of β-arrestin over-expression to influence P2Y1/P2Y2 receptor internalisation appears to rule out this factor (Mundell & Benovic, 2000).

Section 1.2.4: Distribution and function

Several analytical methods have been employed to locate P2Y receptors in diverse areas of the central and peripheral nervous systems where they are thought to participate in a range of excitatory and sedative processes. Importantly, ATP (acting in astrocytes *via* P2Y1/2/4 receptors (Fam *et al*, 2000; Newman, 2001)) can act in concert with a variety of tyrosine kinase-linked growth factor receptors (e.g. EGF, FGF and PDGF). In doing so, it has been shown to stimulate astrocyte proliferation leading to reactive astrogliosis, a hypertrophic/hyperplastic disorder associated with various brain disorders including trauma, stroke, and ischemic/neurodegenerative lesions (Neary *et al*, 1996). Neotrophin (AIT-082), a synthetic purine, can enhance working memory, and restore age-induced memory deficits in mice by elevating production of neurotrophins (Glasky *et al*, 1994; Middlemiss *et al*, 1995).

GPCRs for ATP in optic nerve glia also mediate Ca^{2+} signalling at physiological concentrations of ATP, and therefore appear to participate in physiological functioning of these cells (James & Butt, 2001). P2Y12 and P2Y13 are also both found in brain tissue (Zhang *et al*, 2001; Hollopeter *et al*, 2001; Communi *et al*, 2001; Zhang *et al*, 2002), although their functions have not yet been determined.

P2Y effects in the cardiovascular system are also thought to be profuse. Knockouts of P2Y receptors have suggested a variety of functions for these receptors. P2Y1 knockouts have decreased platelet aggregation and bleeding time, and reduced risk of thromboembolism (Fabre *et al*, 1999; Léon *et al*, 1999). ADP acts as an agonist at P2Y1 receptors to cause transient platelet aggregation and shape changes, and at P2YT/P2Y12 receptors to induce longer-term aggregation and platelet degranulation (Léon *et al*, 1999). ATP can act as an antagonist to limit thrombus formation at lesions primarily by antagonising ADP actions *via* P2Y receptors but also through the release of prostaglandin I_2 and nitric oxide (NO). NO production also mediates arterial vasodilatation in the mesentery and apparently occurs by virtue of Ca^{2+}-stimulated NO synthase activity (Buvinic *et al*, 2002). The antagonism of P2Y12 receptors by ATP was used as a starting point from which to develop AR-C69931 MX, a P2Y12 antagonist with a safer side effect profile than aspirin and better anti-thrombotic properties than its predecessors (see Burnstock and Williams, 2000).

Nucleotides released in response to shear stress may also have a role in bone regenerative functions, potentiating the effects of growth factors released to act upon osteoblasts following bone damage (Dixon & Sims, 2000). However, extracellular nucleotides have also been shown to be inhibitory to bone formation by osteoblasts, and may contribute to certain pathological bone wasting disorders (Hoebertz *et al*, 2002). In the eye, P2Y2 receptors increase the secretion of salt, water and mucus in the ocular mucosa (Li *et al*, 2001). In addition, P2Y2 knockout mice have impaired epithelial chloride secretion (Cressman *et al*,

1999). Similarly, ATP and UTP have been shown to participate in lung function by causing chloride secretion and mucus secretion in airway epithelia *via* activation of P2Y2 (Stutts and Boucher, 1999). UTP has been used to good effect to stimulate mucociliary clearance (Kellerman, 2002).

This study aims to investigate mechanisms of crosstalk between P2Y receptors and chemokine receptors. Therefore, it is important to be aware of the distribution of P2Y receptors in the hematopoietic system and in the brain, the main areas of chemokine receptor expression (described fully below) and therefore the main loci where physiological crosstalks could occur given co-expression of these receptors. Much work in P2Y receptor expression has been performed on the promyelocytic cell line, HL-60. These cells can be differentiated into either neutrophils or monocytes with dimethyl sulfoxide (DMSO), retinoic acid, dibutyryl cAMP (dbcAMP) and granulocyte-colony stimulating factor (G-CSF), although both differentiated and undifferentiated cells were found to be responsive to ATP and extracellular nucleotides in a PLC/Ca^{2+}-mediated signalling pathway that leads to exocytosis (Stutchfield and Cockroft, 1990). Further analysis of differentiated HL-60 cells found that they indeed express two types of P2Y receptors: P2Y2 and P2Y11 (Jiang *et al*, 1997; Communi *et al*, 1997; Martin *et al*, 1997). It has also been shown that differentiation causes the up-regulation of P2Y receptor expression (Communi *et al*, 2000). Such studies have also been performed on native hematopoietic cells (Jin *et al*, 1998), assessing P2Y receptor expression in a variety of promyelocytic cell lines and in human leukocytes. Their findings are summarised in Table 1.3. The apparent absence of P2Y2 receptors from neutrophils is perhaps surprising, but data exist elsewhere supporting the existence of P2Y2 receptors in neutrophils (Boarder *et al*, 1995). Eosinophils have also been proposed to express P2Y receptors (Idzko *et al*, 2001), although some caution must be taken here considering that most eosinophil isolations contain some neutrophil contamination which may generate false positives.

Of the more recently discovered P2Y receptor subtypes, less is known about their distribution due to the short time elapsed since their discovery. However, P2Y11 receptors have been shown to be expressed in lymphocytes (Conigrave *et al*, 2001), HL-60 neutrophil precursors (van der Weyden, 2000a) and monocyte-derived dendritic cells (Wilkin *et al*, 2001). P2Y12 and P2Y13 receptors are both present in brain tissue (Zhang *et al*, 2001; Hollopeter *et al*, 2001; Communi *et al*, 2001; Zhang *et al*, 2002), although whether any tissues co-express them with chemokine receptors such as CXCR2 is not known. P2Y12 expression has also been shown in endothelial cells in the brain (Simon *et al*, 2002).

		P2Y1	P2Y2	P2Y4	P2Y6	P2Y11
Cell lines	U937 (mono)	-	+	-	+	-
	HL60 (neut)	+	+	-	+	+
	K562	+	-	-	-	-
Human cells	PMNs	-	+/-	+	+	ND
	Monocytes	+	+	+	+	+[1]
	Lymphocytes	+	+	+	+	+
	Endothelial	+	+	+	+	ND

Table 1.3: Expression of P2Y receptors in various promyelocytic cell lines and human leukocytes (modified from Jin *et al*, 1998). U937 (mono): U937 is a monocyte-like cell line. HL60 (neut): HL60 is a neutrophil-like ceil line. PMN: polymorphonuclear cells, i.e. neutrophils. [1]: Expressed on monocyte-derived dendritic cells (Wilkin *et al*, 2001). ND: not determined.

Section 1.2.5: Crosstalk interactions

P2Y receptors have been shown to participate in several synergistic crosstalk relationships involving GPCR and non-GPCR targets. There is extensive evidence that P2Y receptors and adenosine receptors are capable of Ca^{2+} signal amplification when co-stimulated (Okajima *et al*, 1987 and 1989a/b; Dickenson & Hill, 1998b; Megson *et al*, 1995). In the context of the potential physiological significance of crosstalk, it is interesting that ATP released at sites of hypoxic or ischemic lesions, or following trauma, is fairly rapidly degraded by a family of 11 ectonucleotidases (Zimmerman, 1999) into ADP, AMP and adenosine. It may be of significance that adenosine and ATP could co-exist in the same lesion, a situation that may encourage crosstalk interactions between their respective receptors. Growth factor signals are potentiated by P2Y receptors in rat osteoblasts (Dixon & Sims, 2000) and HEK-293 cells (Schmidt *et al*, 2000), and nucleotide receptors have been implicated in potentiation of calcium signalling downstream of simultaneously stimulated GPCRs including parathyroid hormone (PTH) receptor (Buckley *et al*, 2001), serotonin receptor 1B ($5HT_{1B}$) (Dickenson & Hill, 1998a) and CCR 4 chemokine receptors (Rosethorne and Charlton, 2002). Finally, cAMP generation downstream of the β2 adrenergic receptor (Meszaros *et al*, 2000) and neutrophil degranulation in response to leukotriene B4 (Kannan, 2001) are both synergistically increased by concomitant stimulation of P2Y receptors. Therefore, these nucleotide receptors are clearly well suited to communicating with a variety of other cellular inputs. This study is targeted at investigating a potential interaction with the chemokine receptor, CXCR2. The following section describes the structure, function, distribution and pharmacology of these receptors, before addressing the potential loci for crosstalk with P2Y receptors.

Section 1.3: Chemokines

The chemokine (chemotactic cytokine) family is a group of naturally occurring peptide agonists with a variety of physiological functions, mainly in the immune system. There have

so far been identified more than fifty chemokines in the family, and these are grouped into four main subsets, based on the possession of one of four identifying motifs within their primary structure. Most chemokines carry four cysteine residues in the primary sequence, and it is the position of the N-terminal pair of these, relative to one another, that allows distinction between chemokine sub-families. In CC chemokines, these residues are directly adjacent to one another, whereas they are separated in the CXC and CX3C families by one and three random amino acids, respectively. The other major class of chemokines is slightly atypical in that it only has two cysteines, and does not have the second residue of the crucial pair, and is thus denoted the C family. C and CX3C chemokines are relatively rare (lymphotactin α and β, and fractalkine are the only known examples of C and CX3C chemokines, respectively). The CXC and CC families are predominant having more than 15 and 25 members, respectively. These ligands mediate their effects *via* 7-TMD GPCRs. As such, the chemokine receptor family has the potential to participate in heterologous interactions with other GPCRs similar to those detailed above. Any modulation of receptor activity or signalling specificity may have profound effects on the type of response seen following stimulation of these receptors, and subsequently on the physiological effects mediated by them.

Section 1.3.1: Receptor pharmacology

There are divisions within the CXC chemokine sub-family based on the presence or absence of a so-called ELR amino acid motif. This Glu-Leu-Arg motif is present in certain chemokine peptides, N-terminal to the first cysteine, and appears to confer some specificity of action on those chemokines containing it. For instance, neutrophils only respond to ELR+ chemokines (Hebert *et al*, 1991). The main CXC chemokines are interleukin-8 (IL-8), growth-related oncogene (GRO)α/β/γ, epithelial-derived neutrophil activating peptide (ENA)-78, granulocyte colony stimulating peptide (GCP)-2 and neutrophil-activating peptide (NAP)-2 (all of which are ELR+), and platelet factor 4 (which is ELR-). Of these

agonists, IL-8, the GRO ligands, NAP-2 and ENA-78 all selectively target CXCR2 (also known as IL-8RB), although IL-8 is not absolutely specific, having also specificity for CXCR1 (as does ENA-78, although minimal) and for Duffy (or Duffy antigen receptor for chemokines/DARC), a non-signalling 7TMD chemokine-binding protein expressed in mammalian systems (Horuk, 1994). The ELR+ motif seems to be crucial for binding to CXCR2. Site-directed mutagenesis of one or more residues in the motif severely diminishes the CXCR2-activating properties of these ligands (Clark-Lewis *et al*, 1991). However, it is not an absolute determinant since short ELR+ peptides show no neutrophil chemotactic activity, suggesting that the secondary structure of the chemokine, especially that in the highly flexible N-terminus, may also play a significant role in receptor binding and activation. This is supported by the observation that reduction of the disulfide bridges, or elimination of any of the cysteine residues that typify chemokines, inactivates them (Baggiolini & Clark-Lewis, 1992).

Further evidence for multiple determinants of receptor binding and activation was provided by Clark-Lewis *et al* (1991), who showed that both C- and N-terminal truncation of the IL-8 molecule itself compromises its ability to act as a CXC receptor ligand. The probable three-dimensional structure of CXC chemokine receptors is the same as for most 7TMD receptors, forming a circular subunit arrangement bringing all the extracellular domains together into a pocket. This pocket will be of similar size to the IL-8 molecule itself (Clark-Lewis *et al*, 1991). This suggests that, in contrast to some hormone receptors (which bind large glycoprotein hormone peptides *via* their abnormally long N-terminal extension) or receptors that bind small molecules (e.g. catecholamines) directly to the intra-membrane segments, IL-8 binding is determined by a combination of several distinct sites in the extracellular pocket. It is therefore of interest that there seem to be distinct binding sites on a single receptor for different chemokine agonists. The construction of chimeric receptors using regions of CXCR1 and CXCR2 illustrated that NAP-2 and GROα agonist activity could be conferred

upon CXCR1 by the insertion of certain sequences from CXCR2 (Ahuja *et al*, 1996). The inference from these data was that the N-terminal of CXCR2 was dominant in conferring CXCR2 binding specificity on this receptor, and could therefore do likewise in CXCR2:CXCR1 chimeras. This study highlighted the fact that high binding affinity and high agonist potency are separable in these receptors, in that agonists that did not appear to bind well still elicited strong functional responses. It was proposed that the high and low affinity binding sites may be connected to different functional responses (Ahuja *et al*, 1996). The Ca^{2+} elevation demonstrated was evident even when agonists appeared to have little binding affinity, so the authors suggest that the high affinity binding site, while being unimportant for Ca^{2+} elevation, may be involved in a distinct transduction mechanism e.g. that which mediates chemotaxis.

Given the evidence that there are distinct binding sites for the different agonists, it is interesting to note that evidence has been presented showing that IL-8 and GROα acting *via* CXCR2 seem to activate different transduction mechanisms (Damaj *et al*, 1996b). The use of EGTA or Ca^{2+}-entry blockers attenuated the Ca^{2+} response to GROα but not that to IL-8, suggesting that Ca^{2+} entry from the extracellular milieu was the predominant Ca^{2+} source for the GROα-induced elevation. This was in contrast to IL-8 induced elevation, which seemed to depend on intracellular Ca^{2+} release. It should be noted that GROα does still appear to have an intracellular Ca^{2+} component, but additionally couples to Ca^{2+} entry, whereas IL-8 couples exclusively to intracellular release.

Some CXCR2 antagonists exist, but there are very few selective ones except SKB RS-145004-000, a small non-peptide antagonist at CXCR2 (Murphy *et al*, 2000), and SB-225002, also a non-peptide inhibitor, exhibiting 150-fold selectivity for CXCR2 over CXCR1 (White *et al*, 1998). Most antagonists are peptides derived from viruses such as the *M. contagiosum* virus from the poxviridae, but this is typical of viral chemokine antagonists

in that it has very little human chemokine receptor specificity. However, N-terminally truncated IL-8 and GROα have been developed that have antagonist activity (Hesselgesser *et al*, 1995) as have selective blocking antibodies (Hammond *et al*, 1995; Green *et al*, 1996).

Section 1.3.2: G-protein/effector coupling

The broad characteristics of chemokine receptor signalling are laid out in a review of chemokine receptor nomenclature by Murphy *et al* (2000). The signalling pathway involves coupling to a PTX-sensitive ($G\alpha_i/G\alpha_o$) G protein (Damaj *et al*, 1996a), presumably leading to inhibition of adenylyl cyclase, but also elicits intracellular Ca^{2+} elevation (Schorr *et al*, 1999). However, this Ca^{2+} flux appears to be cell type specific, and may be due to certain cell types expressing Gβγ-sensitive PLCβ isoforms that are readily activated by G_i-derived Gβγ subunits. It is not clear which functional responses are attributable to the cAMP or Ca^{2+} signalling streams, although some authors attribute all cellular functional effects of chemokines to the Ca^{2+} elevation (Baggiolini & Clark-Lewis, 1992). Using a mutant G-protein that is activated by 'G_i-coupled' GPCRs but that does not impinge upon adenylyl cyclase activity and is not susceptible to PTX, neutrophil chemotaxis to CXCR1 has been shown to be independent of $G\alpha_i$ (Neptune *et al*, 1999). This suggests either that Gβγ or a different Gα subunit (e.g. G_{14}, G_{15} or G_{16} (Wu *et al*, 1993b and 1995)) may mediate chemotaxis. A further level of complexity is apparent in the knowledge that CCR1 is capable of trafficking signals from different agonists, inducing agonist-specific cellular responses (Zhang *et al*, 1999b). It is not known whether CXCR2 is similarly capable of trafficking, but it has been shown to exhibit some dual-coupling capability in that it can couple to the G_q-like G proteins $G\alpha_{14/15/16}$ when co-transfected with these subunits (Wu *et al*, 1993b). It is not known whether this coupling is in addition to that to $G\alpha_i$, or whether it is some effect of over-expression, but it does suggest that CXCR2 may be able, under certain conditions, to orchestrate α-subunit-mediated Ca^{2+} elevation in addition to its normal $G\alpha_i$ signalling. However, Ca^{2+} elevation could be blocked using blocking antibodies specific to $G\alpha_{i2}$ or $G\alpha_t$

(Damaj *et al*, 1996a), and is, in general, PTX-sensitive, suggesting that a significant proportion of the observed Ca^{2+} response is due to G_i-derived $G\beta\gamma$ subunits.

CXCR2 has also been shown to couple to pathways other than inhibition of adenylyl cyclase and elevation of $[Ca^{2+}]_i$. Its PTX-insensitive coupling to activation of extracellular-signal regulated kinases (ERKs) and PI 3-K has been suggested to have some role in the pathophysiology of Alzheimer's disease (Xia & Hyman, 2002). This diversity of coupling supports the idea that these receptors are amenable to changes in their mode of signal transduction, and suggests that minor changes in their environment brought about by a nearby alternative GPCR (perhaps P2Y receptors) may be enough to induce such alterations. It is for this reason that CXCR2 was studied here for potential involvement in crosstalk between heterologous GPCRs.

Section1.3.3: Response desensitisation

CXCR2 desensitisation and internalisation appears to be a complicated issue involving multiple inputs and will not be covered in detail here to avoid deflecting from the topic in hand. Briefly, CXCR2 desensitises by a classical phosphorylation-dependent mechanism (see Section 1.1.2) that almost certainly uncouples it from G-protein activation (Mueller *et al*, 1997; Ben-Baruch *et al*, 1997), but also appears to desensitise CXCR2-directed responses at some other downstream locus, perhaps the effector (Richardson *et al*, 1998). However, CXCR2 internalisation occurs by rather less well-understood mechanisms. Some researchers find that it is dependent on C-terminal phosphorylation sites (Mueller *et al*, 1998; Richardson *et al*, 1998). Others argue that it is independent of phosphorylation (unlike its family member, CXCR1), suggesting that desensitisation and internalisation retain a large degree of mutual exclusivity (Feniger-Barish *et al*, 1999; Schraufstatter *et al*, 1998). This indecision may be due to differences in the cellular backgrounds used to study these receptors, but is almost certain to also reflect some sophistication and diversity in the mode of receptor

deactivation. Given this uncertainty, the precise role of factors such as β-arrestin, GRKs and dynamin, which are central to CXCR1 internalisation (Barlic *et al*, 1999), are unclear for CXCR2, although the failure of PKC inhibition to prevent the phosphorylation of CXCR2 suggests that GRK enzymes may well be involved. Regardless of the underlying mechanism, CXCR2 internalises rapidly, with around 95% of cell surface presentation of receptors being lost within 5mins (Richardson *et al*, 1998). This speed of desensitisation of CXCR2 is interesting in terms of crosstalk in that the receptor may be restricted by this brevity of signalling to a particular type(s) of transduction. Interactions with another GPCR may somehow lift this restraint and allow extended signalling and coupling to pathways to which CXCR2 would not normally couple. For instance, it has been shown previously that CXCR2 is only able to couple to phospholipase D when it has been mutated in a way that also enables it to resist internalisation and to undergo prolonged periods of receptor activation (Richardson *et al*, 1998). Whether these properties are linked in a causative manner, and whether this can happen physiologically downstream of a GPCR, is not clear.

Section 1.3.4: Distribution and function

The patho-physiological roles of CXCR2 are summarised in Fig. 1.3. IL-8 has been shown to be involved in angiogenesis, mediating rat corneal vascularisation and also proliferation and chemotaxis of human umbilical vein endothelial cells (Koch *et al*, 1992). It is also apparent that the ELR motif is central to the angiogenic activity of certain chemokines (Strieter *et al*, 1995), suggesting that these effects are being mediated by CXCR2, which preferentially binds ELR+ ligands. Indeed CXCR2 knockout mice exhibited severely reduced (90-99%) angiogenesis in response to ELR+ chemokines, as did $CXCR2^{+/+}$ mice treated with anti-CXCR2 antibody (Addison *et al*, 2000). This angiogenic role perhaps predisposes CXCR2 to a role in cancer progression. Indeed, over-expression of GRO family (i.e. CXCR2 specific)

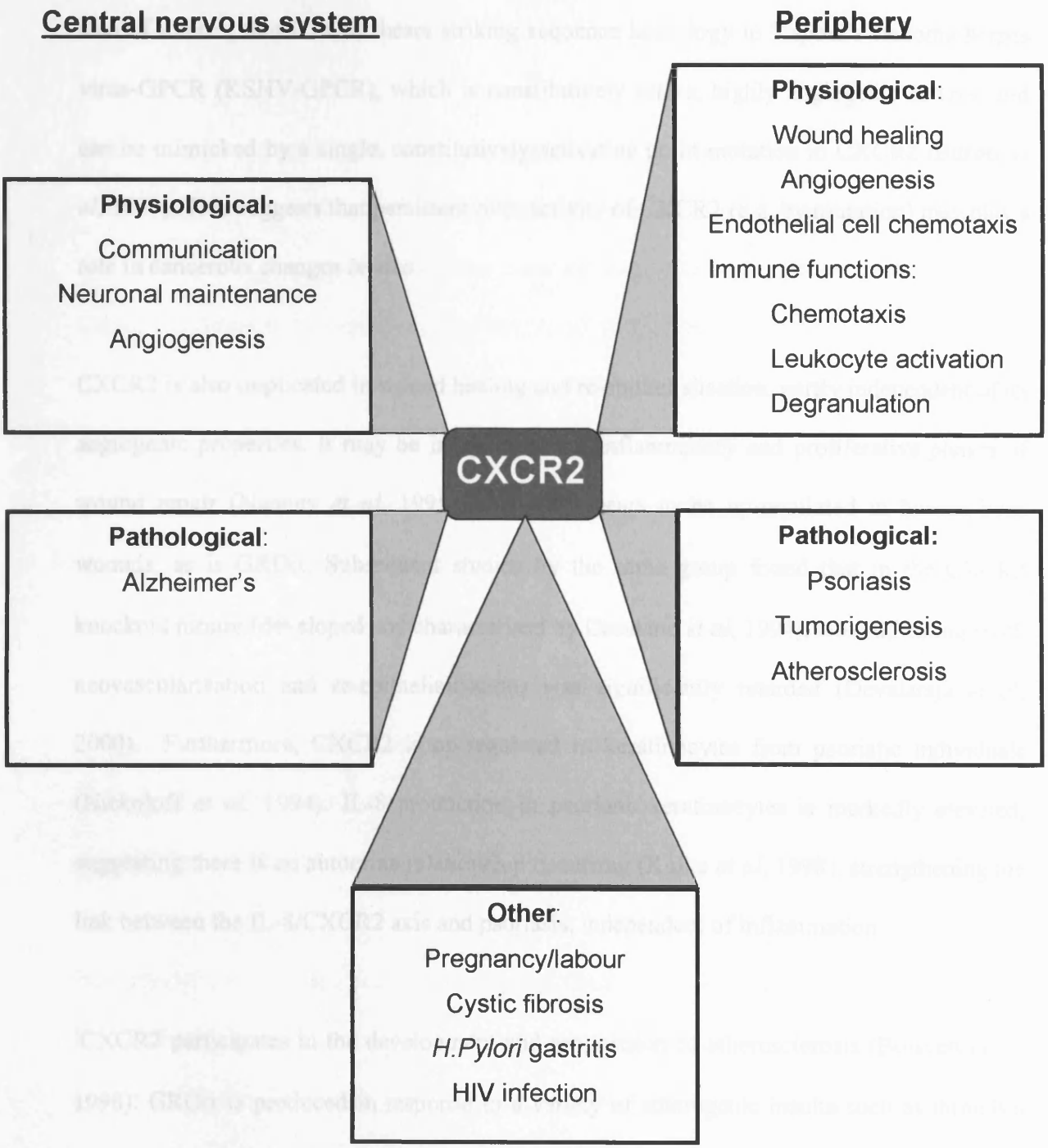


Figure 1.3: Potential patho-physiological functions of CXCR2. Proposed roles of CXCR2 in both the periphery and the central nervous system. References to this Figure can be found in the body of the text (Section 1.3.4)

chemokines has been shown to be tumorigenic (Luan *et al*, 1997; Owen *et al*, 1997). It is also of interest that CXCR2 bears striking sequence homology to Kaposi's sarcoma herpes virus-GPCR (KSHV-GPCR), which is constitutively active, highly angiogenic *in vivo,* and can be mimicked by a single, constitutively-activating point mutation in CXCR2 (Burger *et al*, 1999). This suggests that persistent over-activity of CXCR2 (e.g. by mutation) may play a role in cancerous changes *in vivo*.

CXCR2 is also implicated in wound healing and re-epithelialisation, partly independent of its angiogenic properties. It may be involved in the inflammatory and proliferative phases of wound repair (Nanney *et al*, 1995). CXCR2 appears to be up-regulated in human burn wounds, as is GROα. Subsequent studies by the same group found that in the CXCR2 knockout mouse (developed and characterised by Cacalano *et al*, 1994), wound healing (both neovascularisation and re-epithelialisation) was significantly retarded (Devalaraja *et al*, 2000). Furthermore, CXCR2 is up-regulated in keratinocytes from psoriatic individuals (Nickoloff *et al*, 1994). IL-8 production in psoriatic keratinocytes is markedly elevated, suggesting there is an autocrine relationship occurring (Kulke *et al*, 1998), strengthening the link between the IL-8/CXCR2 axis and psoriasis, independent of inflammation.

CXCR2 participates in the development and progression of atherosclerosis (Boisvert *et al*, 1998). GROα is produced in response to a variety of atherogenic insults such as thrombin and low-density lipoprotein (LDL) *in vitro,* and CXCR2 was detected in human atherosclerotic lesions, expression that was shown to be pro-atherogenic (Boisvert *et al*, 2000). Also, following radiation-induced depletion of bone marrow leukocyte precursor cells in a murine model that is hyper-susceptible to atherosclerosis, replenishment of leukocyte precursor cells with cells positive for expression of the murine homologue of CXCR2 (mIL-8RH) caused increased number and advancement of atherosclerotic lesions (Boisvert *et al*,

1998). Therefore mIL-8RH/CXCR2 can be said to be central in the recruitment of monocytes and macrophages to atherosclerotic plaques.

Neuronal expression of CXCR2 has also been demonstrated in projection neurons in regions of the brain and spinal cord including the hippocampus, dentate nucleus, pontine nuclei, locus coeruleus, paraventricular nucleus, anterior horn, interomediolateral cell column and Clarke's column of the spinal cord (Horuk *et al*, 1997). Small interneurons failed to show CXCR2 expression. The wide expression of CXCR2 in brain areas has spawned several theories as to its function, including communication, maintenance and trophic actions (Coughlan *et al*, 2000; Horuk *et al*, 1997; Araujo & Cotman, 1993. See also review by Hesselgesser and Horuk, 1999). CXCR2 may also have pathological consequences. Alzheimer's disease seems to involve CXCR2 in neurofibrillary tangles, and there is a suggestion that CXCR2-positive neurites in Alzheimer's plaques are aberrantly regenerative (Gitter *et al*, 1995; Xia *et al*, 1997a). Over-expression of CXCR2 is not an absolute precursor of Alzheimer's disease but does seem to play some role, and it has been suggested that this link may be *via* CXCR2-stimulated activity of either ERK or PI 3-K (Xia & Hyman, 2002).

Haematopoietic roles have also been proposed for CXCR2. Evidence exists to suggest that IL-8 and a receptor for it are responsible for negatively regulating myeloid progenitor cell proliferation (Cacalano *et al*, 1994; Broxmeyer *et al*, 1996) although it should be noted that no distinction was made between CXCR2 and CXCR1 in these studies.

There have been studies of IL-8 expression in several other pathological and non-pathological states such as pregnancy (Dame & Juul, 2000) and the initiation of labour (Elliott *et al*, 2001); cystic fibrosis (Brennan *et al*, 2001); *Helicobacter pylori* gastritis (Eck *et al*, 2000); and human immunodeficiency virus (HIV) infection (Cota *et al*, 2000). These

will be discussed no further as there is distinct potential for digression in that IL-8 effects can be mediated by CXCR1 in addition to CXCR2.

Section 1.4: Crosstalk between nucleotide- and chemokine receptors

CXCR2 has been shown to be involved in Ca^{2+}-potentiating crosstalk with ionotropic glutamate receptors, modulating GluR1 channel open probability and increasing the apparent affinity of this channel for glutamate (Lax *et al*, 2002). However, as yet, CXCR2 has not been shown to participate in interactions with any G-protein coupled 7-TMD receptors. Such crosstalk may have physiological or patho-physiological significance in tissues where specific combinations of receptors are co-expressed. As demonstrated above, CXCR2 has been implicated in numerous disease states. It would be of great interest to this study if CXCR2 and any P2Y receptors could be shown to be co-expressed in disease tissue from any of these states, especially if the expression of one or both could be shown to be dysfunctionally regulated. In this respect, it is of note that the P2Y1 receptor, which is normally expressed in human brain associated with neuronal tissue, becomes more specifically associated with neurofibrillary tangles and neuritic plaques in Alzheimer's disease (Moore *et al*, 2000). This is of interest given the abnormal expression of CXCR2 in this disease, although it should be highlighted that it is not yet known whether CXCR2 up-regulation in Alzheimer's disease is causative to the disease or symptomatic of it. However, the significance of the altered expression profiles of these two receptors in Alzheimer's disease is unknown, and the potential of the two to interact, perhaps deleteriously, has yet to be investigated.

Not all CXCR2-expressing cells give an intracellular Ca^{2+} response *in vivo*, and the reasons for this are unclear. In dendritic cells, both CXCR2 and CXCR1 are expressed at appreciable levels, but in terms of $[Ca^{2+}]_i$ elevation and chemotaxis are inactive (Sozzani *et al*, 1995 and 1997). An identical paradigm exists in monocytes (Baggiolini *et al*, 1994; Ben-Baruch *et al*,

1995; Sozzani *et al*, 1996). This suggests that either they have functions that are entirely mediated by $G\alpha_i$ or that they are not strong enough activators of PLCβ that they give an observable rise in $[Ca^{2+}]_i$. The possibility that these cells do not express any Gβγ-sensitive PLCβ isoforms seems unlikely (given that other chemokine receptors are expressed in dendritic cells and do mediate Ca^{2+} responses (Sozzani *et al*, 1997). Similarly, the possibility that CXCR2 is not properly presented at the cell membrane can also be discounted since there are significant numbers of ^{125}I-IL-8 binding sites on the cell surface (Xu *et al*, 1996). Promyelocytic cell lines have been shown to express chemokine receptors on the cell surface in an uncoupled state (Xu *et al*, 1996; McColl *et al*, 1993). This raises the possibility that monocytes, dendritic cells or any other cell that expresses non-Ca^{2+} signalling CXCR2 could be reprogrammed to couple to Ca^{2+} by the presence of secondary non-chemokine agonists. Alternatively, any interaction between CXCR2 and P2Y receptors may be representative of GPCR interactions in general rather than a property of CXCR2 alone, and thus may have wider implications for GPCR signalling without necessarily being relevant to any CXCR2-mediated physiological role. The aim of this thesis is to explore the interaction between certain GPCRs at the level of Ca^{2+} signalling (primarily P2Y2 and CXCR2, but also the P2Y1 nucleotide receptors, muscarinic M3 receptors, and β2 adrenoceptors), to identify the nature of the relationship(s) and the mechanism(s) underlying this convergence of signalling. It also aims to determine whether there is any apparent relevance to cells naturally expressing CXCR2, and therefore, by extension, to CXCR2-related patho-physiologies.

Chapter 2 - Materials and Methods

Section 2.1: Abbreviations

ADP, adenosine 5'-diphosphate; (±)-arterenol, noradrenaline; ATP, adenosine 5'-triphosphate; BSS, balanced salts solution; Ca^{2+}, calcium ion; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; cAMP, cyclic adenosine-5'-monophosphate; CTX, cholera toxin; CXCR2, CXC chemokine receptor 2 or IL-8 receptor B; DAG, diacylglycerol; dbcAMP, dibutyryl cyclic adenosine monophosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; eGFP-$PH_{PLC\delta}$, enhanced green fluorescent protein–tagged phospholipase Cδ pleckstrin homology domain construct; ERK, extracellular-signal regulated kinase; FLIPR, fluorescent light imaging plate reader; fluo-3/AM, fluo-3 acetoxymethyl ester; fura-2/AM, fura-2 acetoxymethyl ester; GAP, GTPase activating protein; GPCR, G-protein-coupled receptor; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GTP, guanosine triphosphate; HEK, human embryonic kidney cell; HEK-CXCR2, CXCR2-transfected HEK cell; HEK-WT, wild-type HEK cell; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HTS, high-throughput screening; IL-8, interleukin-8; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3$/BM, inositol 1,4,5-trisphosphate hexakis(butyryloxymethyl) ester; $InsP_3$-BP, inositol 1,4,5-trisphosphate-binding protein; $InsP_x$, inositol phosphates; 2MeSADP, 2'-methyl-thio-adenosine 5'-diphosphate; 2MeSATP, 2'-methylthioadenosine 5'triphosphate; NAADP, nicotinic acid-adenine dinucleotide phosphate; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol-(4)-phosphate; PtdIns(4,5)P_2, phosphatidylinositol-(4,5)-bisphosphate; PI 3-K, phosphatidylinositol 3-kinase; PI 4-K, phosphatidylinositol 4-kinase; PtdIns(4)P 5-K, phosphatidylinositol-(4)-phosphate 5-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMSF,

phenylmethylsulfonylfluoride; 8-PST, 8-(p-sulfophenyl)theophylline; PTH, parathyroid hormone; PTX, pertussis toxin; RGS protein, regulator of G-protein signalling protein; TCA, trichloroacetic acid; 7TMD receptor, seven transmembrane domain receptor; UDP, uridine 5'-diphosphate; UTP, uridine 5'-triphosphate.

Section 2.2: Materials

Buffer ingredients were from either Sigma Aldrich (Poole, Dorset, UK) or Fisher Scientific (Loughborough, UK). Receptor ligands and other test agents were all obtained from Sigma Aldrich (Poole, Dorset, UK) or Calbiochem (through CN Biosciences, Nottingham, UK) unless stated otherwise. All cell culture reagents were obtained from Invitrogen (Inchinnan, Scotland), including various cell culture media, serum (foetal calf/goat), phosphate buffered saline (PBS), poly-D-lysine and fibronectin. Polymorphprep and Nycoprep for leukocyte isolation procedures (see below) also came from Invitrogen. Ethylenediamine tetra-acetic acid (EDTA)-coated tubes for monocyte isolation procedure obtained from Corning Life Sciences Ltd. Radiochemicals were either from Amersham ([^{3}H]-*myo*-inositol (TRK912); [^{3}H]-cAMP (TRK498); [^{3}H]-*myo*-$InsP_3$ (TRK999)) or NEN ($^{45}Ca^{2+}$ (NEZ013); [^{35}S]-GTPγS (NEG030H); [γ^{32}P]-ATP (NEG502A)). Phytoerythritin-conjugated anti-human CXCR2 monoclonal antibody from Becton Dickinson (Bedford, MA, USA). Rabbit polyclonal antibodies against ERK and $G\alpha_{i1}$ were obtained from Santa Cruz (CA, USA). Lipofectamine transfection reagent and the expression plasmids, pRc/CMV and pTracer were supplied by Invitrogen. Genejuice® transfection reagent was from Novagen (through CN Biosciences). Molecular weight markers for use in Western blotting procedure were supplied by BioRad Ltd (CA, USA). ECL+® reagents and Hyperfilm® for Western blotting procedure were bought from Amersham (Bucks, UK). Cell culture plastics were obtained from Nalgene (Europe) Ltd (Hereford, UK), glass coverslips (No. 1.5 thickness, 22 or 25mm diameter) were from Fisher, and black poly-D-lysine-coated 96-well plates for use in the fluorescent light imaging plate reader (FLIPR) were from Becton Dickinson (Bedford, MA, USA). Fura-2 acetoxymethyl ester (fura-2/AM) and apyrase (Grade III) were obtained from Sigma Aldrich. Fluo-3 acetoxymethyl ester (fluo-3/AM) was from TEF Labs (Austin, TX, USA) and pluronic F-127 was from Molecular Probes Ltd (Leiden, Holland). IL-8 and GROα were supplied by R & D Systems (Abingdon, UK). AR-C67085 (2-propylthio-β,γ-

dichloromethylene-D-ATP) was provided by the Medicinal Chemistry Department, AstraZeneca R & D Charnwood, Loughborough, UK. Folin-Ciocalteu reagent, tri-n-octyl-amine, 1,1,2-trichloro-trifluoroethane, Triton-X-100 detergent (Octylphenoxy-polyethoxyethanol) were all obtained from Sigma. Safefluor organic cocktail for liquid scintillation counting was from Lumac.LSC (Groningen, Holland). Protein-A-sepharose beads were bought from Amersham Biosciences (Bucks, UK). The synthetic peptide substrate corresponding to amino acids 662–681 of the epidermal growth factor receptor (for use in ERK activation assays) was a gift from Dr J B lank (Dept. of Cell Physiology and Pharmacology, Leicester University) prepared following a previously published method (Gardner *et al*, 1994). P81 cation-exchange paper was from Whatman (Maidstone, UK).

Buffers:

Balanced salt solution (BSS) – composition (mM): NaCl, 130; KCl, 5.4; $NaHCO_3$, 16; NaH_2PO_4, 1.3; $MgCl_2$, 0.8; $CaCl_2$, 1.8; HEPES, 10; D-glucose, 5.5; pH 7.4. Nominally Ca^{2+}-free BSS is identical to above, but with no added $CaCl_2$.

Tyrodes solution – composition (mM): NaCl, 140; KCl, 2.7; $K_2H_2PO_4$, 0.26; $CaCl_2$, 1.8; $MgCl_2$, 1; D-glucose, 10; HEPES, 10; pH 7.4.

Section 2.3: Methods

Section 2.3.1: Generation of cell line and cell culture

HEK-CXCR2 cell line: The coding sequence for human CXCR2 was cloned into the expression plasmid pRc/CMV and transfected into HEK-293 cells using Lipofectamine in accordance with the manufacturer's instructions. Cells were selected using geneticin and positive clones expressing CXCR2 identified using [^{125}I]-IL-8 binding. Expression of CXCR2 in the clone used for these studies was approximately 50,000 sites/cell as assessed by radioligand binding (data not shown). Establishment of HEK-CXCR2 cell line was performed by AstraZeneca R&D Charnwood (Dr I.A. Dainty). HEK-CXCR2 cells were maintained routinely in Dulbecco's Modified Eagle's Medium (containing 25mM D-glucose, 4mM L-alanyl-L-glutamine and 1mM sodium pyruvate) and supplemented with 10% foetal calf serum, 1% non-essential amino acids, 50μg/ml gentamicin and geneticin (400μg/ml). Cells were grown in 175cm^2 tissue culture flasks at 37°C in a 5% CO_2 humidified atmosphere.

Primary human microvascular endothelial cell (HMVEC): Primary cultures of HMVECs were obtained from Clonetics (*via* www.cambrex.com) (Cat. no: CC-2543) and maintained in EGM-2-MV medium (Cat. no: 3202) containing foetal calf serum (5%), ascorbic acid, hydrocortisone, hEGF, VEGF, hFGF-b, R^3-FGF-1, gentamicin and amphotericin supplied by Clonetics (Cat. no: CC-3202). The exact concentrations are not known due to restrictions on access to proprietary information. Cells were grown in 175cm^2 tissue culture flasks at 37°C in a 5% CO_2 humidified atmosphere. Cultures were discarded after passage 10 after which CXCR2 expression disappeared from the cells, as analysed by flow cytometry, using detection of binding of phytoerythritin-tagged antibodies against CXCR2 (Glen Andrews and David Nicholls, AstraZeneca R&D Charnwood, personal communication).

Experimentation on adherent cells

HEK cells are not tenaciously adherent to plasticware or glassware, and the use of perfusion systems (Ca^{2+} imager/confocal microscope) and injection additions (FLIPR) threatens the integrity of the monolayer during experiments of this nature. Therefore, where required, plasticware and glass coverslips were coated with either poly-D-lysine (using 1ml of a 1mg/ml solution placed onto cell culture surface for >5min) or bovine plasma fibronectin (using a similar length exposure to 1ml of a 10μg/ml solution). Solution was then removed, and cells were added onto the cell culture surface and incubated as normal. Coverslips for the study of HMVECs were also prepared with fibronectin coating.

Section 2.3.2: Immunocytochemistry for visualisation of CXCR2 expression

HEK-CXCR2 or HEK-WT cells were plated onto fibronectin-coated 22mm diameter/No. 1.5 thickness circular borosilicate glass coverslips. Cells were then allowed to adhere and grow for 24 - 48 hours. Coverslips were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 3min before washing copiously with PBS. Cells were then incubated with goat serum (10% in PBS) and Triton-X-100 (0.5%) for 30mins, followed by copious washing with PBS. Phytoerythritin-conjugated anti-human CXCR2 monoclonal primary antibody (0.7μg/ml in goat serum) was added to the samples and incubated overnight at 4°C. The cells were then washed with PBS. Coverslips were dried, placed onto Citifluor AF1 immersion fluid (glycerol/PBS solution containing an antifadent; Citifluor, London, UK) on glass microscope slides, and imaged using confocal microscopy. A krypton/argon laser was used to excite the fluorescent moiety at 488nm and emitted light was collected above 510nm. Confocal images were captured by cooled CCD camera, and converted into on-screen images using Ultra*View* 4.0 spatial imaging software (PerkinElmer Life Sciences, Cambridge, UK).

Section 2.3.3: $[Ca^{2+}]_i$ measurement

FLIPR

Cells were seeded at 50,000 cells per well in black-walled 96-well FLIPR plates and grown overnight to approximately 80% confluence. Preliminary experiments indicated that this density was optimal for detection of agonist-induced changes in fluorescence in subsequent FLIPR assays (data not shown). Cells were incubated for 1hr at room temperature in BSS buffer containing 5μM fluo-3/AM and 0.044% pluronic F-127. Cells were washed once with BSS at 37°C, 100μl of BSS added to each well and the plate transferred to a FLIPR (Molecular Devices Ltd, USA) for assay at 37°C. Addition protocols were performed robotically by the FLIPR. All additions were made in a 50μl volume at a rate of 40μl/s. Fluo-3-loaded cells were excited at 505nm, with emission recorded at 530nm every 2 seconds.

Fluorimetry

HEK-CXCR2 cells were grown to confluence, harvested with Ca^{2+}-free PBS and washed once with BSS. Viable cells (>95%) were counted using exclusion of 0.2% trypan blue, re-suspended in BSS at $5x10^6$ viable cells per ml and loaded with 5μM of fura-2/AM in the presence of 0.044% pluronic F-127. Aliquots of $3.5x10^6$ cells were taken, centrifuged at 7000rpm for 5s and re-suspended in 2ml BSS at 37°C in a cuvette. Following a 5-10min incubation at 37°C to enhance intracellular de-esterification of fura-2/AM, cuvettes were transferred to a Fluoromax I fluorimeter (Jobin-Yvon Ltd, Middlesex, UK) in which the cell suspension was maintained at 37°C and mixed continually using a magnetic follower. Cells were alternately excited at wavelengths of 340 nm and 380 nm with emission determined at 510 nm. Data are presented as a ratio of the 340/380 values that were collected every 1.5 seconds.

Confocal microscopy

Cells were seeded onto 22mm diameter glass coverslips and cultured for 48hrs. Cells were then loaded with fluo-3/AM (using the conditions described above for FLIPR-based $[Ca^{2+}]_i$ measurement) and the coverslips mounted in a chamber on the stage of an Olympus IX70-S1F inverted microscope. The chamber was perfused at a rate of 5ml/min with BSS or drug solutions and the temperature maintained at 37°C using a peltier unit. Using an Ultra*VIEW* confocal imaging system (PerkinElmer Life Sciences, Cambridge, UK), cells were excited with a krypton/argon laser at 488nm and emitted light collected above 510nm. Confocal images were captured by cooled CCD camera at a rate of approximately 1 frame per second.

Merlin Ca^{2+} imager

Coverslips were prepared as for confocal microscopy and connected to an identical perfusion system. Cells were excited at 488nm by light from a xenon lamp filtered through a monochromator (Perkin Elmer Life Sciences). Fluorescence emissions (510nm) were detected by a cooled CCD camera at a rate of 0.75 frames per second and converted into on-screen images by UltraVIEW imaging software (PerkinElmer Life Sciences, Cambridge, UK).

Section 2.3.4: Extracellular-regulated kinase (ERK) activation assay

Stimulation of ERK activity was assessed by measuring the incorporation of ^{32}P into an ERK substrate peptide using the method of Wylie *et al* (1999). Cells were grown to ~80-90% confluence in 6-well plates, washed with PBS and the medium replaced with serum-free medium to bring the cells to a state of quiescence. After 24hrs serum-starvation, cells were washed with PBS and stimulated by the addition of test agents. After the required time, the reaction was stopped by aspiration and the cells were scraped and solubilized in 300µl of lysis buffer (composition: Tris-HCl, 20mM; Triton-X-100, 1% (v/v); glycerol, 10% (v/v);

NaCl, 137mM; EDTA, 2mM; β-glycerophosphate, 25mM; Na_3VO_4, 1mM; PMSF, 1mM; leupeptin, 10μg/ml; pH 7.4). Rabbit polyclonal anti-ERK antiserum was added (5μg/ml) and incubated for 1hr. The antigen/antibody complex was immunoprecipitated with 70μl of a 15% (w/v) slurry of protein-A-sepharose beads for 1hr at 4°C on a roller to ensure maximal immunoprecipitation was achieved. Protein-A-sepharose beads were then washed twice in lysis buffer and twice in kinase buffer (composition: HEPES, 25mM; β-glycerophosphate, 25mM; $MgCl_2$, 25mM; DTT, 2mM; Na_3VO_4, 100μM; pH 7.2) by centrifugation (13000g, 30s) and resuspension. After washing, the supernatant was aspirated and 40μl of kinase buffer containing 20 μM [γ-^{32}P]ATP (2.5 μCi/nmol) and 200 μM of a synthetic peptide substrate corresponding to amino acids 662–681 of the epidermal growth factor receptor (EGFR peptide; Gardner *et al*, 1994) was added to each sample. This mix was incubated for 20mins at 30°C. The reaction was stopped with 25μl 25% TCA, and samples were centrifuged (13400g, 20s). The supernatant (45μl) was dripped onto P81 cation-exchange paper. After three 5min washes with 0.5M orthophosphoric acid, papers were washed for 2mins with acetone and left overnight to dry. Papers were then added to scintillation tubes containing 5ml scintillation fluid, and the amount of ^{32}P incorporated into EGFR peptide by ERK was determined by liquid scintillation counting.

Section 2.3.5: $InsP_3$ mass assay

Preparation of $InsP_3$ binding protein ($InsP_3$ BP)

An $InsP_3$-binding protein was purified from bovine adrenal glands as described previously (Challiss *et al*, 1990). Glands were obtained fresh from an abattoir and kept on ice. The glands were halved longitudinally, with the medulla then being removed by dissection and discarded. The cortical region was separated from the capsule and the capsule discarded. Cortical tissue was minced with a scalpel blade, mixed with ~5ml buffer (composition: $NaHCO_3$, 20mM; DTT, 1mM; pH 8.0 at 4°C) in centrifuge tubes and homogenised using a

Polytron homogeniser (20-30s, half-speed). The tubes were topped up with buffer, centrifuged (3000g, 10mins), and the supernatant collected into a beaker on ice. The pellet was re-suspended in 5ml buffer and homogenisation and centrifugation steps were repeated. The supernatants from both spins were combined and centrifuged (40,000g, 20mins) to pellet the binding protein. The pellet was then re-suspended in buffer, re-homogenised and centrifuged (40,000g, 20mins). The pellet was re-suspended (by further homogenisation, if necessary) at a concentration of 15-25mg protein/ml and stored at –20°C.

Stimulation of $InsP_3$ generation in intact cells and extraction of $InsP_3$ from samples

Cells were grown to confluence in 24-well plates. After aspirating the media, cells were washed with BSS, which was then also aspirated. Agonists were added to the cell monolayer in a 200µl total volume. Reactions were stopped at the desired time with 200µl of ice-cold 1M TCA and placed on ice for ~20mins. An aliquot of 320µl was then removed and added to 80µl of 10mM EDTA. A 1:1 (v/v) mixture of tri-n-octyl-amine and 1,1,2-trichloro-trifluoroethane was freshly prepared and 400µl added to the sample, which was then vortexed thoroughly. After centrifuging at high speed (11000g, 2min), a 200µl sample from the upper aqueous layer was removed and added to 100µl of 60mM $NaHCO_3$. Samples were stored (at 4°C for a week, or –20°C for up to a month) or processed immediately for determination of $InsP_3$ content by [^{3}H]-$InsP_3$ competition binding assay.

$InsP_3$ competition binding assay

$InsP_3$ mass was determined using a previously described method (Challiss *et al*, 1990). Standard concentrations of $InsP_3$ (range: 0.3nM – 10µM) were prepared in extracted buffer, produced by processing fresh BSS buffer through the phase separation protocol above. [^{3}H]-$InsP_3$ (specific activity 34Ci/mmol) was diluted in water to a concentration of 3.6nM. On ice, assay constituents were added to a 1.5ml eppendorf tube in this order: 30µl of sample or

standard; 30µl of [^{3}H]-$InsP_3$; 30µl of assay buffer (composition (mM): Tris-base, 100; EDTA, 4; pH 8.0 at 4°C). The binding reaction was started by the final addition of 30µl of binding protein. This mix was incubated on ice for 30mins, then collected on GF-B filter paper (Whatman, Maidstone, UK) using a Brandel cell harvester. Filters were rapidly washed twice with 5ml ice-cold wash buffer (composition (mM): Tris-base, 25; EDTA, 1, $NaHCO_3$, 5; pH 8.0 at 4°C) using the cell harvester. Filters were added to 5ml scintillation fluid and the radioactivity of trapped $InsP_3$-BP-bound [^{3}H]-$InsP_3$ was determined by liquid scintillation counting.

Section 2.3.6: Total [^{3}H]-inositol phosphate ([^{3}H]-$InsP_x$) generation

Lithium ions (Li^+) block the metabolism of inositol-1-monophosphate to inositol by inositol monophosphatase (Berridge *et al*, 1982), causing the accumulation of inositol phosphate species ($InsP_x$) against this block. The extent of this accumulation is proportional to the rate of generation of these species i.e. to PLC activity. Thus, this technique can be used as a measure of PLC activity independent of the metabolism of $InsP_x$. Cells were plated into 24 well plates and grown for 48hrs in the presence of 3µCi/ml [^{3}H]-*myo*-inositol. Cells were washed once with BSS containing 10mM LiCl (BSS/Li^+), then incubated for 20mins at 37°C with 250µl BSS/Li^+. Agonists (made up at two times the desired final concentration in BSS/Li^+) were added in a 250µl volume at the appropriate time. The reaction was stopped with 500µl of ice-cold 1M trichloroacetic acid (TCA) and the cells left on ice for ~20mins. The entire contents of each well (1ml) were removed and added to 250µl 10mM EDTA in a 5ml polypropylene tube (Greiner Bio-One, Stonehouse, Gloucs, UK). Following this, 1ml of a freshly-prepared 1:1 (v/v) mixture of tri-n-octyl-amine and 1,1,2-trichloro-trifluoroethane was added and the tube vortexed to ensure thorough mixing. Samples were stood for ~5min to partition into aqueous and organic layers. A 700µl aliquot of the upper aqueous layer was removed and added to 50µl 250mM $NaHCO_3$. This sample was then either stored frozen or

processed through strongly basic Dowex chloride anion exchange columns (8% cross linkage, 100-200 dry mesh; Sigma no. 1X8-200). After washing the columns with H_2O, the sample was added to the column, and then washed through with a further 10ml H_2O, and then 12ml 25mM ammonium formate. Elution of [^{3}H]-$InsP_x$ from the columns was performed by washing the columns through with 10ml 1M HCl. The eluate was collected into scintillation vials. A 5ml aliquot was mixed with 15ml scintillation fluid and radioactivity determined by scintillation counting. Dowex chloride columns were regenerated by washing with 10ml 2M HCl.

Section 2.3.7: [^{35}S]-GTPγS binding assay

GTP binding to an activated G-protein is a critical moment in the initiation of signal transduction. Binding of the radio-labelled, slowly-hydrolysable GTP analogue, [^{35}S]-GTPγS, to activated G-proteins in membrane preparations was performed to quantify the activation of G-proteins by GPCR agonists, alone or in combination. The method used was a modification of a protocol described previously (Sim *et al*, 1997, and refs therein).

Membrane preparation

Cells were grown to ~80-90% confluence in cell culture flasks. After removal of growing medium, cells were washed with 10ml lifting buffer (composition: HEPES, 10mM; EDTA, 5mM; NaCl, 150mM; pH 7.4), then incubated with 10ml fresh lifting buffer to detach cells. All subsequent actions were performed at 4°C. The suspension was centrifuged (350g, 5mins) and the pellet re-suspended in 25ml lysis buffer (composition: HEPES, 10mM; EDTA, 10mM; pH7.4). This suspension was crudely homogenised with a Polytron homogeniser at high speed for ~20s, centrifuged at 40,000g, 15min) and the pellet re-suspended in 20ml storage buffer (composition: HEPES, 10mM; EDTA, 0.1mM; pH 7.4). The homogenisation and centrifugation steps were repeated once. The pellet was re-

suspended in ~1ml storage buffer per flask of cells and homogenised with a smaller homogeniser probe for a further ~20s. Protein concentration was assessed using the method of Lowry *et al* (1951) (see below) and the sample was diluted in storage buffer to a concentration of 2mg/ml before freezing (-80°C).

Binding assay

All solutions for this binding assay were made up in assay buffer (composition (mM): HEPES, 10; NaCl, 100; $MgCl_2$, 10; pH 7.4). Ingredients for the reaction mix were as follows: non-specific binding - 10μM GDP, 100pM [^{35}S]-GTPγS, 10μM GTPγS, 100μl membrane homogenate (to a final volume of 1ml); stimulations - 10μM GDP, 100pM [^{35}S]-GTPγS, 100μl 10-fold concentrated agonist stock (or assay buffer for basals), 100μl membrane homogenate (final volume of 1ml). Membranes were added last to initiate the reaction. The reaction was performed at 30°C, and stopped by the addition of 1ml ice-cold assay buffer. Membrane-bound [^{35}S]-GTPγS was collected onto GF-B filter paper and washed with ~5mls ice-cold assay buffer using a Brandel cell harvester. Filters were added to 6ml scintillation fluid and radioactivity determined by liquid scintillation counting.

Section 2.3.8: Transfection of HEK cells with cDNA encoding RGS2 or $G\alpha_{t1}$

Expression of recombinant DNA encoding human RGS2 in HEK cells was achieved by transfection using pTracer expression vector. Complementary DNA encoding RGS2 (a kind gift from Dr. C. Doupnik, University of South Florida, Tampa, FL, USA) was removed from pRC/CMV vector and re-ligated into pTracer using a BstX1 restriction cut site. The pTracer vector was delivered to the cells using Genejuice® transfection reagent at a ratio of 3μl of Genejuice® to 1μg of DNA following the manufacturer's instructions. Briefly, Genejuice® reagent was added to 100μl of cell culture medium (no additives) and incubated at room temperature for 5mins. DNA was then added, mixed by gentle pipetting, and incubated for a

further 15mins. After further mixing, 100µl of transfection mixture (approximately 1µg DNA) was added to each well of a 6-well plate containing adherent HEK cells at approximately 40% confluence. Cells were incubated for a minimum of 48hrs at normal cell culture conditions. pTracer also contains DNA encoding a protein product that confers resistance to blasticidin. Transfected cells were semi-stably selected by growing for 3-4weeks in media containing 5µg/ml blasticidin, by which time all untransfected control cells had died. Expression of RGS2 was not determined due to lack of a suitable antibody.

Complementary DNA encoding bovine $G\alpha_{t1}$ (a kind gift from Prof. G. Milligan, University of Glasgow, UK) was transfected into HEK-CXCR2 cells in an identical manner to that described above for RGS2 transfection, using the pTracer expression plasmid. $G\alpha_{t1}$ was removed from the pcDNA3 vector and re-ligated into pTracer using EcoR1 and Xho1 restriction cut sites. $G\alpha_{t1}$-expressing cells were then selected using the blasticidin-resistance method. $G\alpha_{t1}$ expression was verified by Western blotting, using a rabbit polyclonal anti-$G\alpha_{t1}$ antibody. This procedure is described below.

Section 2.3.9: Protein solubilisation and Western blotting for $G\alpha_{t1}$ expression

Western blot analysis was used to check the successful expression of the $G\alpha_{t1}$ construct in HEK-CXCR2 cells using methods described elsewhere (Harlow & Lane, 1988). Briefly, cells (adherent or recently spun gently out of suspension) were transferred into 200-300µl protein solubilisation buffer (composition: Tris-HCl, 10mM; EDTA, 1mM; octylphenoxypolyethoxyethanol (NP40), 1% (v/v); NaCl, 0.15mM; sodium dodecyl sulfate (SDS), 0.1%; deoxycholate, 5mg/ml; leupeptin, 1µg/ml; benzamidine, 100µg/ml; phenylmethylsulfonylfluoride (PMSF), 1mM)) on ice. The cell extract was then removed into microfuge tubes and spun (16,000g, 10min, 4°C). Avoiding aggregated DNA, supernatant

was removed into a fresh tube and stored at –20°C. The concentration of protein was assessed using the method of Lowry *et al* (1951) (see below).

A 10% polyacrylamide running gel was mixed (per 10ml: 4ml deionised water; 3.3ml 30% polyacrylamide; 2.5ml 1.5M Tris (pH 8.8); 0.1ml 10% SDS; 0.1ml 10% ammonium persulfate; 0.004ml N,N,N',N'-tetramethyl-ethylenediamine (TEMED)). The stacking gel was mixed (per 1ml: 0.68ml H_2O; 0.17ml 30% polyacrylamide; 0.13ml 1M Tris (pH 6.8); 0.1ml 10% SDS; 0.1ml 10% ammonium persulfate; 0.001ml TEMED) and poured on top of the solidified running gel using BioRad minigel apparatus.

Samples were equalised for protein content and approximately 20µg of protein loaded onto each lane of the gel after being mixed 1:1 (v/v) with sample buffer (composition: Tris-HCL (pH6.8), 100mM; dithiothreitol (DTT), 200mM; SDS, 2% (w/v); bromophenol blue, 0.1% (v/v); glycerol, 10% (v/v)) and 'cracked' by incubating at ~90°C for 3-4mins. Gels were immersed in running buffer (composition: Tris base, 25mM; glycine, 250mM; SDS 0.1% (w/v)) and run at a constant 200V for 40-60mins. Molecular weight markers were run alongside samples (molecular weight range: 250kDa – 10kDa).

After separation, proteins were transferred onto nitrocellulose paper (pre-soaked in transfer buffer (composition: glycine, 39mM; Tris-HCl, 48mM; SDS, 0.37mg/ml, methanol, 20% (v/v); pH 8.3)) at 0.65mA/cm^2 for 1hr. The nitrocellulose paper was then incubated for 1-2hrs with Tris-buffered saline containing Tween® 20 (polyoxyethylene(20)sorbitan monolaurate), (TBST buffer, composition (mM): NaCl, 300; KCl, 2.7; Tris-HCl, 25; 0.05% Tween® 20, and also containing 5% (w/v) powdered low fat skimmed milk (Marvel®), pH 7.4), to block non-specific protein binding sites. After washing copiously with TBST, nitrocellulose was incubated overnight with a rabbit polyclonal anti-Gα_{t1} primary antibody

(0.2μg/ml) (Santa Cruz Biotechnologies Inc, CA, USA), before again washing copiously with TBST. Nitrocellulose paper was then incubated for 4hrs with a horseradish peroxidase (HRP)-coupled mouse anti-rabbit secondary antibody (Sigma Aldrich). The nitrocellulose was then further washed with TBST, dried, developed with ECL+® reagents (according to manufacturer's instructions) and exposed to Hyperfilm®.

Section 2.3.10: Primary cell isolation

Neutrophils

Approximately 100ml peripheral venous blood was collected from healthy human donors into tubes containing heparin (final concentration ~ 10U/ml). This blood was layered onto Polymorphprep and spun at 450g for 30mins at room temperature to cause the blood to separate into a red cell pellet, a layer of polymorphonuclear cells, a fatty layer, and a layer of plasma. The plasma and fatty layers were removed and discarded, while the polymorphonuclear cells were transferred into a fresh tube. The volume was made up to 50ml with PBS containing 0.2% glucose and centrifuged at 660g for 10mins at room temperature. The pellet was resuspended in hypotonic (0.2%) saline, incubated at room temperature for 3min to lyse red blood cells before correcting the osmolarity by adding an equal volume of hypertonic (1.6%) saline (final isotonic solution – 0.9% saline). The suspension was made up to 50ml with PBS containing 0.2% glucose and spun at 350g for 10min at room temperature. The red blood cell lysis step was repeated if there was visible red blood cell contamination. Final neutrophil purity was determined using Alamar Blue dye recognition, and found to be consistently >90%. The major contaminant was T-cells, with minor impurities being monocytes and eosinophils. The pellet was then re-suspended in Tyrodes buffer, and loaded with fluo-3/AM using the same protocol as for HEK-CXCR2 cells, except that pluronic acid was not required. Loaded cells were counted by an automated cell counter (Coulter Ltd, Miami, FL), seeded at 200,000 cells per well in a 96-well FLIPR

plate and centrifuged onto the well floor prior to assay (460g, 5min). Ca^{2+} responses were then assayed using the FLIPR following the same protocol as for HEK cells.

Monocytes

Peripheral venous blood (200ml) was collected from healthy human donors into a series of EDTA-coated 9ml tubes. To each of these tubes was added 1ml 6% dextran/0.9% NaCl, the contents mixed by inversion and left at room temperature for ~30mins for the red blood cells to settle out. The upper plasma layer was removed to a fresh tube, layered upon 2.5ml Nycoprep (composition: Nycodenz, 13%w/v; NaCl, 0.58%w/v; tricine/NaOH, 5%w/v; pH 7.4), and spun at 600g for 10min at room temperature (without brake). The plasma layer and the leukocyte-containing layer were separated and both retained. Both collections were centrifuged at 700g for 10min at room temperature (without brake). The plasma supernatant was used to make 5% autologous plasma (1 part plasma:19 parts Ca^{2+}/Mg^{2+}-free PBS). The cell supernatant was removed, and the pellet resuspended in 5% autologous plasma, before spinning at 78g for 10mins at room temperature. This spin, to remove platelet contamination, was repeated 2-3 times. Assessment of final monocyte purity achieved using this protocol was determined previously by quantifying the proportion of cells expressing antigenicity for CD14, a marker for monocytic phenotype, using fluorescently-labelled antibody and flow cytometric cell sorting (Jill Theaker, AstraZeneca R&D Charnwood), and found to be consistently >90%. Contamination was mainly due to a small presence of T-cells and platelets. Purity was not assessed for the preparations used in these studies, and assumed to be the same as previous samples. The collected cells were counted, prepared for FLIPR assay (fluo-3/AM loaded for 1hr as for neutrophils, plated at 200,000 cells/well) and assayed using the same protocol as for neutrophils and HEK cells.

Section 2.3.11: Data analysis

Use of Fluo-3 as a Ca^{2+} indicator

A calibration of fluo-3 in terms of its *in situ* properties has been performed previously (Thomas *et al*, 2000). The K_d of fluo-3 is 450nM at 37° and the region of the Ca^{2+} binding curve that approximates to linearity (20-80%) will, therefore, be within the magnitude of the $[Ca^{2+}]_i$ responses in these cells. Furthermore, in fluo-3-loaded HEK cells, ionomycin (by elevation of $[Ca^{2+}]_i$) causes an increase in fluorescence that is twice that seen with a maximal concentration of UTP (data not shown). Thus, agonists produce elevations of $[Ca^{2+}]_i$ that are considerably below dye saturation. Additionally, over the range of fluorescence values within the experiments herein, the relationship between [fluo-3] and fluorescence in nominally Ca^{2+}-free BSS is linear as measured by FLIPR (data not shown) indicating linearity of the fluorescence detection system. Thus, the fluo-3 fluorescence values should be directly proportional to $[Ca^{2+}]_i$ over the range encountered in these experiments and allow direct determination of both pEC_{50} and E_{max} values.

Ca^{2+} measurement: FLIPR

The general protocol used in FLIPR assays is described above. The response to all agonists was expressed as the initial peak increase in fluo-3 fluorescence that occurred following addition of agonist (see Fig. 3.1). These measurements provide an index of the increase in $[Ca^{2+}]_i$ calculated as the difference between the lowest point on the baseline in the ten seconds preceding agonist addition and the highest point of elevation in the thirty seconds following the addition. Unless stated otherwise, data were normalised against the response achieved by a maximal concentration of the pre-stimulating receptor agonist (≥100μM for ATP or UTP; ≥1μM for 2MeSADP; ≥100μM for carbachol). Concentration-response curves were fitted using a four-parameter logistic equation with equal weighting to all points using GraphPad Prism (GraphPad Software, San Diego, CA, USA). The pEC_{50} value (negative

logarithm of the molar concentration of agonist giving 50% of the maximal response to that agonist) and E_{max} values (the maximal response relative to the maximal response achieved by the pre-stimulating agonist unless otherwise stated) were determined from these curves.

Ca^{2+} measurement: Fluorimetry, confocal microscopy and Ca^{2+} imaging systems

Where required, a protocol was used that provided a more flexible approach to addition of agonists and test agents than was possible when using the FLIPR. In these circumstances, data analysis was altered slightly to reflect this. Data were, like those from FLIPR assays, generally normalised against a reference value (usually the peak response to a maximal concentration of pre-stimulating agonist (see above)). Like FLIPR, peak Ca^{2+} responses were measured as the difference between the lowest point in the ten seconds preceding addition of agonist and the highest point in the 20-30s following agonist addition. Occasionally, raw fluo-3 fluorescence values (measured in 'grey levels') were used to describe Ca^{2+} responses.

The use of the lowest value in the 10s preceding agonist addition in assays using small numbers of cells (confocal microscopy, Merlin Ca^{2+} imager) tends to create some small false positive results as a consequence of large degrees of background fluctuation. Thus, responses less than 5-10% in these systems were considered to be due to background fluctuation rather than being genuine agonist-induced Ca^{2+} elevations.

Statistical analysis

Except where stated, data from three or more identical experiments are presented as the mean±s.e.m. Where statistical analyses were required, the format of the data was taken into account in the selection of an appropriate test. Two-tailed, unpaired Student's t-test was used for direct comparison of a test value with a control, with $P<0.05$ accepted as statistical significance. However, for comparison of multiple data sets, one-way or two-way analysis of

variance (ANOVA) was used. If $P<0.05$ using ANOVA, data were, where appropriate, further analysed using a two-tailed, unpaired Student's t-test as a subsidiary *a priori* multiple range test, ensuring that there were no more than $(k-1)$ individual comparisons (where k is the number of data sets).

Chapter 3 – Characterisation of an interaction between differentially-coupled GPCRs that results in enhanced intracellular Ca^{2+} signalling

Section 3.1: Introduction

The phenomenon of crosstalk between GPCRs has been demonstrated previously with numerous receptor combinations, many of which include members of the P2Y sub-family of nucleotide receptors in a variety of cell settings (Okajima *et al*, 1987 and 1989a/b; Megson *et al*, 1995; Dickenson & Hill, 1998a and b; Buckley *et al*, 2001). Chemokine receptors have also been shown to be involved in crosstalk that result in enhanced Ca^{2+} signalling (Rodríguez-Frade *et al*, 1999; Rodríguez-Frade *et al*, 2001; Mellado *et al*, 2001a and b), mainly by oligomerization between these receptors. This allowed the stimulation of a PTX-resistant Ca^{2+} response to chemokine receptor agonists (Mellado *et al*, 2001b), suggesting that oligomerization was causing alternative G-protein coupling to that in the monomeric form. However, there are instances where non-chemokine receptors have been demonstrated to potentiate Ca^{2+} signalling in response to chemokine agonists. Unpublished data also suggests that $G\alpha_q$-coupled receptors endogenously expressed in human embryonic kidney (HEK)-293 cells (i.e. muscarinic M3 receptors) mediate potentiation of Ca^{2+} responses downstream of recombinant chemokine receptors (Dr. G.F. Wilkinson, AstraZeneca, personal communication).

However, any interaction specifically between nucleotide receptors and the CXC chemokine receptor 2 (CXCR2) at the level of Ca^{2+} signalling has not been reported. Such an interaction may be of great importance physiologically, in that both ATP (a P2Y receptor agonist) and

chemokines are frequently co-localised in sites of infection and tissue damage. Any co-operativity between these receptors in terms of the magnitude of Ca^{2+} elevations may be fundamental to co-ordinating an appropriate immunological and/or reparative response in leukocytes that co-express receptors for these ligands (e.g. neutrophils/monocytes). This further highlights the relevance of an examination of CXCR2 interactions with P2Y2 receptors. These studies have used an HEK cell line recombinantly expressing CXCR2, and endogenously expressing the P2Y2 and P2Y1 receptor subtypes, both coupled to $G\alpha_q$ and thereon to intracellular Ca^{2+} release. These cells also express another $G\alpha_q$-coupled receptor, the muscarinic M3 receptor, and thus provide the opportunity to investigate any differences between Gq-coupled receptors in terms of their abilities to mediate crosstalk with CXCR2 (see Chapters 4 and 5). Throughout this study, the focus is mainly on the characterisation of the interaction between P2Y2 receptors and CXCR2, and the elucidation of the mechanism underlying it. P2Y1 and muscarinic M3 receptors were not as extensively characterised in terms of their interaction with CXCR2, except to establish crucial differences between their mechanisms of interaction in comparison with that of P2Y2 receptors. Additional to this rationale was the practical drawback of using other $G\alpha_q$-coupled GPCRs to pre-stimulate the cells. The P2Y receptor-mediated Ca^{2+} signal was more robust than the muscarinic response in these cells. Furthermore, preliminary studies showed that the muscarinic M3 receptor was not a good choice for pre-stimulation since potentiation of CXCR2 signalling by muscarinic M3 receptors was not at all reproducible, whereas potentiation by P2Y receptors was. The first aim of this thesis is to use an HEK cell line expressing recombinant human CXCR2 (rhCXCR2) to characterise any interaction between these GPCRs and quantify the basic pharmacological parameters describing it.

Wild-type HEK (HEK-WT) cells endogenously express both muscarinic M3 receptors and β2 adrenoceptors (Premont *et al*, 1992). These receptors are co-expressed in a multitude of

neuronal cell types, and crosstalk between them may have significance to the functioning of these cells following co-stimulation. Curiously, preliminary results using this receptor combination showed that the muscarinic M3 receptor caused reproducible potentiation of the β2 adrenoceptor Ca^{2+} signals, unlike its rather more erratic effects of the CXCR2 signals. This may be due to some clonal difference between the wild-type cells and the CXCR2-transfected cell line, since the response to the muscarinic receptor agonist, carbachol, was rather more robust in wild-type cells compared to that in the HEK-CXCR2s (whereas the opposite is true for the nucleotide-mediated response, which is considerably larger in the HEK-CXCR2 cell line). Alongside the characterisation of the interaction between P2Y receptors and CXCR2, a further interaction between P2Y receptors and β2 adrenoceptors is also studied in the HEK-CXCR2 cell line. However, this gives way in the later stages of this thesis to the examination of a mechanism of crosstalk between muscarinic M3 receptors and β2 adrenoceptors, and the fundamental differences between the two phenomena.

This chapter details the initial characterisation of both the P2Y receptor-CXCR2 and P2Y receptor-β2 adrenoceptor interactions. It defines the pharmacological parameters describing them (maximal achievable responses, pEC_{50} values), the P2Y receptor subtype specificity in mediating the potentiation between CXCR2 and P2Y receptors, and some of the basic properties of the interaction that may give an early indication as to how this phenomenon is brought about mechanistically. The data from this chapter are intended to provide a platform for continuation of this research aimed at elucidating the exact mechanism(s) underlying these examples of crosstalk, and whether the two examples are identical or display fundamental differences.

Section 3.2: Results

Section 3.2.1: Establishment of a HEK-293 cell line expressing recombinant human CXC chemokine receptor 2 (CXCR2)

The HEK-CXCR2 cell line used in much of this study was kindly provided by Dr Ian Dainty (AstraZeneca R&D Charnwood). The human chemokine receptor, CXCR2, was recombinantly expressed in HEK-293 cells using the method described in Chapter 2. Briefly, following transfection using the Lipofectamine® transfection reagent, CXCR2-expressing cells were clonally selected, grown under geneticin selection, and assessed for CXCR2 expression levels by radioligand binding. Cells expressing levels similar to those in neutrophils (50,000-90,000 sites per cell) were chosen to avoid much of the uncertainty surrounding the validity of systems where recombinant receptors are massively over-expressed. Visualisation of these receptors at the cell surface was achieved by immunolabelling monolayers of Triton-X-100-permeabilised HEK-CXCR2 cells with a phytoerythritin-conjugated anti-CXCR2 monoclonal antibody. Images of immunolabelled cells were captured using confocal microscopy. CXCR2 was found to be abundantly expressed at the cell surface (Fig. 3.1a) in HEK-CXCR2 cells incubated with anti-CXCR2 antibody. Incubation of HEK-WT cells with the CXCR2 antibody (Fig. 3.1b) confirmed that these cells do not endogenously express CXCR2, and that the antibody does not cross-react with any other receptor protein in HEK cells.

Section 3.2.2: Demonstration of crosstalk between P2Y receptors and CXCR2

Initial studies using the FLIPR demonstrated that challenge of HEK-CXCR2 cells with 30nM IL-8 alone was unable to elevate $[Ca^{2+}]_i$ (Fig. 3.2). In contrast, challenge with the same concentration of IL-8, 150s following (and in the continued presence of) 100μM ATP resulted in a substantial elevation of $[Ca^{2+}]_i$ (Fig. 3.2). The initial challenge with ATP

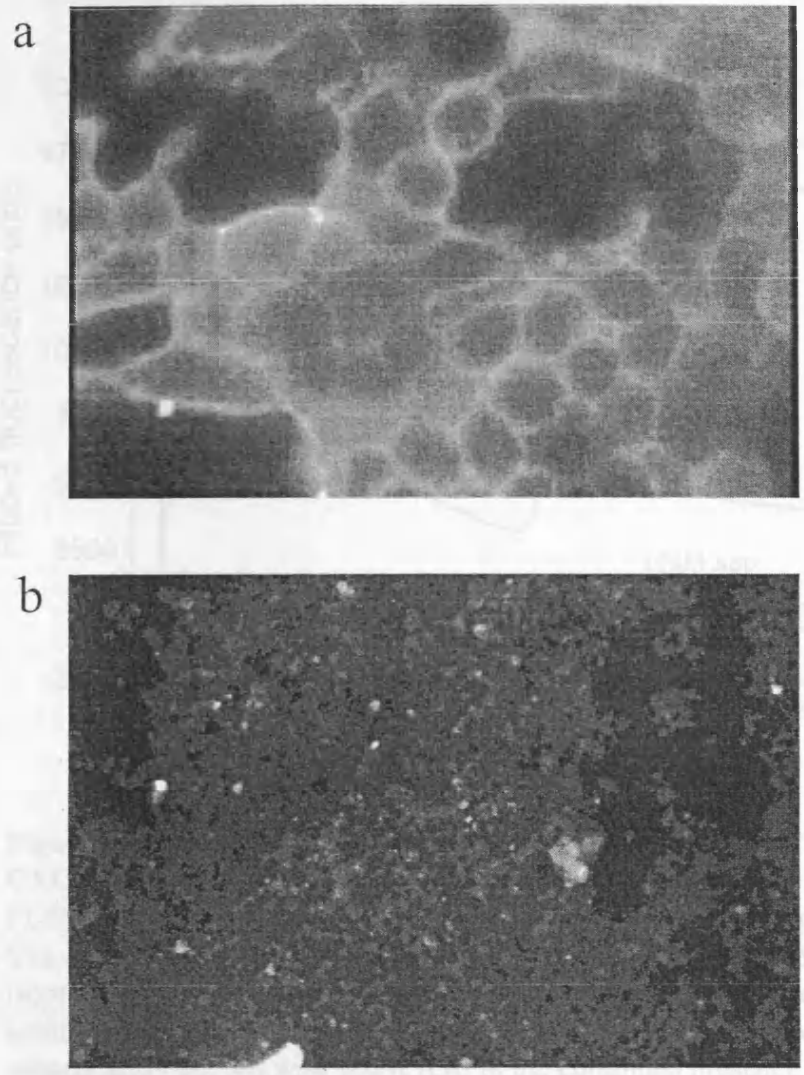


Figure 3.1: CXCR2 expression in HEK-CXCR2 cells and HEK-WT cells. Cells were grown on 22mm glass coverslips to approximately 70% confluence, fixed with paraformaldehyde, and probed with either a PE-tagged CXCR2 antibody (see Materials and Methods section). Fluorescence was visualised using confocal microscopy (see Materials and Methods). a) CXCR2 Antibody on CXCR2 cells. b) CXCR2 antibody on HEK-WT cells.

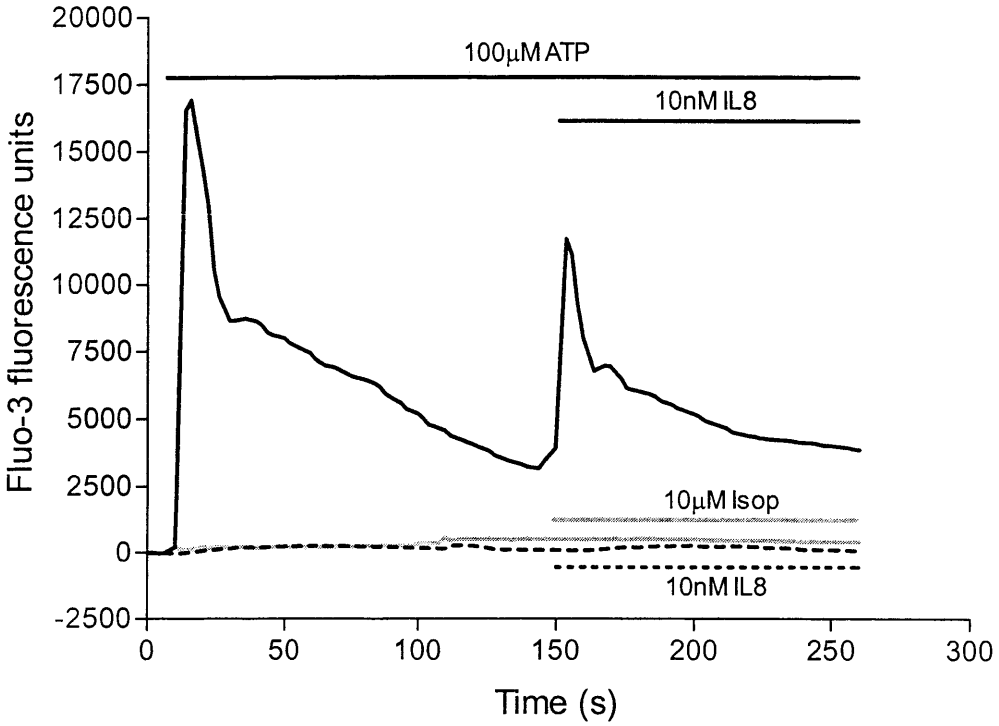


Figure 3.2. Potentiation of IL-8-mediated elevation of $[Ca^{2+}]_i$ in HEK-CXCR2 cells following ATP pre-stimulation. Representative traces from FLIPR experiments showing fluo-3 fluorescence as an index of $[Ca^{2+}]_i$. The dashed and grey lines represent cells to which 10nM IL-8 and 10μM isoprenaline, respectively, was added at 150s without any pre-addition. The solid line represents cells pre-stimulated at 10s with 100μM ATP and subsequently at 150s with 10nM IL-8 in the continued presence of ATP.

produced an increase in $[Ca^{2+}]_i$ consisting of a rapid, transient peak followed by a slower declining phase such that $[Ca^{2+}]_i$ was still elevated at the point of IL-8 addition. Similarly, challenge of endogenously expressed adrenoceptors with (±)-arterenol[§] gave qualitatively identical results (Fig. 3.2).

Concentration-response curves were generated to IL-8 and (±)-arterenol following either no addition, addition of 1mM ATP or vehicle (buffer, BSS) addition. In the absence of any pre-addition, neither IL-8 nor (±)-arterenol elevated $[Ca^{2+}]_i$ at any of the concentrations used (Fig. 3.3a, b). Following addition of 1mM ATP, IL-8 evoked a concentration-dependent increase in $[Ca^{2+}]_i$ with a pEC_{50} of 9.7±0.1, and E_{max} 83±8% compared to the maximal response to 1mM ATP (Fig. 3.3a; n=3), whilst (±)-arterenol elevated $[Ca^{2+}]_i$ with a pEC_{50} of 6.8±0.1 and E_{max} of 67±3% (Fig. 3.3b; n=3). Addition of vehicle alone also revealed $[Ca^{2+}]_i$ responses to IL-8 and (±)-arterenol although the magnitude and potency of these were significantly less than the responses observed in the presence of 1mM ATP ($P<0.05$ for both pEC_{50} and E_{max} values) (Fig. 3.3a, b; n=3). Vehicle pre-treatment caused IL-8 to elevate $[Ca^{2+}]_i$ with a pEC_{50} of 8.4±0.4 and E_{max} of 34±3% whilst arterenol elevated $[Ca^{2+}]_i$ with a pEC_{50} of 6.2±0.1 and E_{max} of 11±1%.

Section 3.2.3: Effects of apyrase on buffer-induced potentiation in HEK-CXCR2 cells

Ca^{2+}-signalling downstream of $G\alpha_i$-coupled δ-opioid receptors is known to be sensitive to PTX, indicating that it is probably mediated by $G\beta\gamma$ subunits from these receptors. It has been shown previously that these responses in stirred suspensions of SH-SY5Y cells are ablated by apyrase (ATP diphosphohydrolase, which converts nucleotide polyphosphates to nucleoside monophosphates (Curdova *et al*, 1982)). This suggests that endogenous

[§] NB (±)-arterenol is used throughout this thesis to refer to noradrenaline

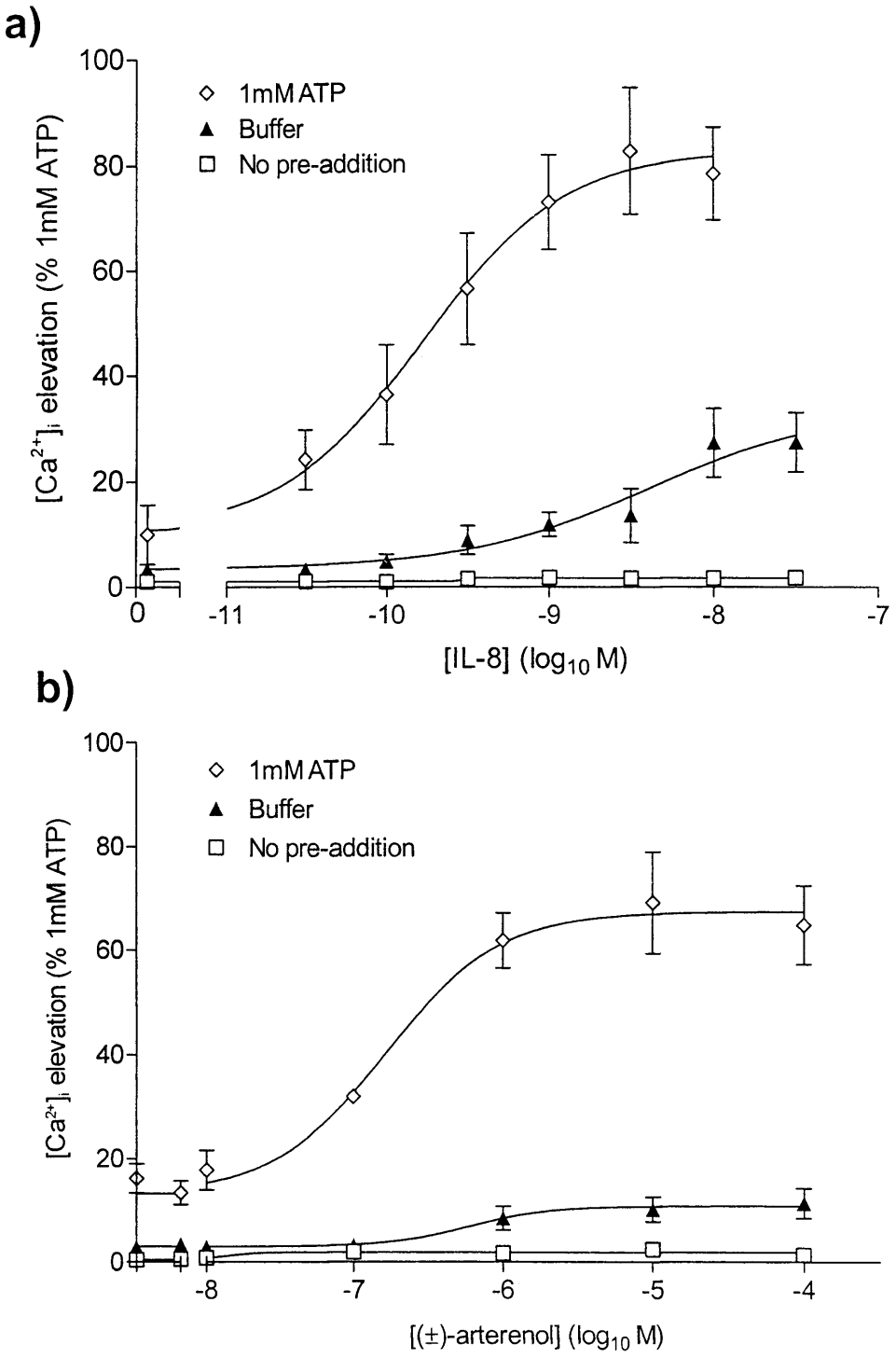


Figure 3.3. Potentiation by ATP of IL-8-mediated (a) and (±)-arterenol-mediated (b) $[Ca^{2+}]_i$ responses in HEK-CXCR2 cells. Using the FLIPR, cells were pre-stimulated at t=10s with either 1mM ATP, buffer control or were not pre-stimulated, and at t=150s, either IL-8 (a) or (±)-arterenol (b) was added. Changes in fluo-3 fluorescence were measured as an index of $[Ca^{2+}]_i$. Data are expressed as the percentage of the response to 1mM ATP and are the mean ± s.e.m. of three experiments, each performed in duplicate.

nucleotides released following cell damage due to stirring may act as a potentiating factor that is permissive to these opioid-induced responses (Yeo *et al*, 2001). The possibility was investigated that release of endogenous nucleotides from the cell monolayer following addition of buffer by the FLIPR was responsible for the effects of vehicle pre-treatment. In the absence of apyrase, the addition of vehicle revealed responses to IL-8 or (±)-arterenol that were 28.4±1.4% and 26.9±2.0% (n=3), respectively, of the response to 1mM ATP (Fig. 3.4). These responses were reduced to 10.4±0.2% and 5.5±0.6% (n=3), respectively, in the presence of 10U ml^{-1} apyrase (Fig. 3.4). Pre-addition of buffer did not reveal a response to a subsequent addition of buffer in either the presence or absence of apyrase (Fig. 3.4).

Section 3.2.4: Characterization of P2Y nucleotide receptor expression in HEK-CXCR2 cells

In order to further characterise the receptor type that revealed IL-8- and (±)-arterenol-mediated $[Ca^{2+}]_i$ responses, concentration-response curves were constructed for a number of P2Y receptor agonists. The rank order of potency was determined using the standard FLIPR protocol as: 2MeSADP>2MeSATP>ADP>ATP=UTP (Fig. 3.5 and Table 3.1). UDP (10nM – 1mM) did not elevate $[Ca^{2+}]_i$ (data not shown). These data are consistent with previous studies (Schachter *et al*, 1997b) and demonstrate the expression of P2Y1 and P2Y2 receptors in HEK cells. AR-C67085, a potent P2Y11 agonist (Communi *et al*, 1999), did not elevate $[Ca^{2+}]_i$ in the HEK-CXCR2 cell line confirming the absence of P2Y11 receptor expression.

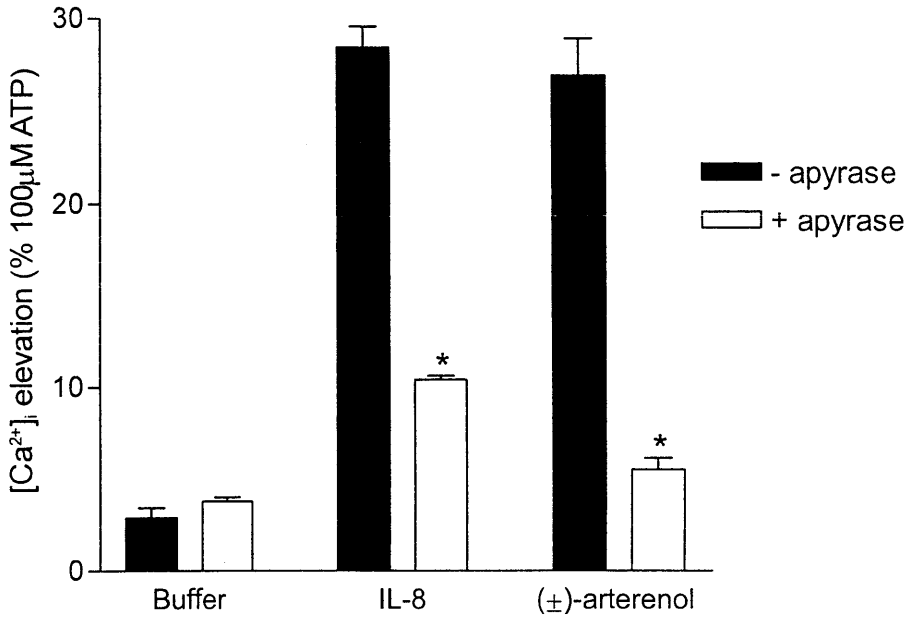


Figure 3.4. Effects of apyrase on buffer-induced potentiation of IL-8- and (±)-arterenol-mediated Ca^{2+} signalling. Using the FLIPR, cells were exposed to an addition of buffer (t=10s) followed (t=150s) by either an addition of buffer, 10nM IL-8 or 10μM (±)-arterenol. Apyrase (10U/ml, grade III) was either absent or present for ~5mins prior to the start of the experiment and for the entire duration of it. Fluo-3 fluorescence was recorded as an index of $[Ca^{2+}]_i$. The elevation of $[Ca^{2+}]_i$ in response to the second addition (t=150s) is expressed as a percentage of the response to a maximal concentration of ATP (100μM) in the absence of apyrase. Data are mean ± s.e.m., n=3. *: $P<0.001$, by Student's unpaired t-test *vs.* controls in the absence of apyrase.

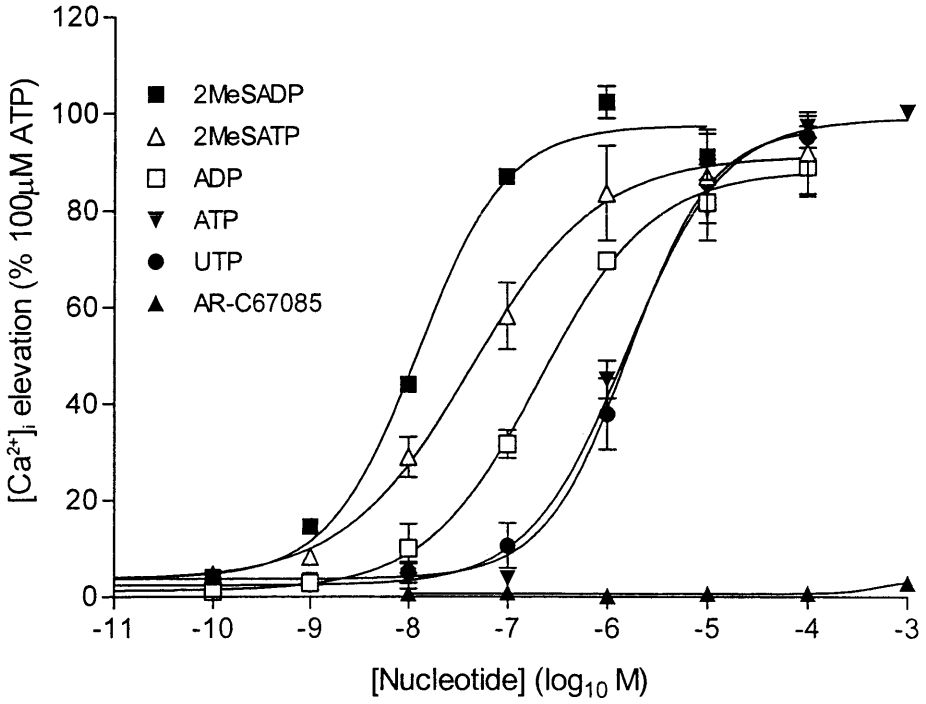


Figure 3.5. $[Ca^{2+}]_i$ elevation in HEK-CXCR2 cells challenged with P2Y nucleotide receptor agonists. Using the FLIPR, changes in fluo-3 fluorescence was recorded as an index of $[Ca^{2+}]_i$ following the addition of different P2Y nucleotide receptor agonists. The maximal change was determined in the 30s following agonist addition and expressed as a percentage of $[Ca^{2+}]_i$ response to 100μM ATP. Data are mean ± s.e.m, n≥3.

	2MeSADP	2MeSATP	ADP	ATP	UTP
pEC_{50}	7.9±0.1	7.4±0.1	6.7±0.1	5.9±0.1	5.8±0.1
Slope	1.0±0.2	0.7±0.1	0.8±0.1	1.0±0.2	1.0±0.1
E_{max} (%)	97±4	91±2	90±2	100	97±2

Table 3.1: The pEC_{50}, slope and E_{max} values for stimulation of $[Ca^{2+}]_i$ elevation by various P2Y receptor agonists. E_{max} values are expressed as a percentage of the maximal $[Ca^{2+}]_i$ elevation in response to 100μM ATP. Data are mean ± s.e.m.; $n \geq 3$.

Section 3.2.5: Failure of IL-8 to initiate reciprocal potentiation of UTP-induced responses

The use of ATP was considered to be relatively non-specific between P2Y1 and P2Y2 nucleotide receptors. The choice of UTP in some experiments was an attempt to increase the P2Y2 receptor selectivity of pre-stimulation, negating any of the partial agonist effects of ATP at P2Y1 receptors. Substitution of UTP in place of ATP is highly convenient given that both agonists are approximately equi-potent at the P2Y2 receptor, so identical concentrations could be used, and the maximal effects of each are practically identical (Fig. 3.5 and Table 3.1). The effects of IL-8 pre-stimulation on UTP-mediated responses were tested to assess whether the relationship between UTP and IL-8 was a reciprocal one in which total Ca^{2+} signalling was potentiated in the combined presence of the two agonists, regardless of the order of addition. Using a FLIPR assay, cells were stimulated firstly with either 10nM IL-8 or vehicle, and subsequently, 150s later, with 3μM UTP. (The use of 3μM UTP was to create a window in which potentiated Ca^{2+} signals could be observed. Pre-stimulation with 100μM UTP was likely to disguise any potentiation since this concentration of UTP is not on the linear part of the concentration-response curve and evokes a maximal response in the absence of any assistance, leaving little room for potentiation). Shown are responses to the

addition of 3μM UTP, expressed as an average of 20 time-matched observations (Fig. 3.6). It was found that the response to 3μM UTP was not significantly changed by pre-stimulation with IL-8.

Section 3.2.6: P2Y1 and P2Y2 receptor activation evoke different temporal patterns of $[Ca^{2+}]_i$ elevation and have differential effects on Ca^{2+} signalling by IL-8 and (±)-arterenol.

Using the FLIPR, the profiles of $[Ca^{2+}]_i$ elevation following addition of maximal concentrations of the P2Y receptor-selective agonists 2MeSADP (P2Y1 selective; 1μM) or UTP (P2Y2 selective; 100μM) (Nicholas *et al.*, 1996b) were studied and found to be markedly different. 2MeSADP caused a rapid, transient elevation of $[Ca^{2+}]_i$ followed by a rapid fall back to basal levels (Fig. 3.7; $t_{½}$: 17±1s after peak $[Ca^{2+}]_i$ elevation, n>10). In contrast, UTP caused a rapid, transient elevation of $[Ca^{2+}]_i$ followed by a much slower declining phase ($t_{½}$: 72±4s after peak $[Ca^{2+}]_i$ elevation, n>10) such that the $[Ca^{2+}]_i$ was still elevated above basal (26±6% of maximum, n>10) at the time at which the addition of either IL-8 or (±)-arterenol was made (i.e. 150s after UTP addition) (Fig. 3.7). A second addition of a maximal concentration of either 2MeSADP or UTP following an initial maximal addition of the same agonist did not cause a further elevation of $[Ca^{2+}]_i$ (Fig. 3.7).

To test the P2Y subtype selectivity of the potentiation, cells were exposed to a range of concentrations of either 2MeSADP (0.1nM-10μM) or UTP (1nM-1mM), before being stimulated with a sub-maximal concentration of IL-8 (1nM). The response to IL-8 was measured and expressed as a percentage of the response to 100μM UTP. This concentration of UTP is maximal for $[Ca^{2+}]_i$ elevation and gives a response equivalent to that of 1mM ATP (Fig. 3.5). Pre-stimulation with UTP increased the $[Ca^{2+}]_i$ response to 1nM IL-8 with a pEC_{50} for the potentiation of 5.0±0.1 (Fig. 3.8a). The E_{max} achieved by 1nM IL-8 following

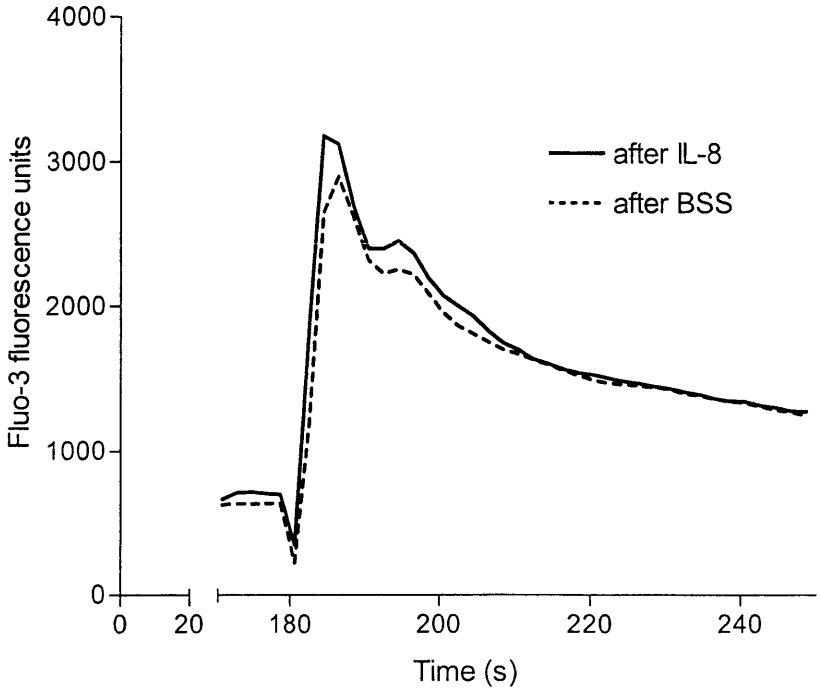


Figure 3.6. Potentiation is not a reciprocal process. IL8 does not potentiate UTP-mediated responses. Using the FLIPR, cells were stimulated initially with either 10nM IL-8 or vehicle control, followed, 150s later, by an addition of 3μM UTP. Increases in fluo-3 fluorescence in response to the addition of UTP were measured as an index of $[Ca^{2+}]_i$. Shown are the responses to 3μM UTP following pre-addition of vehicle (dashed line) or 10nM IL-8 (solid line). Traces shown are average of 20 time-matched observations.

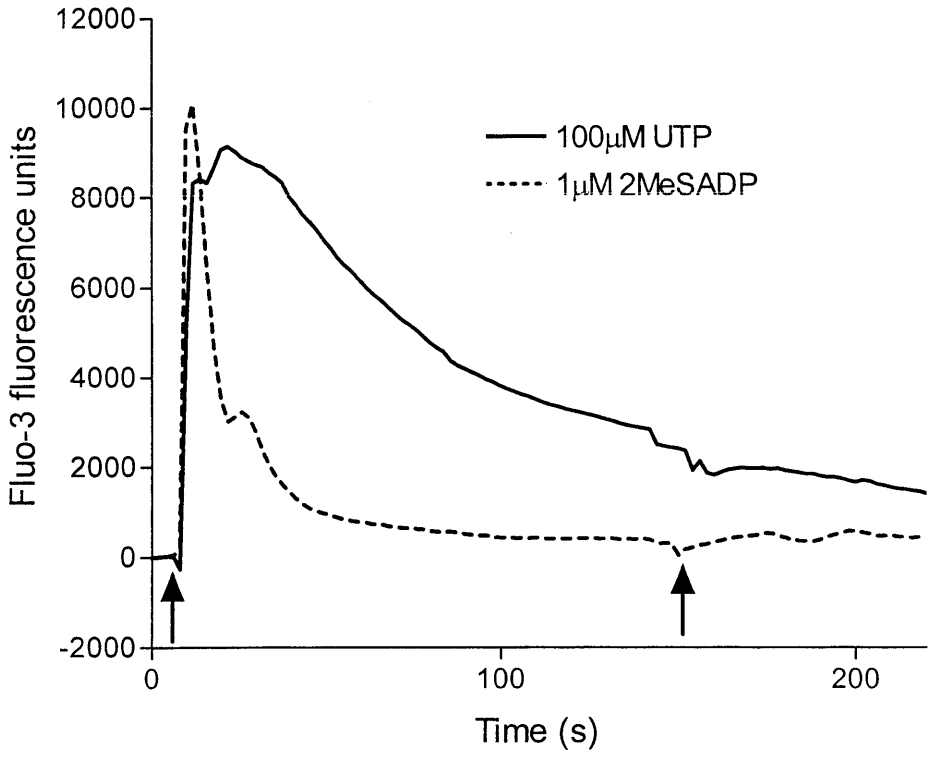


Figure 3.7. Comparison of $[Ca^{2+}]_i$ elevation profile for UTP and 2MeSADP. Cells were stimulated in the FLIPR at t=10s with either 100μM UTP or 1μM 2MeSADP and the change in fluo-3 fluorescence recorded as an index of $[Ca^{2+}]_i$. The cells were then re-stimulated with an identical concentration of the same agonist at t=150s, without prior washout. Each trace is the average of at least ten traces from three separate experiments.

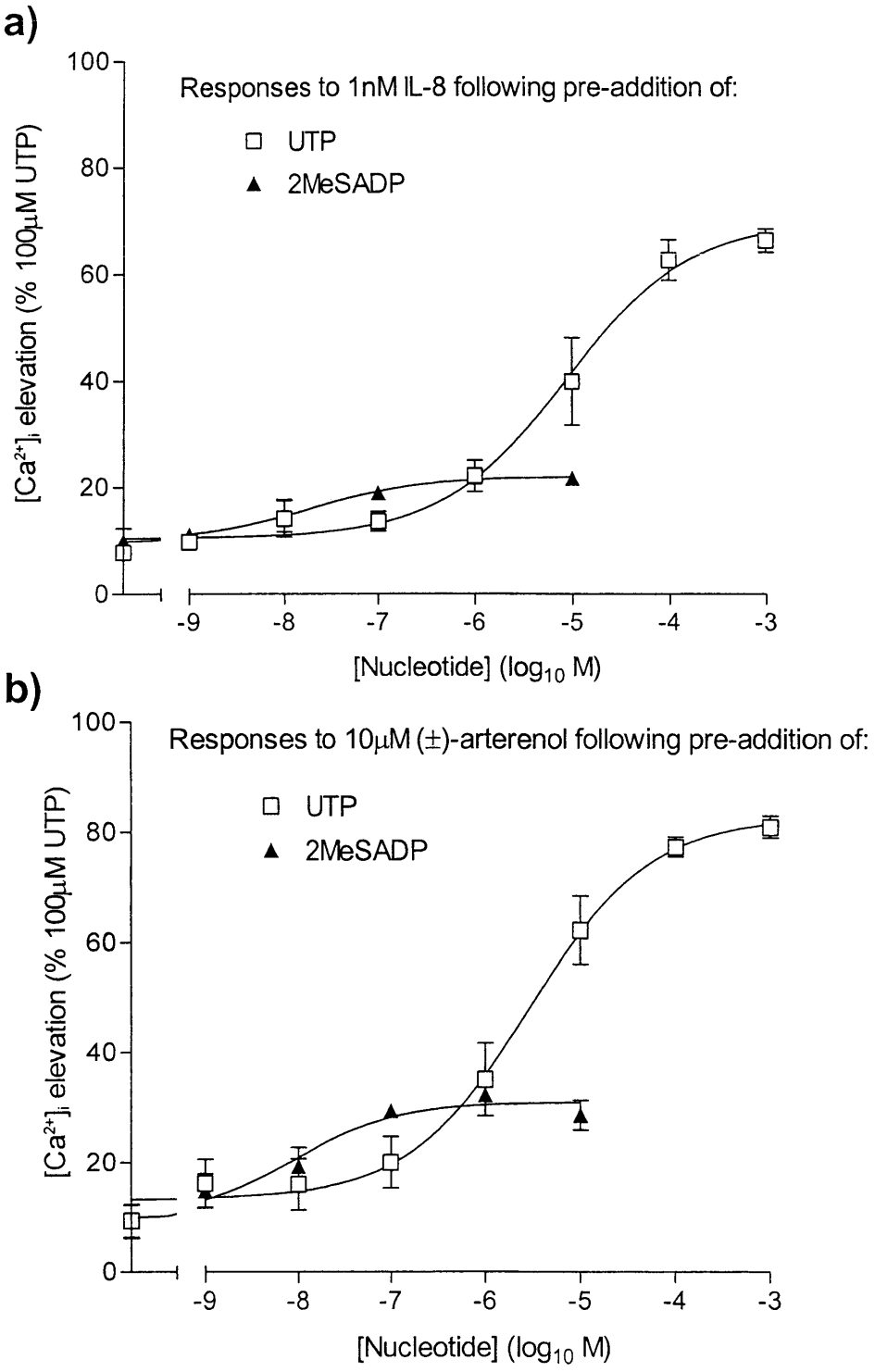
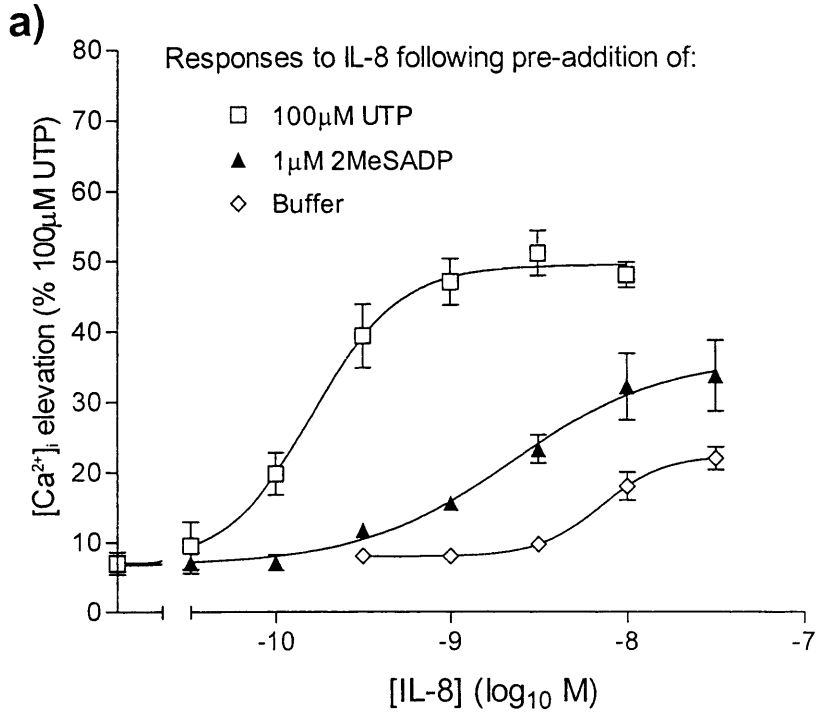


Figure 3.8. Ca^{2+} signalling following CXCR2 or β2-adrenoceptor activation is potentiated by both P2Y2 and P2Y1 receptor activation. Using the FLIPR, cells were exposed to 1nM IL-8 (a) or 10μM (±)-arterenol (b) at t=150s following pre-stimulation at t=10s with increasing concentrations of a subtype-selective P2Y receptor agonist (P2Y2-selective: UTP; P2Y1-selective: 2MeSADP). Changes in fluo-3 fluorescence were recorded as an index of $[Ca^{2+}]_i$ in response to the IL-8 or (±)-arterenol addition and expressed as a percentage of the maximal Ca^{2+} response to 1mM ATP. All data are mean ± s.e.mean, n=3.

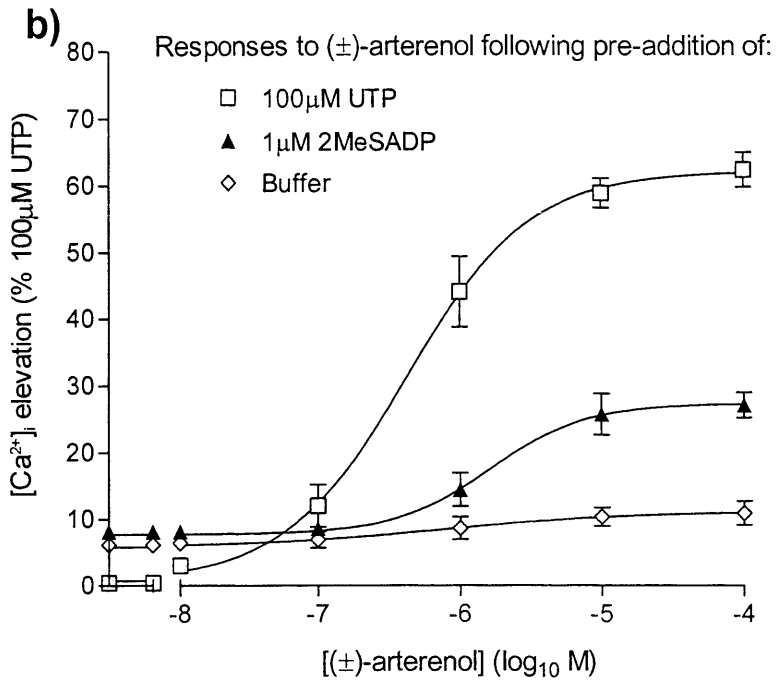
stimulation with a maximal concentration of UTP (100μM) was 73±3%. 2MeSADP also increased the response to 1nM IL-8 (pEC_{50} for the potentiation = 7.6±0.1, E_{max} = 22±1%, n=3) (Fig. 3.8a). Both pre-stimulating agonists caused statistically significant potentiation of the IL-8 response above that caused by buffer alone ($P<0.05$, by unpaired Student's t-test comparing E_{max} values for IL-8 responses in the presence of a maximal concentration of nucleotide or following buffer addition). However, there was a significant difference in the relative ability of these nucleotides to cause potentiation ($P<0.001$, by two-way ANOVA comparing potentiation curves for each nucleotide). This may be expected for the pEC_{50} values, as the concentration-response curve for Ca^{2+} release by 2MeSADP lies some way to the left of that for UTP, and the curve for potentiation may reasonably be expected to do the same. However, the maximal achievable response (E_{max}) to 10nM IL-8 following pre-stimulation with each agonist was also significantly different ($P<0.01$, by unpaired Student's t-test). Similarly, UTP and 2MeSADP both significantly ($P<0.001$, unpaired Student's t-test) potentiated the Ca^{2+} response to 10μM arterenol (pEC_{50} values for the potentiation = 5.5±0.1 and 7.8±0.1 (n=3), respectively; E_{max} 82±1% and 30±1% (n=3), respectively) (Fig. 3.8b). However, there again existed a statistically significant difference between the two agonists in terms of mediating potentiation relative to one another ($P<0.001$, one-way ANOVA; $P<0.01$, Student's unpaired t-test on E_{max} values).

To further demonstrate the relative abilities of P2Y1 and P2Y2 receptors to potentiate $[Ca^{2+}]_i$ signalling by IL-8 and (±)-arterenol, concentrations of either 2MeSADP (1μM) or UTP (100μM) were used for the pre-stimulation that were maximal and equi-effective in terms of the spike $[Ca^{2+}]_i$ response (Fig. 3.6). Both the potency and E_{max} of IL-8- and (±)-arterenol-mediated $[Ca^{2+}]_i$ elevations were significantly greater following pre-stimulation with 100μM UTP compared to pre-stimulation with 1μM 2MeSADP (Figs. 3.9a, b. See tables accompanying Fig. 3.9 for values).



	IL-8		
	2MeSADP	UTP	*P* value (2MeSADP vs. UTP)
E_{max} (%)	36±2	50±1	<0.01
pEC_{50}	8.7±0.2	9.8±0.1	<0.01

Figure 3.9. (a) Potentiation of IL-8-mediated responses by concentrations of P2Y receptor subtype-selective ligands that elicit equivalent maximal increases in $[Ca^{2+}]_i$. Cells were pre-stimulated with concentrations of UTP or 2MeSADP that gave equivalent maximal elevation of $[Ca^{2+}]_i$ (100μM and 1μM, respectively), or with vehicle. Cells were subsequently stimulated with increasing concentrations of IL-8. Changes in $[Ca^{2+}]_i$ in response to IL-8 were measured by increases in fluo-3 fluorescence and are presented as a percentage of the maximal Ca^{2+} response to 100μM UTP. Data are mean ± s.e.m, n=3.



	(±)-arterenol		
	2MeSADP	UTP	*P* value (2MeSADP vs. UTP)
E_{max} (%)	27±1	62±1	<0.001
pEC_{50}	5.8±0.2	6.4±0.2	<0.05

Figure 3.9b Potentiation of (±)-arterenol-mediated responses by concentrations of P2Y receptor subtype-selective ligands that elicit equivalent maximal increases in $[Ca^{2+}]_i$. Identical to Fig. 1.9a, except using (±)-arterenol in place of IL-8.

A cuvette-based spectrofluorimeter assay was used to examine the temporal characteristics of the potentiation and found that the elevation of $[Ca^{2+}]_i$ by IL-8 was potentiated irrespective of the time of addition of IL-8 between 50s and 270s (the longest time point used) after the addition of 100μM UTP (Fig. 3.10). However, subsequent studies on more extended periods of UTP pre-stimulation (up to 15mins) showed that the potentiation does decrease to some extent, and that this is probably due to P2Y2 receptor desensitization (see Chapter 5).

Section 3.2.7: Potentiation requires continued presence of nucleotide

In order to assess the requirement for continued presence of the pre-stimulating agonist, a confocal microscope linked to a perfusion system was used instead of the FLIPR to analyse changes in $[Ca^{2+}]_i$. This provided the useful capability to wash out both exogenously added and endogenously released ligands. In the absence of a P2Y nucleotide receptor agonist, neither IL-8 (10nM) nor (±)-arterenol (10μM) elevated $[Ca^{2+}]_i$ (Fig. 3.2). In the presence of UTP (100μM), IL-8 (10nM) stimulated an $[Ca^{2+}]_i$ elevation (Fig. 3.11). However, if UTP was washed out for 90s before the addition of IL-8, no $[Ca^{2+}]_i$ elevation was seen in response to IL-8 (Fig. 3.11). Qualitatively identical results were seen when (±)-arterenol rather than IL-8 was used.

Section 3.2.8: Sensitivity of Ca^{2+} signalling to pertussis and cholera toxins

Treatment of cells for 20 h with 100ng/ml PTX had no effect on $[Ca^{2+}]_i$ responses to 100μM UTP. However, it abolished $[Ca^{2+}]_i$ responses to 10nM IL-8 following UTP pre-stimulation (47±4% in the absence of PTX, 1±0.5% in the presence of PTX; Fig. 3.12a). In contrast, PTX had no effect on the response to 10μM (±)-arterenol in the presence of 100μM UTP (Fig. 3.12a). Treatment of cells for 20h with 2μg/ml CTX (which ADP-ribosylates and, with extended exposure, down-regulates $G\alpha_s$-mediated responses (Seidel *et al*, 1999)) had no

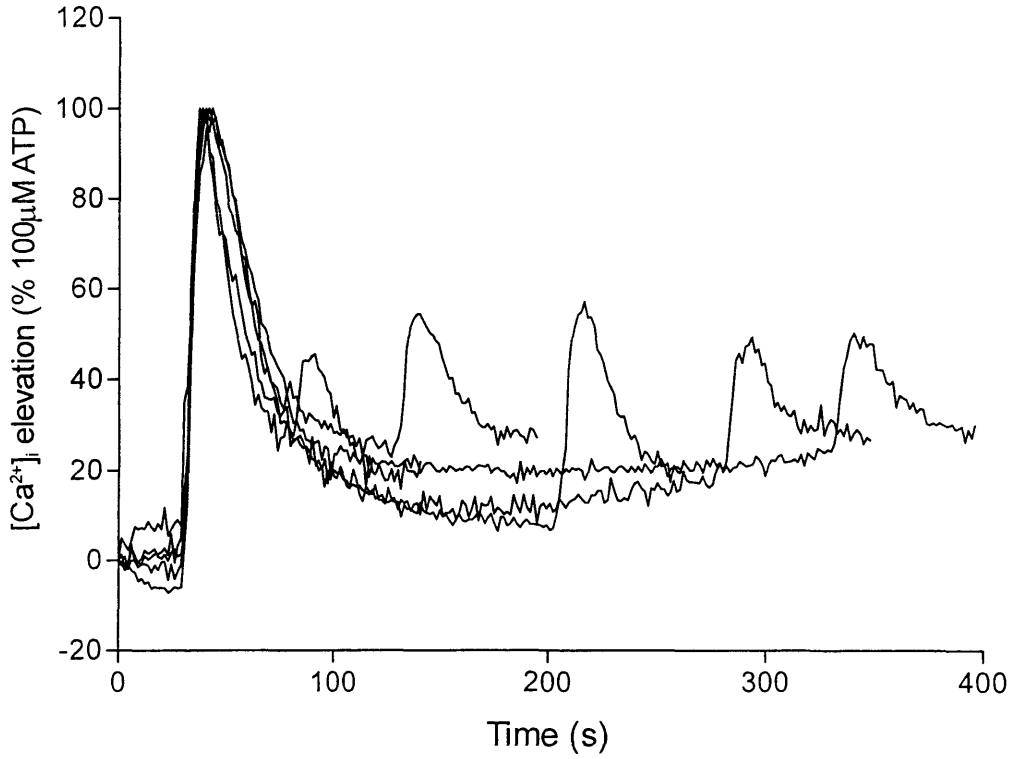


Figure 3.10. The Ca^{2+} response to stimulation of CXCR2 is potentiated by P2Y2 receptor activation independently of the time of addition of IL-8 following UTP. Fura-2-loaded HEK-CXCR2 cells were pre-stimulated at 25s with 100μM UTP followed, after varying time intervals, by 10nM IL-8. Using a cuvette-based fluorimeter, cells were excited alternately at 340 and 380nm, with emission collected at 510nm as an index of $[Ca^{2+}]_i$. Graph shows a representative trace for each pre-stimulation time period (n=3) showing changes in $[Ca^{2+}]_i$ expressed as the 340/380 ratio. In each case the $[Ca^{2+}]_i$ response to IL-8 occurred within 10s of its addition.

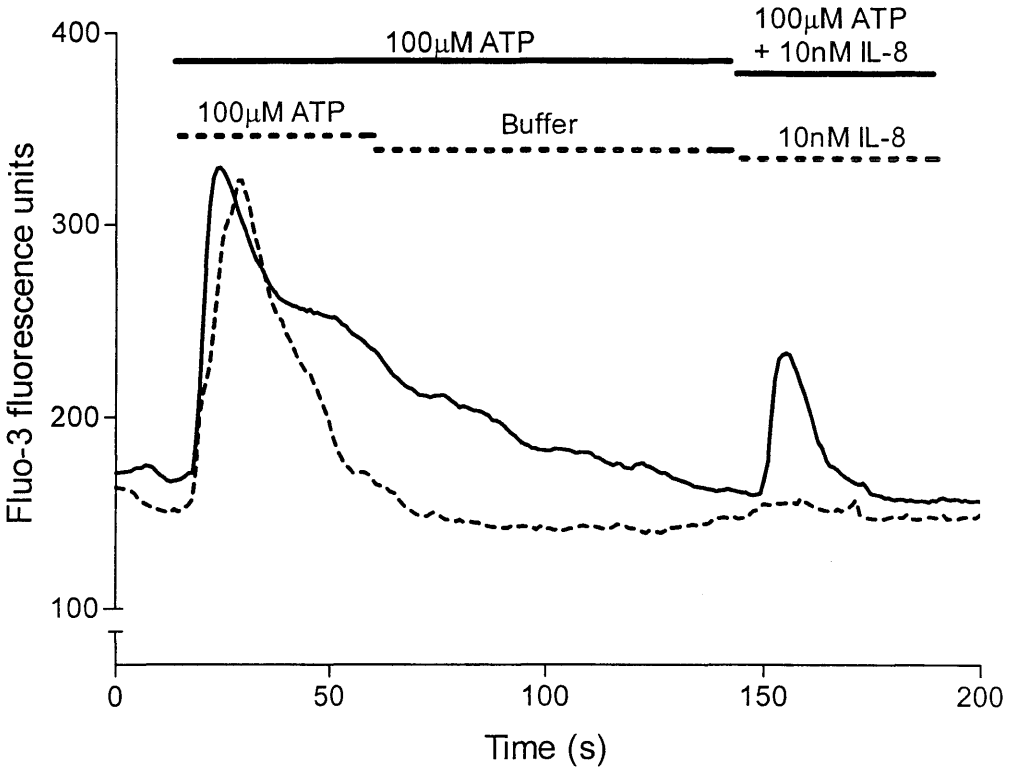


Figure 3.11. The potentiation of CXCR2-mediated Ca^{2+} signalling requires the continued activation of nucleotide receptors. Changes in $[Ca^{2+}]_i$ were determined in fluo-3-loaded HEK-CXCR2 cells on 22mm coverslips by confocal microscopy. The solid line represents an initial stimulation with 100μM UTP (10s) followed by a second stimulation (in the continued presence of UTP) with 10nM IL-8 (150s). The dashed line represents the same protocol except that UTP was washed out between additions by perfusing with buffer in the 90s prior to the second addition (represented by solid bar). Data shown are representative of at least 3 experiments.

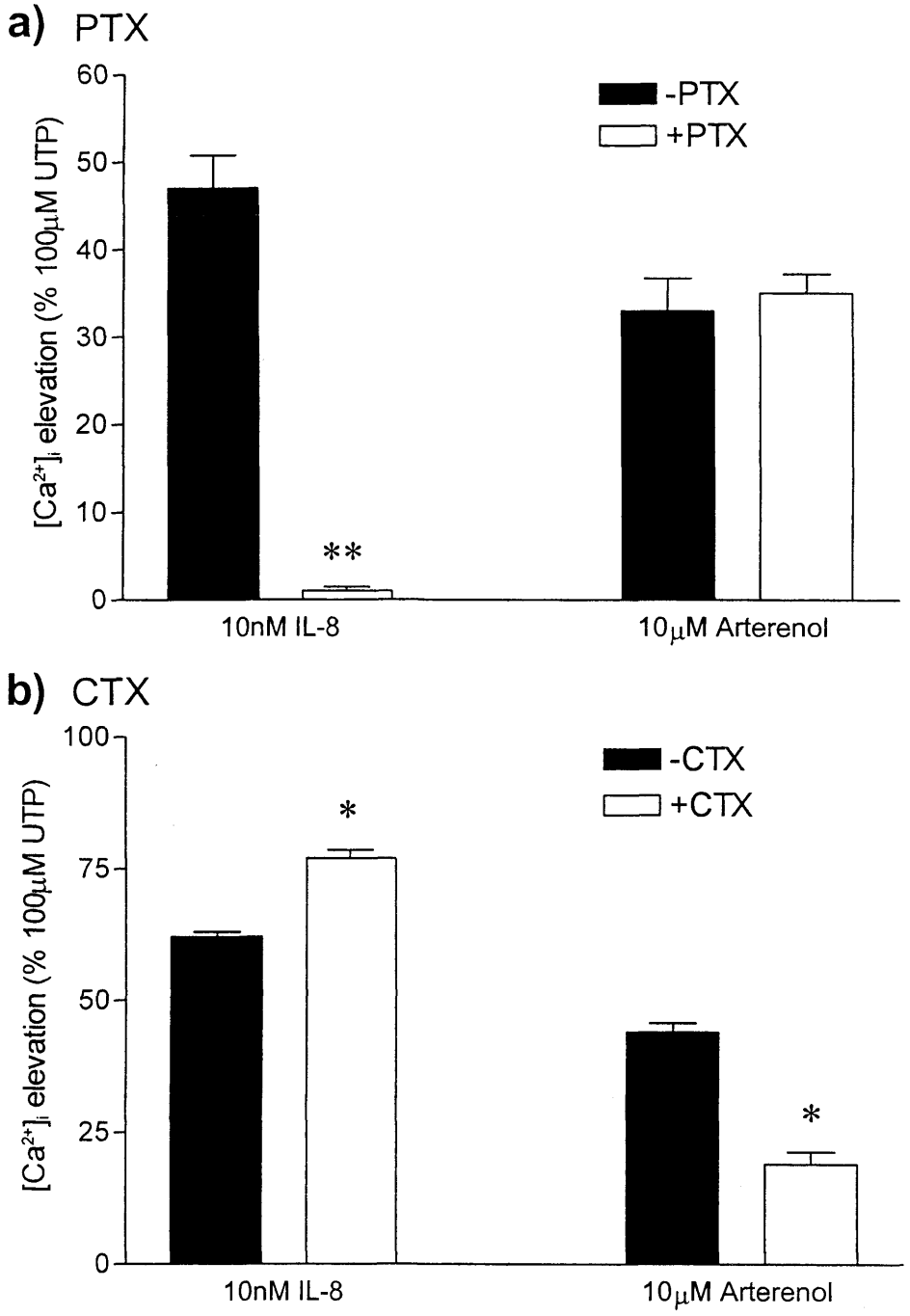


Figure 3.12. Effects of PTX and CTX on the potentiation of CXCR2- and adrenoceptor-mediated Ca^{2+} signalling by UTP. a) HEK-CXCR2 cells were cultured with or without 100ng/ml PTX for 20h prior to FLIPR assay. The data represent $[Ca^{2+}]_i$ responses to either 10nM IL-8 or 10μM (±)-arterenol following pre-stimulation with 100μM UTP. b) Identical experiment to that shown in (a) with the exception that the cells were cultured with or without 2μg/ml CTX for 20h prior to FLIPR assay. Results are mean ± s.e.m, n=3. *: $P<0.01$; **, $P<0.001$, by Student's unpaired t-test *vs.* controls in the absence of toxin.

effect on $[Ca^{2+}]_i$ responses to 100μM UTP. However, it reduced the $[Ca^{2+}]_i$ response to 10μM (±)-arterenol in the presence of 100μM UTP from 44±2% to 19±2% (Fig. 3.12b). CTX pre-treatment caused a small but significant increase in the response to 10nM IL-8 in the presence of 100μM UTP (15±1.0%; $p<0.05$).

Section 3.2.9: Lack of dependence of the potentiation effect and IL-8-mediated Ca^{2+} signalling on extracellular Ca^{2+}

To determine if Ca^{2+} flux across the plasma membrane was required for the ability of P2Y receptor activation to potentiate IL-8- or (±)-arterenol-mediated $[Ca^{2+}]_i$ signalling, FLIPR experiments were performed in BSS without added Ca^{2+}. Under these conditions, the magnitude of the spike $[Ca^{2+}]_i$ responses to either UTP or 2MeSADP were unaffected (compare Figs. 3.13a and 3.13b). However, the $[Ca^{2+}]_i$ returned quickly to basal levels following stimulation with either nucleotide (compare Figs. 3.13a and 3.13b). In the absence of extracellular Ca^{2+}, the addition of IL-8 in the presence of either UTP or 2MeSADP still provoked an elevation of $[Ca^{2+}]_i$ that was not significantly decreased compared to that in the presence of added extracellular Ca^{2+} (Fig. 3.14a). Similarly the absence of extracellular Ca^{2+} had no effect on the ability of UTP to potentiate the $[Ca^{2+}]_i$ response to (±)-arterenol (Fig. 3.14b).

Section 3.2.10: Drainage of thapsigargin-sensitive Ca^{2+} stores abolishes the potentiation

To begin to understand where the 'extra' Ca^{2+} released by IL-8 following pre-stimulation originates from, cells were treated for 5mins with the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitor, thapsigargin (2μM), before stimulating with a single addition of either 100μM UTP, 10nM IL-8, or a co-addition of 100μM UTP and 10nM IL-8 (Fig. 3.15). Responses were measured by detection of changes in fluo-3 fluorescence by a Ca^{2+} imaging set-up. Since IL-8 elicited no appreciable Ca^{2+} response, thapsigargin had no

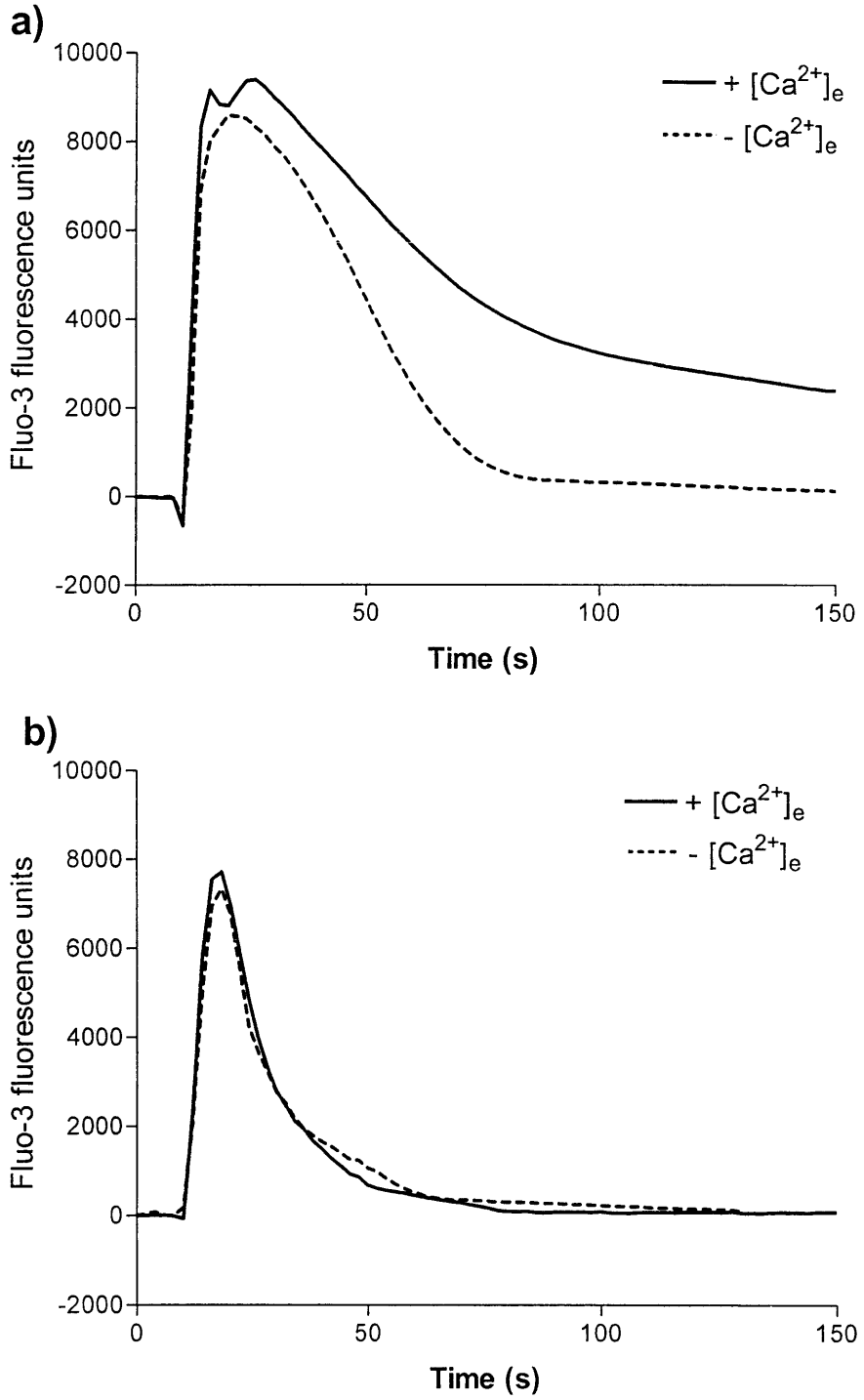


Figure 3.13. Effects of removal of extracellular Ca^{2+} on intracellular Ca^{2+} signalling by a) P2Y2 and b) P2Y1 receptors. Following loading with fluo-3, cells were washed with BSS containing no added Ca^{2+} and then transferred to the FLIPR with each well of the plate containing 100µl of BSS or BSS with no added Ca^{2+}. Following collection of basal fluorescence values for 10s, cells were stimulated by the addition of either UTP (a) or 2MeSADP (b) in either BSS or in BSS with no added Ca^{2+}. Profiles are the average of twenty or more similar traces over three different experiments.

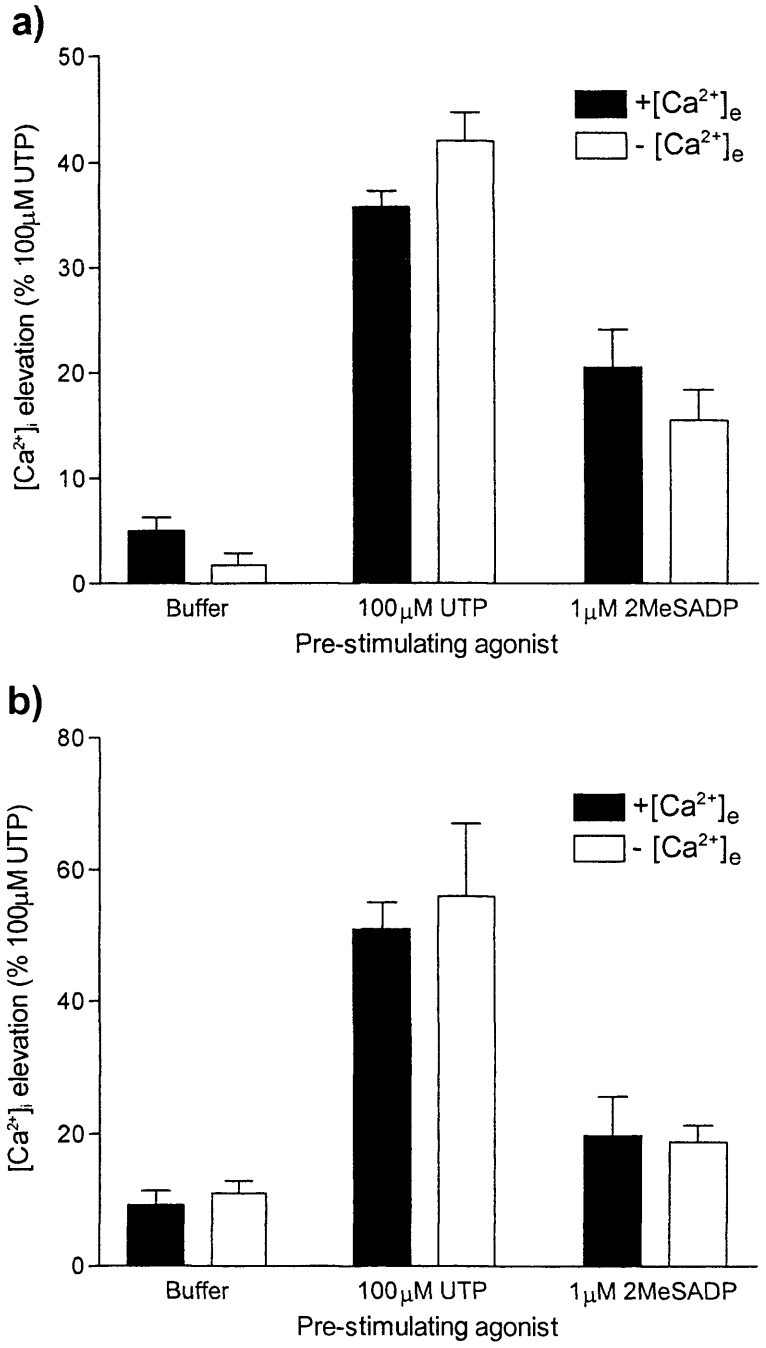


Figure 3.14. Effects of removal of extracellular Ca^{2+} on the nucleotide receptor-mediated potentiation of signalling by CXCR2 (a) and β-adrenoceptors (b). To determine the effect of extracellular Ca^{2+} on the potentiation response, cells were pre-treated in a FLIPR assay with either 100μM UTP, 1μM 2MeSADP or buffer at t=10s, followed at t=150s by 10nM IL-8 (a) or 10μM (±)-arterenol (b) in the presence or absence of extracellular Ca^{2+}. The data show the maximal response to the second addition (IL-8 or (±)-arterenol) as a percentage of the maximal response to 100μM UTP. Data are mean ± s.e.m., n=3.

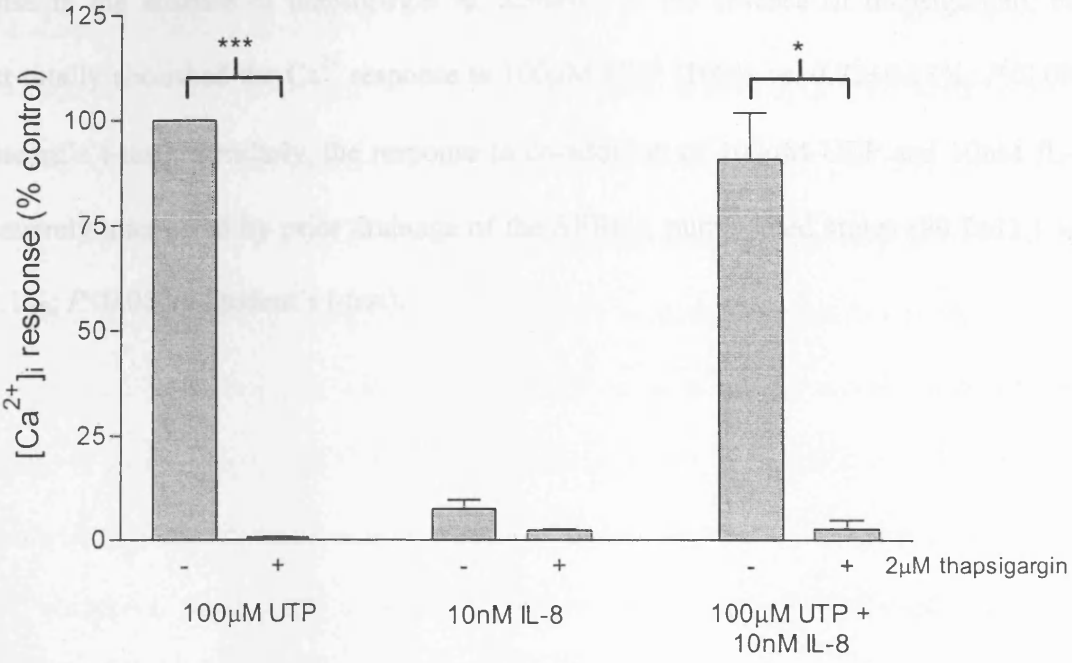


Figure 3.15. Ca^{2+} for both the 'UTP' and 'UTP+IL-8' co-addition responses comes from a thapsigargin-sensitive store. HEK-CXCR2 cells were grown on 22mm coverslips and loaded (for 1hr prior to assay) with fluo-3/AM. Using a single cell imaging system, changes in fluo-3 fluorescence were monitored as an index of changes in $[Ca^{2+}]_i$. Cells were initially treated either with or without a 5min perfusion of 2μM thapsigargin, then stimulated with 100μM UTP or 10nM IL-8 alone, or with both agonists in combination. Data are mean±s.e.m., n=4. *: $P<0.05$; ***; $P<0.001$ by Student's unpaired t-test.

significant inhibitory effect on IL-8-mediated signalling (7.53±2.2% of maximal UTP response in the absence of thapsigargin *vs.* 2.36±0.3 in the absence of thapsigargin), but almost totally abolished the Ca^{2+} response to 100μM UTP (100% *vs.* 0.72±0.18%; $P<0.001$ by Student's t-test). Similarly, the response to co-addition of 100μM UTP and 10nM IL-8 was severely attenuated by prior drainage of the SERCA pump-filled stores (90.7±11.1 *vs.* 2.7±2.1%; $P<0.05$ by Student's t-test).

Section 3.3: Discussion

Section 3.3.1: Summary of data

This chapter provides evidence that P2Y nucleotide receptors and CXCR2 do indeed interact at the level of $[Ca^{2+}]_i$, and quantifies this interaction. It shows that potentiation of Ca^{2+} signalling is seen when cells are stimulated with CXCR2 agonists (e.g. IL-8 and GROα) in the presence of a ligand activating a $G\alpha_q$-coupled P2Y receptor, but that this phenomenon is not reciprocal - pre-incubation with IL-8 does not cause a similar enhancement of P2Y signalling. It also details the P2Y receptor expression profile in the HEK-CXCR2 cell line, and demonstrates that the ability to induce potentiation is not a general familial characteristic of P2Y receptors, since P2Y1 and P2Y2 receptors have markedly different abilities to enhance CXCR2-mediated Ca^{2+} signalling. Also illustrated here is the dependence of the phenomenon on various conditions: the continued activation of P2Y receptors, the activation of Gi-proteins (CXCR2) and Gs-proteins (β2 adrenoceptor), and the integrity of thapsigargin-sensitive intracellular Ca^{2+} stores. In contrast, the observed synergy is not dependent on the presence of extracellular Ca^{2+} or, up to five minutes following pre-stimulation, on the time duration between pre-stimulation with P2Y agonist and the time of addition of secondary agonist (CXCR2- or β2 adrenoceptor ligand).

Section 3.3.2: Differential effects of P2Y receptors on potentiation

Although this HEK cell line expresses both endogenous P2Y1 and P2Y2 nucleotide receptors, it is shown here that potentiation of CXCR2 and β-adrenoceptor[§] Ca^{2+} signalling is mediated predominantly by P2Y2 receptors. The differential effect of P2Y1 and P2Y2 receptors is unrelated to the magnitude of the initial $[Ca^{2+}]_i$ elevation as a concentration of

[§] It should be noted that although (±)-arterenol is not a β-subtype-selective adrenoceptor agonist, isoproterenol (which does specifically activate β adrenoceptors) can be used to the same effect, consistent with the expression of β adrenoceptors in HEK cells (Premont *et al*, 1992). Isoproterenol was substituted into all experiments studying the adrenoceptor-mediated effects from this point on to eliminate any unexpected contributions from unrecognised adrenoceptor subtypes.

UTP that gives an equivalent spike response to 2MeSADP causes greater potentiation of both the potency and E_{max} of the subsequent response to IL-8 or (±)-arterenol. A possible explanation of this differential effect is that P2Y1 and P2Y2 receptors desensitize to different extents over the time-frame of these experiments. Thus, the relatively rapid return of $[Ca^{2+}]_i$ to basal levels following activation of P2Y1 receptors with 2MeSADP indicates that, at least at this level of signalling, this receptor fully desensitizes. In contrast, P2Y2 receptor activation results in a sustained $[Ca^{2+}]_i$ elevation. Although differences in the cellular handling of Ca^{2+} during P2Y1 and P2Y2 receptor stimulation could account for the different profiles of $[Ca^{2+}]_i$ elevation (for example, differential coupling to the plasma membrane Ca^{2+}-ATPase), these data suggest that P2Y1 receptors may desensitize more fully than P2Y2 receptors over the time-frame of our experiments. Further study of the profiles of P2Y receptor desensitization is covered in Chapter 5. The consequence of these two different profiles of receptor desensitization would be that at the point of IL-8 or (±)-arterenol addition, the P2Y1 receptor would be fully desensitized, whilst signalling *via* the P2Y2 receptor would be maintained. This need for sustained receptor signalling is in agreement with the finding that washout of UTP results in a loss of potentiation of IL-8-mediated Ca^{2+} signalling. Furthermore, removal of extracellular Ca^{2+} does not affect the ability of P2Y receptor activation to potentiate Ca^{2+} signalling by CXCR2 or β-adrenoceptors. This, together with the evidence that thapsigargin completely abolishes the revealed Ca^{2+} response to IL-8, indicates both that influx of extracellular Ca^{2+} is not required to mediate the potentiation effect, and that activation of CXCR2 or β-adrenoceptors, in the presence of P2Y receptor stimulation, can cause mobilization of Ca^{2+} from an intracellular store. This store is likely to be the endoplasmic reticulum, although the Ca^{2+} could be derived from elsewhere and released from the endoplasmic reticulum following a GPCR-mediated transfer from this alternate source. The identity of this source is unknown, and this issue will be addressed in Chapter 4.

The potentiating effect of P2Y receptor activation on CXCR2-mediated Ca^{2+} signalling is not a consequence of the over-expression of a recombinant receptor as the level of CXCR2 expression (approximately 50,000 sites per cell) is similar to that in neutrophils (approximately 50-90,000 sites per cell; Lee *et al.*, 1992). Furthermore, signalling by endogenously expressed β-adrenoceptors is also potentiated. The Ca^{2+} signalling evoked by (±)-arterenol in the presence of P2Y receptor activation is inhibited by CTX but not PTX whereas CXCR2 signalling is inhibited by PTX and not CTX. Thus, (±)-arterenol signalling appears to be through a $G\alpha_s$-coupled β-adrenoceptor. This is consistent with evidence demonstrating the expression of these but not other adrenoceptor sub-types in HEK cells (Premont *et al.*, 1992). Further, our data are consistent with other reports (Hall *et al.*, 1999; Damaj *et al.*, 1996a) that CXCR2 is coupled *via* $G\alpha_i$. Activation of $G\alpha_{q/11}$-coupled receptors is, therefore, able to potentiate Ca^{2+} signalling through both $G\alpha_i$- and $G\alpha_s$-coupled receptors.

Although UTP and 2MeSADP produce similar increases in the initial peak Ca^{2+} elevation, and their relative potencies for this response are similar to those for their ability to facilitate CXCR2- and β-adrenoceptor-mediated Ca^{2+} signalling, the mechanism of potentiation appears to be independent of the size of the initial $[Ca^{2+}]_i$ 'spike'. Concentrations of the two nucleotide agonists that elevate $[Ca^{2+}]_i$ to equivalent levels are markedly different in terms of their ability to potentiate CXCR2 signalling. In addition, removal of Ca^{2+} from the extracellular milieu, which prevents sustained P2Y2 receptor-mediated Ca^{2+} signalling, has no effect on the ability of P2Y2 receptors to mediate potentiation of Ca^{2+} signalling by CXCR2. This suggests that neither the initial peak Ca^{2+} elevation nor the sustained plateau of Ca^{2+} elevation is required (or at least are not sufficient) for this potentiation of CXCR2 signalling. In support of this, Yeo *et al* (2001) show that a similar type of crosstalk occurring between δ-opioid receptors and muscarinic M3 receptors in SH-SY5Y cells cannot be mimicked by a lysophosphatidic acid-induced elevation of $[Ca^{2+}]_i$ *via* sphingosine kinase,

indicating that potentiation cannot be brought about simply by evoking an intracellular Ca^{2+} response.

Section 3.3.3: Non-reciprocal nature of potentiation

It is slightly surprising that the potentiation of Ca^{2+} signalling is not reciprocal i.e. that pre-stimulation with IL-8 does not cause any significant increase in the magnitude of UTP signalling. Although a full concentration-response relationship was not investigated, the use of an EC_{50} concentration of UTP would be the optimal concentration at which to see changes. This may represent a desensitization issue – CXCR2 has been shown to desensitize and internalize rapidly (~95% in 3-5mins (Richardson *et al*, 1998)), suggesting that at 150s, a significant proportion of CXCR2 receptors may be inactive. It is interesting to note, however, that when UTP and IL-8 are co-applied, without either having had any period of privileged access (i.e. no receptor can be desensitized), that there is no change in the size of the peak Ca^{2+} response compared to UTP alone (Fig. 3.15). It is possible that following co-addition, the 'extra' response seen due to crosstalk is absorbed in the existing robust response to UTP, and does not translate to an increase in peak $[Ca^{2+}]_i$ elevation, rather to a less tangible increase in the total amount of Ca^{2+} released.

Despite this non-reciprocation, the protocol for cell stimulation after thapsigargin drainage (no pre-stimulation, simply a co-addition of both agonists; Fig. 3.15) is still valid in identifying the source from which the potentiated response derives its Ca^{2+}. Given that UTP-stimulated $[Ca^{2+}]_i$ elevation is essentially 100% sensitive to thapsigargin, anything less than 100% inhibition of the response to UTP/IL-8 co-addition would represent the derivation of Ca^{2+} from some other source in the combined presence of these ligands.

Section 3.3.4: The "buffer effect"

An addition of vehicle prior to CXCR2 stimulation, in the absence of any washout (i.e. in the FLIPR assay protocol) causes an apparent potentiation of responses to IL-8, and the fact that this is inhibited by the inclusion of apyrase in the assay medium suggests that a nucleotide polyphosphate is involved. It seems reasonable to conclude that fluid movement over the cell monolayer causes ATP release, and that it is this ATP that causes the observed potentiation of IL-8 signalling. This raises two concerns. Firstly, given that the potentiation induced by the released ATP is equivalent to that induced by exogenous ATP in the micromolar range, this suggests that large quantities of ATP are being released, which may cause significant over-estimation of the effects of exogenously added nucleotides. Secondly, this type of crosstalk, when not accounted for, may have resulted in the over-estimation of the Ca^{2+} signalling ability of a potentially large number of agonists by investigators using systems where ATP release is high (e.g. stirred cell suspensions).

Section 3.3.5: Potential mechanisms

The mechanism by which nucleotide receptors allow the activation of CXCR2 or β-adrenoceptors to elevate $[Ca^{2+}]_i$ is unclear. There is a multitude of mechanisms by which GPCRs could interact to enhance $[Ca^{2+}]_i$ elevation, although some of these seem highly unlikely given the data contained in this chapter. For instance, it would seem that any interaction that positively influences the flow of extracellular Ca^{2+} through plasma membrane Ca^{2+} channels is irrelevant given that this phenomenon is independent of the presence of extracellular Ca^{2+} and appears to be entirely dependent on intracellular stores. Furthermore, given the requirement for continued P2Y receptor activation, it seems improbable that these receptors cause irreversible intracellular changes such as protein cleavage. It has been suggested that intense Ca^{2+} release in response to $G\alpha_q$-coupled receptors may be sufficient to activate the proteolytic enzyme, calpain (Banno *et al*, 1995).

This enzyme may, in certain circumstances (such as PKC phosphorylation of phospholipase C), cleave the C-terminal of phospholipase C, creating a truncated fragment that is hypersensitive to stimulation by Gβγ subunits (Pontremoli *et al*, 1987; Banno *et al* 1994). Such truncated fragments have been shown to exist in physiological circumstances (Blank *et al*, 1993). However, such radical changes would be expected to persist for some time after removal of the primary agonist, suggesting that this theory is not relevant here. Furthermore, the theory that increased receptor re-sensitisation is responsible is also unlikely to be applicable here. Re-activation of desensitized receptors would allow a second round of signalling in response to the primary agonist (as has been suggested for bradykinin B2 receptors with angiotensin-converting enzyme inhibitors (Minshall *et al*, 1997; Marcic *et al*, 1999)). Although this B2 receptor-ACE crosstalk is now proposed to involve prevention of desensitisation rather than receptor re-sensitisation (Tom *et al*, 2002), the theory underlying this mechanism still holds. This represents a neat way of stimulating a secondary response, and would explain the requirement for continued presence of primary agonist, but given that P2Y1 receptors appear to desensitize more fully and more rapidly than P2Y2, it would be expected that this type of mechanism would more noticeably affect these P2Y1 receptors. That this is not the case suggests that this mechanism is perhaps unlikely to play a part in the crosstalk between P2Y2 receptors and CXCR2. However, this mechanism is not immediately ruled out here since a) P2Y1 and P2Y2 receptors may be inducing potentiation by different mechanisms and cannot therefore be compared, and b) differences in the dynamics of P2Y1 receptor re-sensitisation may prevent it from being as strongly re-activated as the P2Y2 receptor. Further study of the potential involvement of phosphatases in this crosstalk is covered in Chapter 5.

In addition to the model describing nucleotide receptor re-sensitisation, there are a number of other mechanisms that cannot be dismissed based on the data contained in this chapter. It

was therefore considered necessary to assess the relative effects of inhibitors of a variety of intracellular factors such as kinases, phosphatases, catalytic enzymes and receptor channels in an attempt to determine some of the components required for this crosstalk in order to select the most plausible model of functional receptor interaction.

Chapter 4 – Initial exploration of the mechanism underlying the GPCR crosstalk that causes enhanced intracellular Ca^{2+} signalling

Section 4.1: Introduction

The previous chapter characterised the interactions between two pairs of GPCRs, finding that receptors that couple preferentially to different signalling pathways can communicate to positively influence Ca^{2+} signalling downstream of these receptors. Thus, P2Y2 nucleotide receptors interact with CXCR2 chemokine receptors to cause potentiated $[Ca^{2+}]_i$ elevations, and while P2Y1 receptors are involved in an analogous function, this was to a markedly lesser degree. Similarly, potentiated Ca^{2+} signalling was observed following concurrent stimulation of P2Y receptors and β2-adrenoceptors.

Pre-stimulation of cells with UTP facilitates Ca^{2+} release in response to a subsequent addition of IL-8 or (±)-arterenol, respectively, increasing both the potency and E_{max} of Ca^{2+} signalling by these agonists. Both of these interactions were found to be independent of extracellular Ca^{2+}, dependent on thapsigargin-sensitive Ca^{2+} stores, and were absolutely dependent on the continued presence of the pre-stimulating agonist.

There are a number of potential mechanisms by which GPCR-mediated Ca^{2+} signalling could be potentiated, few of which have been conclusively proven to occur, but each of which will be addressed in some detail in this study. Receptor oligomerization leading either to alternative binding or coupling properties, or to the prevention of receptor internalisation, may allow increased potency of agonists to stimulate signalling, or prolong their access to

receptor. Infidelity of the coupling of GPCRs to Gα proteins, or of Gβγ proteins to effectors, may allow a diversity of coupling that could be exploited by a secondary GPCR to enhance coupling to Ca^{2+} signals. Modulation of the enzymes responsible for receptor desensitization could defend against signal termination, as could stimulation of phosphatase activity, or inhibition of GAPs such as the RGS proteins. Gβγ/PI 3-K-mediated stabilisation of PLC presence at the cell membrane may facilitate the activation of this enzyme, as may the removal of any restraint on the rate of substrate supply by phosphoinositide synthetic enzymes. Co-operative actions between $G\alpha_q$ and Gβγ at the effector level (i.e. phospholipase C) could enhance second messenger production, a situation that could also occur subsequent to the inhibition of $InsP_3$ metabolising enzymes. The receptors for the Ca^{2+}-signalling second messenger, $InsP_3$, could also be a target for modulation, where increases in sensitivity cause potentiated Ca^{2+} signalling in the absence of any changes in $InsP_3$ mass. Finally, the size and/or diversity of the Ca^{2+} stores could be altered such that more prolific Ca^{2+} release is possible. These potential mechanisms are performed by a bewildering array of kinases, phosphatases, synthetic and metabolic enzymes, and by a gamut of other factors. Furthermore, it is difficult to rule out any of these mechanisms based on the data presented in Chapter 3. It is therefore clear that direct testing of specific mechanisms is somewhat futile until more is known about the basic components of the pathway underlying this interaction. This chapter is aimed at identifying some of this crucial machinery so as to support or refute particular models of crosstalk, and to generate hypotheses on the most likely model in terms of the interaction between P2Y2 receptors and CXCR2 for direct testing in the subsequent sections of this study.

This necessity for an initial assessment of the possible pathways involved has enforced the use of an only semi-rational approach to the selection of pharmacological tools in this chapter. Using two basic protocols, a range of commonly used inhibitors of some of the

central elements of the well-recognised Ca^{2+} signalling pathways (e.g. $InsP_3$ receptors, PLC, PKC, sphingosine kinase, ryanodine receptors) and of alternative signalling pathways (e.g. PI 3-K) was tested for activity against the potentiation of Ca^{2+} signalling by GPCR crosstalk. Measurements of factors other than Ca^{2+} (e.g. inositol phosphates) were also made to assess whether potentiation was also apparent at a level upstream of Ca^{2+}. This section finds that the mechanism underlying crosstalk between P2Y2 receptors and CXCR2 appears to involve the PLCβ/$InsP_3$ pathway, and that enhanced phosphoinositide production in co-stimulated cells is at the root of the potentiation of Ca^{2+} signalling.

Section 4.2: Results

Section 4.2.1: Development of a more flexible protocol to study changes in $[Ca^{2+}]_i$.

The FLIPR protocol used to generate much of the initial data regarding this potentiation was restricted in the number of additions that could be made and also by the inability to remove a drug once added. To circumvent these limitations, a new protocol was developed using a Ca^{2+}-imaging system and utilising a perfusion system for flexible addition and removal of ligands. A typical trace from this protocol is shown in Fig. 4.1. Fluo-3-loaded cells were stimulated (in the presence of extracellular Ca^{2+}) with a maximal concentration of UTP (100μM) for 30s. The change in fluo-3 fluorescence following this stimulation was used as an internal control against which all subsequent responses were normalised. After a 90s period of perfusion in the absence of agonist to allow refilling of intracellular Ca^{2+} stores, cells were perfused with nominally Ca^{2+}-free buffer to prevent any further refilling following subsequent stimulations. UTP-sensitive intracellular Ca^{2+} stores were drained by repeatedly stimulating the cells with 20s pulses of 100μM UTP separated by a 20s period of washout with buffer, until no further Ca^{2+} response was visible to this agonist. In this store-depleted state, potentiation responses could be viewed more clearly, and without interference from significant entry of Ca^{2+} from the extracellular milieu. Importantly, while reinforcing the evidence that the potentiation response is independent of Ca^{2+} entry, this assay also shows that co-stimulation of these cells with UTP and IL-8 is able to access Ca^{2+} from a UTP-insensitive store. In some experiments, a 5min incubation period with a test agent was incorporated at Point A (Fig. 4.1), before continuing with single- and co-additions. UTP was unable to elicit any further Ca^{2+} response after a 5min incubation in the absence of any test agents, indicating that there was no significant store refilling during this period from the low level of Ca^{2+} still present in the buffer (data not shown).

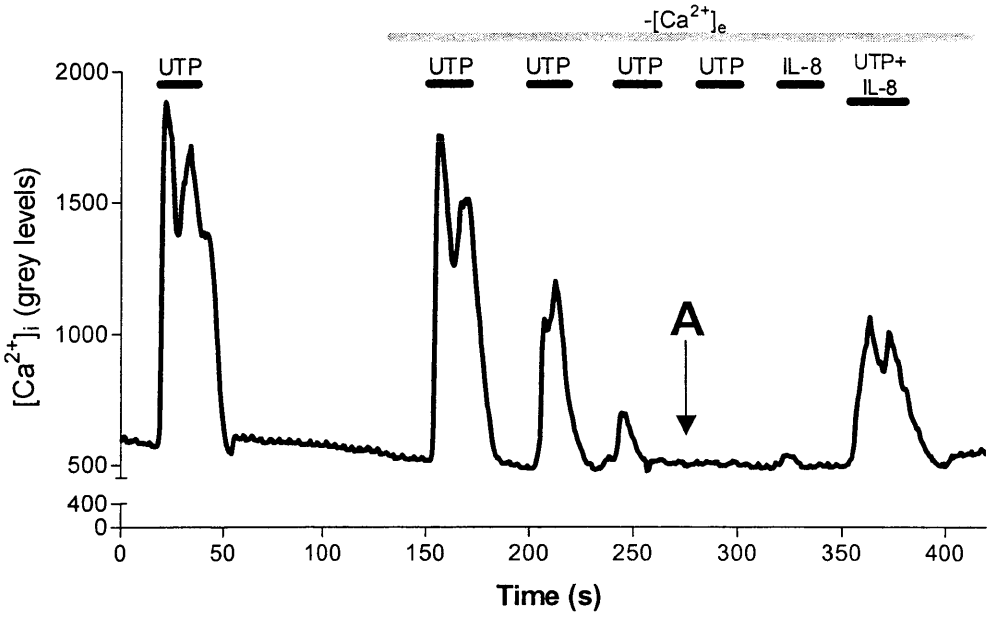


Figure 4.1: Representative trace from calcium imager showing a typical protocol for studies in a perfusion system. Cells were grown on poly-D-lysine-coated glass coverslips, and loaded with fluo-3/AM. Fluo-3 was excited using light from a xenon lamp filtered to 488nm using a monochromator, and its fluorescence was used as a direct measure of $[Ca^{2+}]_i$. After stimulating initially with a maximal concentration of a Ca^{2+}-releasing agonist (100μM UTP or 100μM carbachol), extracellular Ca^{2+} was withdrawn from the perfusion buffer to prevent store refilling, and the $[Ca^{2+}]_i$ response to a maximal concentration of agonist (UTP or CCh) was reduced to zero by repeated 20s pulses of agonist. Subsequent responses to a co-addition of UTP or CCh with another, non-Ca^{2+} releasing agent could then be studied without having to take into account any response to UTP or CCh. Where required, the experiment was stopped at Point A to allow incubation with inhibitors before proceeding with single- and co-additions.

Section 4.2.2: Effects of staurosporine

Staurosporine has potent activity in inhibiting protein tyrosine kinases (Nixon, 1997), but is marketed mainly as a broad specificity inhibitor of PKC. For this property, it was utilised to assess the involvement of these PKC enzymes in the potentiation phenomenon documented here. Cells were pre-treated for 20mins prior to assay with 3μM staurosporine (an incubation that has been shown previously to be effective in HEK-293 cells (Qian *et al*, 1997; Ferrari *et al*, 1999)). Subsequently, using the store depletion protocol detailed in Fig. 4.1, cells were repeatedly stimulated with 100μM UTP in the continued presence of staurosporine until no further response could be seen to this agonist. Subsequent responses to either 100μM UTP or 10nM IL-8 alone (data not shown) or a co-addition of UTP and IL-8 (Fig. 4.2a) were unaffected by pre-treatment. Qualitatively identical results were obtained using an EC_{50} concentration of UTP (3μM; Fig. 4.2b).

Section 4.2.3: Effects of inhibition of PI 3-K

Activation of PI 3-K is blocked by wortmannin at a concentration of 100-300nM (Meier *et al*, 1997; van der Kaay *et al*, 1997; Sweeney *et al*, 2001). To investigate whether PI 3-K played a role in the potentiation response, cells were treated with 300nM wortmannin for 20mins prior to assay. Ca^{2+} responses to co-addition were studied using the Ca^{2+} imaging protocol featured in Fig. 4.1. Wortmannin had no significant effect on the response to co-addition of UTP (100μM or 3μM) and IL-8 (10nM) (Fig. 4.3.1). As a positive control to the action of 300nM wortmannin, activation of ERK (a PI 3-K-mediated process) was measured. Quiescent cells were stimulated with foetal calf serum (FCS) in the presence or absence of wortmannin. FCS produced a modest activation of ERK (approximately 2 fold-of-basal), an activation that was almost entirely inhibited in the presence of wortmannin (Fig. 4.3.2).

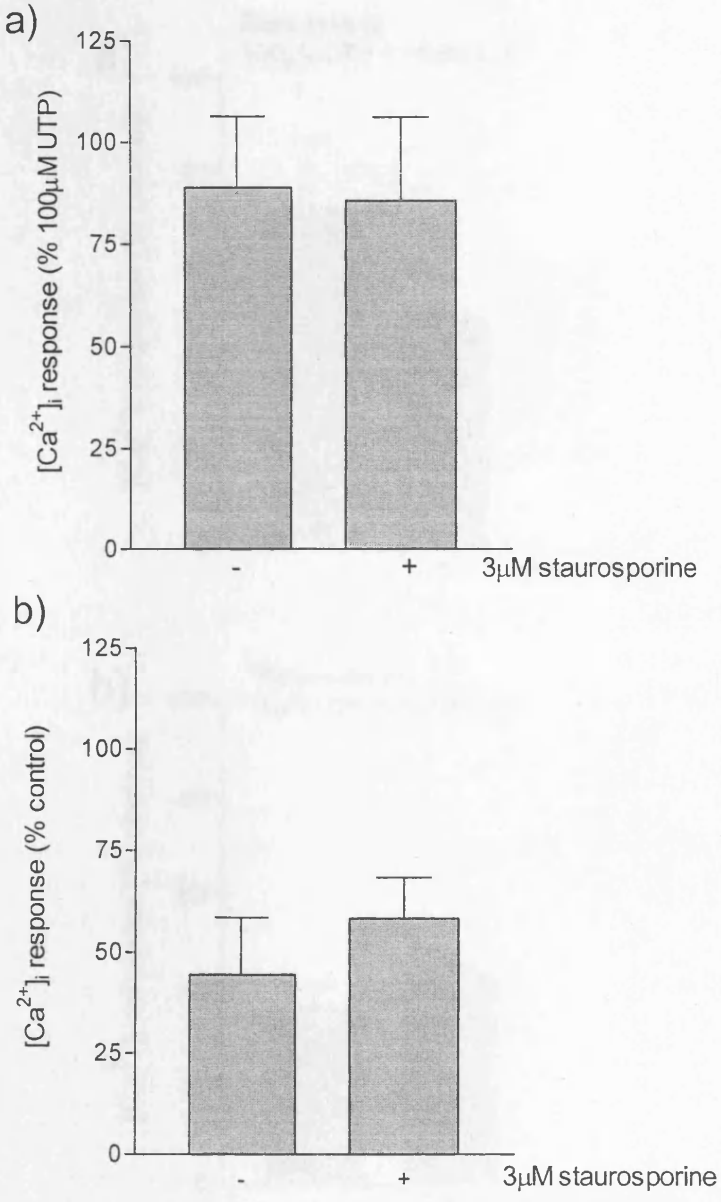


Figure 4.2: Effect of PKC inhibition on potentiation. Cells were grown on poly-D-lysine-coated 22mm glass coverslips for two days, then washed and incubated for 1hr with a solution of 5μM fluo-3/AM. Following a 20min incubation with or without 3μM staurosporine, cells were stimulated using the same protocol as in Figure 4.1. Responses shown are responses to the co-addition of UTP (100μM (a) or 3μM (b)) and 10nM IL-8, in the presence and absence of staurosporine, expressed relative to the control maximal response (to 100μM UTP in the presence of extracellular Ca^{2+}). Data shown are mean±s.e.m, n=4.

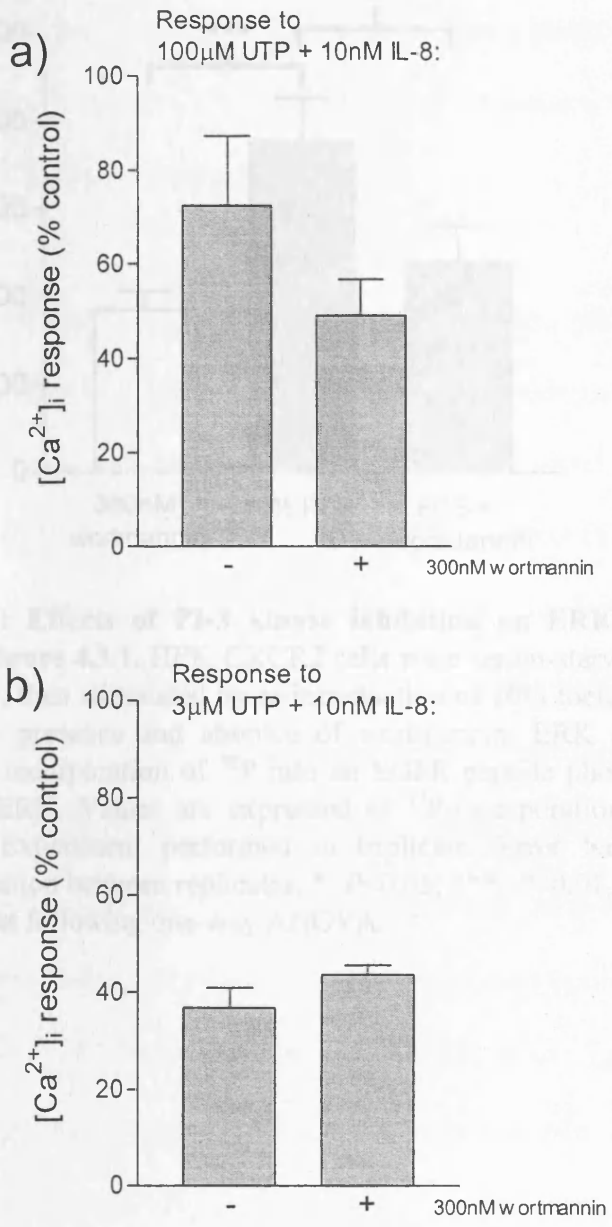


Figure 4.3.1: Effects of PI-3 kinase inhibition on potentiation. HEK-CXCR2 cells were grown on glass coverslips and loaded as described in Materials and Methods. Cells were then stimulated as in Fig. 4.1. Where indicated, a 20 min incubation with 300nM wortmannin was included prior to start of assay. a) Response to co-addition of 100μM UTP and 10nM IL-8 in the presence and absence of wortmannin. b) As a, but with 3μM UTP. Values are expressed as changes in $[Ca^{2+}]_i$ in relation to the maximal response achieved with 100μM UTP, as measured by fluo-3 fluorescence. Data are mean±s.e.m., n=4.

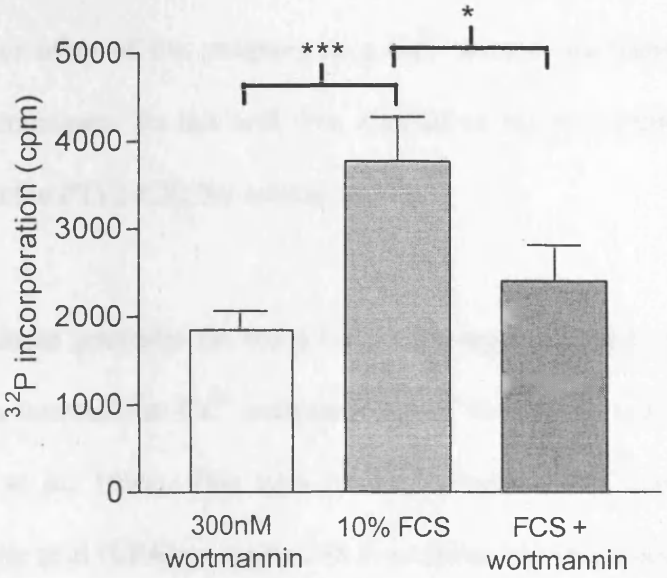


Figure 4.3.2: Effects of PI-3 kinase inhibition on ERK activation. Control to Figure 4.3.1. HEK-CXCR2 cells were serum-starved for 24hrs prior to assay, then stimulated by re-introduction of 10% foetal calf serum (FCS) in the presence and absence of wortmannin. ERK activity was measured by incorporation of ^{32}P into an EGFR peptide phosphorylation substrate of ERK. Values are expressed as ^{32}P-incorporation relative to basal, n=1. Experiment performed in triplicate. Error bars represent standard deviation between replicates. *: $P<0.05$; ***: $P<0.01$, by unpaired Student's t-test following one-way ANOVA.

Section 4.2.4: Involvement of alternative Ca^{2+} signalling pathways

One potential method by which GPCR interaction could lead to Ca^{2+} signal potentiation is by coupling one or other of the receptors to a Ca^{2+} release mechanism to which it does not normally communicate. To this end, two alternative release pathways were examined for involvement in the P2Y2-CXCR2 interaction.

Sphingosine kinase generates the novel Ca^{2+}-releasing messenger, sphingosine-1-phosphate, which releases intracellular Ca^{2+} independently of G-proteins (Meyer zu Heringdorf *et al*, 1998; Young *et al*, 1999). This type of Ca^{2+} response can occur downstream of both lysophosphatidic acid (LPA) receptors and muscarinic M3 receptors (Young *et al*, 1999). To test whether the Ca^{2+} responses revealed by co-stimulation with UTP and IL-8 involved sphingosine kinase and/or sphingosine-1-phosphate, the sphingosine kinase inhibitor, dimethylsphingosine (DMS), was included in co-addition experiments. In fluo-3-loaded HEK-CXCR2 cells, a 5min incubation with 30μM DMS reduced the response to 10μM LPA by 91±7% compared to the response to 10μM LPA prior to incubation with DMS (Fig. 4.4.1). However, this incubation had no significant effect on the response to a co-addition of 100μM UTP and 10nM IL-8 in store-depleted HEK-CXCR2 cells (Fig. 4.4.2a). In contrast, the same incubation appeared to potentiate the response to a co-addition of 3μM UTP and 10nM IL-8, significantly ($P<0.05$) (Fig. 4.4.2b).

Ryanodine receptors (RyR) mediate Ca^{2+}-induced Ca^{2+} release (CICR), acting as a receptor for Ca^{2+} in the cytosol derived either from intracellular store release by e.g. $InsP_3$, or from the extracellular environment through plasma membrane Ca^{2+} channels. This CICR is inhibited by the antagonistic action of ryanodine at these receptors. To test whether RyR-gated stores were involved in this GPCR crosstalk, store-depleted cells were incubated for

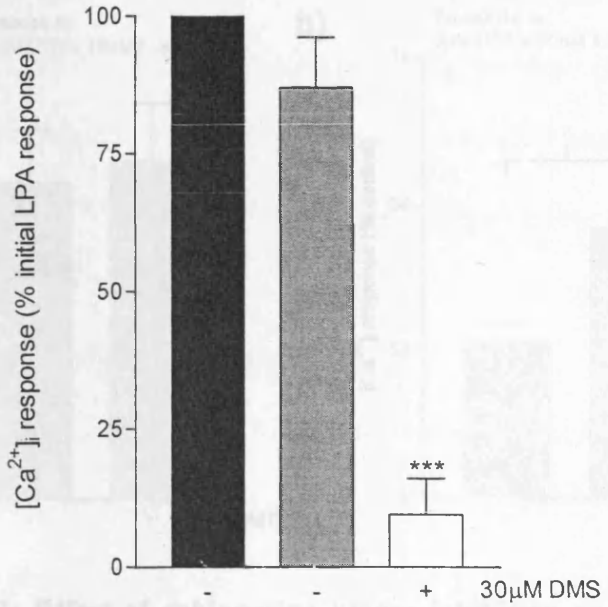


Figure 4.4.1: Effect of sphingosine kinase inhibition on LPA-induced Ca^{2+} responses. Control to Figure 4.4.2. HEK-CXCR2 cells were grown on glass coverslips and loaded as described in Materials and Methods, then stimulated twice with 10μM lysophosphatidic acid (LPA) (to ensure reproducibility) with a 90s period of Ca^{2+} store refilling after each addition. After a 5min incubation with 30μM dimethylsphingosine (DMS) or vehicle, cells were re-stimulated with 10μM LPA in the absence (grey bar) or presence (open bar) of DMS. Shown are the responses to LPA relative to the initial control response (black bar). Data are mean±s.e.m., n=3. ***: $P<0.001$ by Student's t-test (*vs.* initial response) following one-way analysis of variance.

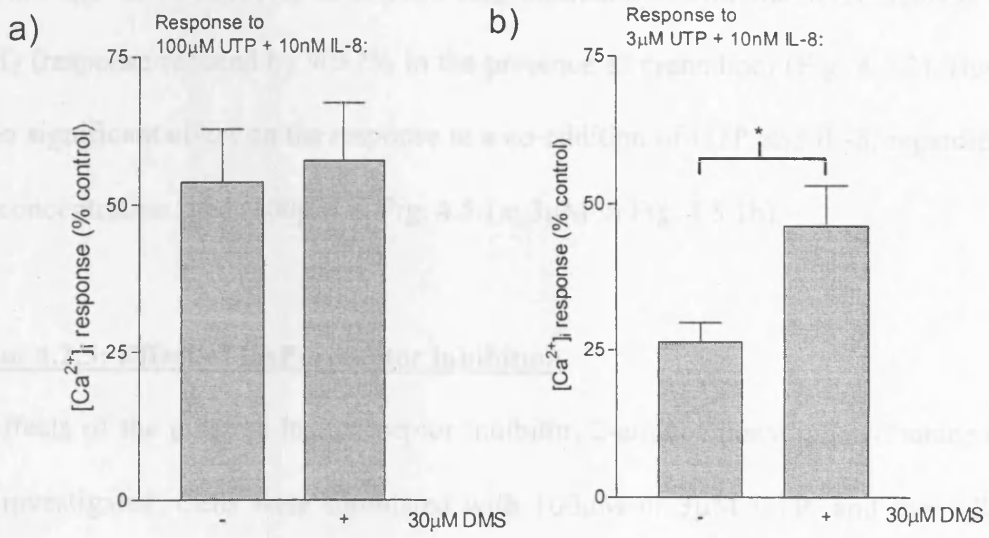


Figure 4.4.2: Effect of sphingosine kinase inhibition on potentiation. HEK-CXCR2 cells were grown on glass coverslips and loaded as described in Materials and Methods. Cells were then stimulated as in Fig. 4.1, with a 5min incubation with 30μM dimethylsphingosine (DMS) at Point A (Fig 4.1). Responses shown are those to co-addition of 10nM IL8 with a) 100μM UTP or b) 3μM UTP, expressed relative to the maximal response to 100μM UTP. Data are mean±s.e.m., n=4. *: $P<0.05$ by unpaired Student's t-test *vs.* response in the absence of DMS.

5mins with 30μM ryanodine before stimulating with 100μM UTP and 10nM IL-8 (Fig. 4.5.1a). This concentration of ryanodine was shown to be effective at blocking RyR since it inhibited the Ca^{2+} elevation seen following stimulation with the RyR agonist, caffeine (1mM) (response reduced by 90±2% in the presence of ryanodine) (Fig. 4.5.2). However, it had no significant effect on the response to a co-addition of UTP and IL-8, regardless of the UTP concentration used (100μM in Fig. 4.5.1a; 3μM in Fig. 4.5.1b).

Section 4.2.5: Effect of $InsP_3$ receptor inhibition

The effects of the putative $InsP_3$ receptor inhibitor, 2-aminoethoxydiphenylborane (2-APB) were investigated. Cells were stimulated with 100μM or 3μM UTP, and then allowed to refill Ca^{2+} stores for 5mins, during which time 100μM 2-APB was added, where required. Subsequent responses to the same concentration of UTP were studied in the presence and absence of 2-APB (Fig. 4.6a and Fig. 4.6b). At a concentration of 100μM, 2-APB significantly reduced responses to both 100μM UTP and 3μM UTP (Fig. 4.6a and Fig. 4.6b, respectively).

Using the protocol detailed in Fig. 4.1, cells were exposed to either buffer or 100μM 2-APB at Point A for 5mins, then stimulated sequentially (with a 20s washout between each addition) with 100μM UTP, 10nM IL-8 and a co-addition of both agonists, in the continued presence or absence of 2-APB. The presence of 2-APB reduced the response to co-addition from 57±10% in control cells to 17±5% in 2-APB treated cells (Fig. 4.7a). Qualitatively identical results were obtained when using an EC_{50} concentration of UTP (3μM), where the response to co-addition was reduced from 36±7% in control cells to 1.3±0.3% in the presence of 2-APB (Fig. 4.7b).

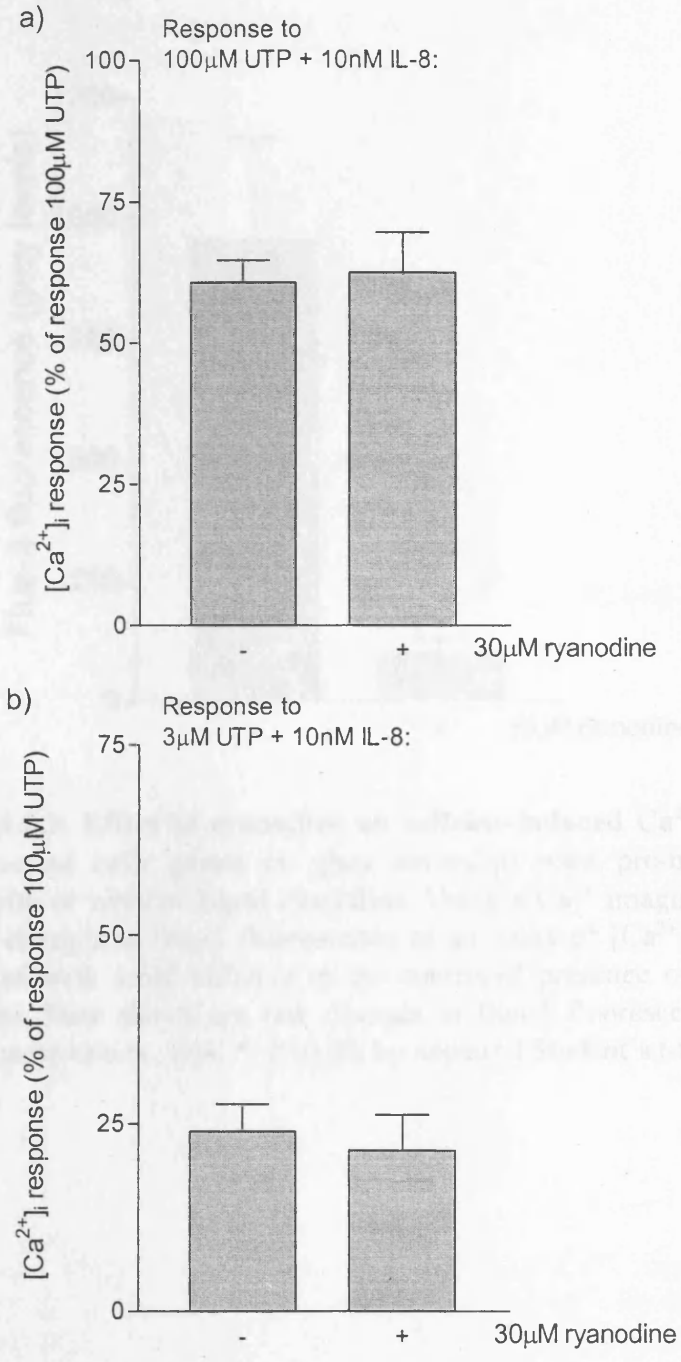


Figure 4.5.1: Effect of ryanodine receptor inhibition on potentiation. HEK-CXCR2 cells were grown on glass coverslips and loaded as described in Materials and Method, then stimulated as in Fig. 4.1 except, where indicated, with a 5min incubation with 30μM ryanodine at Point A (Fig. 4.1). Responses shown are those to co-addition of 10nM IL-8 with a) 100μM UTP or b) 3μM UTP, expressed relative to the maximal response to 100μM UTP. Data are mean±s.e.m., n=4.

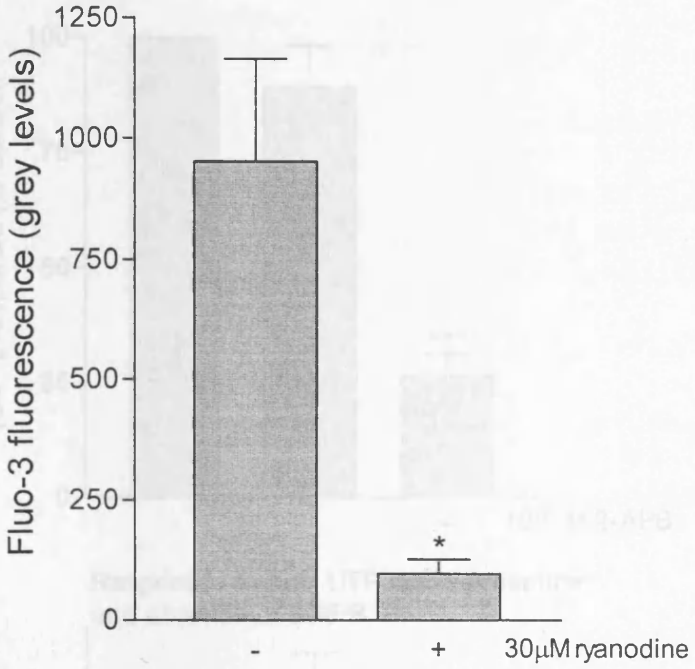


Figure 4.5.2: Effect of ryanodine on caffeine-induced Ca^{2+} responses. Fluo-3-loaded cells grown on glass coverslips were pre-incubated for 5mins with or without 30μM ryanodine. Using a Ca^{2+} imaging system to analyse changes in fluo-3 fluorescence as an index of $[Ca^{2+}]_i$, cells were stimulated with 1mM caffeine in the continued presence or absence of ryanodine. Data shown are raw changes in fluo-3 fluorescence (in grey levels), mean±s.e.m., n=4. *: $P<0.05$, by unpaired Student's t-test.

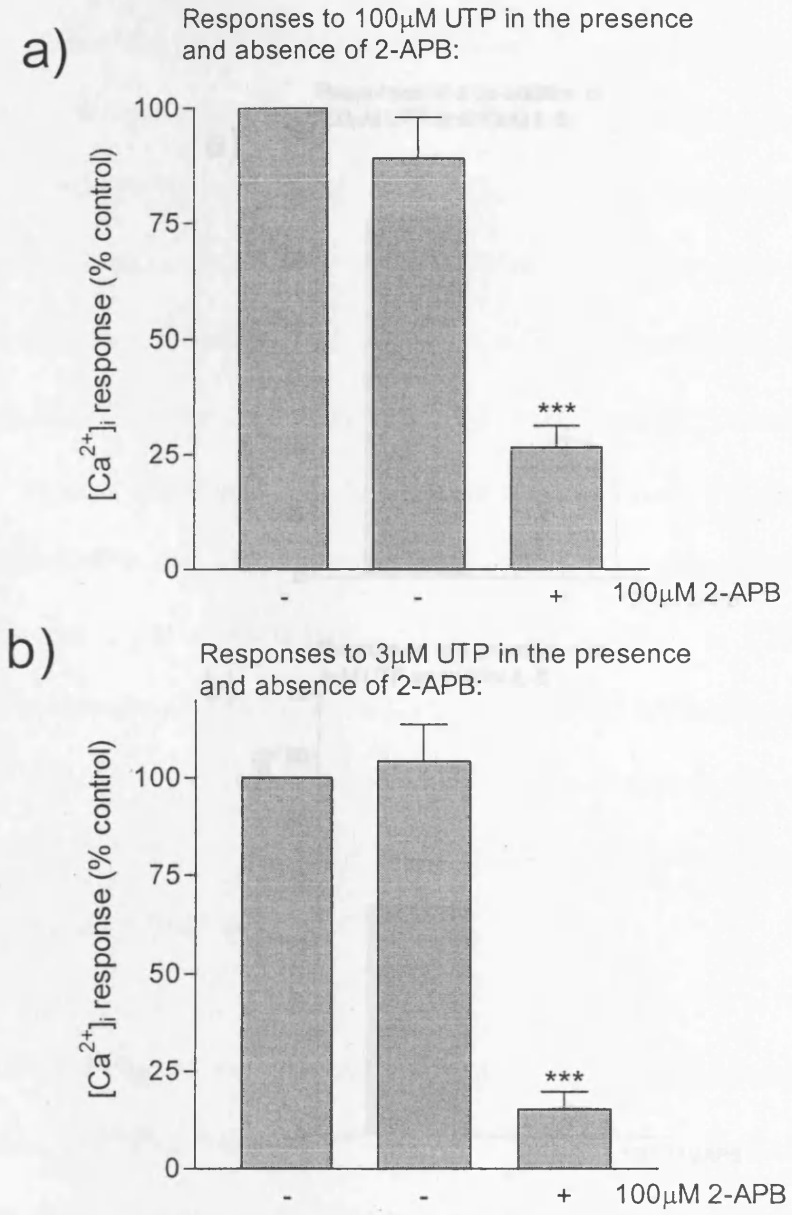


Figure 4.6: Effects of 2APB on UTP-mediated Ca^{2+} responses. HEK-CXCR2 cells grown on glass coverslips were loaded with fluo-3/AM and stimulated with UTP (100μM (a); or 3μM (b)) followed by removal of agonist and refilling of intracellular Ca^{2+} stores. Cells were then incubated with or without 100μM 2-aminoethoxydiphenylborane (2-APB) and re-stimulated with the same concentration of UTP in the presence or absence of 2-APB. Fluo-3 fluorescence was measured as a direct indicator of $[Ca^{2+}]_i$. Values are expressed as a percentage of the maximal response achieved during stimulation with 100μM UTP. Data are mean±s.e.m., n=5. ***: $P<0.001$, by Student's unpaired t-test.

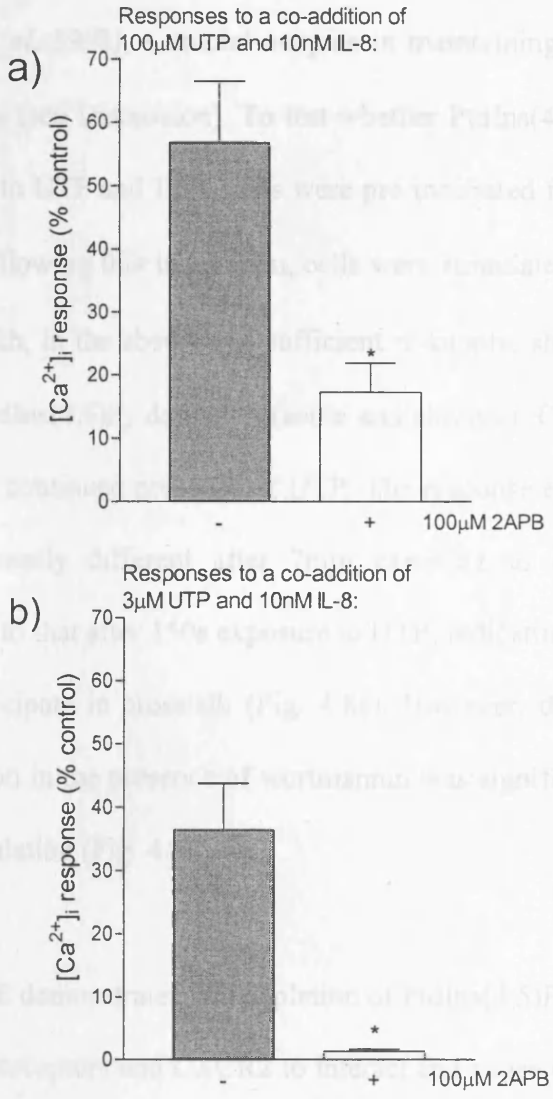


Figure 4.7: Effects of 2-APB on potentiation. Cells were prepared and stimulated as in Fig. 4.1, with an incubation with or without 2-APB (100μM, 5min) at Point A. Cells were then stimulated with a co-addition of 10nM IL-8 and UTP (100μM (a) or 3μM (b)). Values are expressed as changes in $[Ca^{2+}]_i$ in relation to the maximal response achieved to a control stimulation with 100μM UTP, as measured by fluo-3 fluorescence. Data are mean±s.e.m., n=5. *: $P<0.05$, by Student's unpaired t-test).

Section 4.2.6: Dependence of potentiation on PtdIns(4,5)P_2

At higher concentrations than those used previously to block PI 3-K, wortmannin is also effective at blocking phosphatidylinositol 4-kinase (PI 4-K) (Nakanishi *et al*, 1995; Downing *et al*, 1996; Willars *et al*, 1998), a crucial enzyme in maintaining the supply of the PLCβ substrate, PtdIns(4,5)P_2 (see Discussion). To test whether PtdIns(4,5)P_2 is involved at all in the observed response to UTP and IL-8, cells were pre-incubated for 20min with or without 10μM wortmannin. Following this incubation, cells were stimulated for either 150s or 7min with 100μM UTP which, in the absence of sufficient re-supply, should cause two distinctly different degrees of PtdIns(4,5)P_2 depletion (acute and chronic). Cells were then stimulated with 10nM IL-8 in the continued presence of UTP. The response elicited by this addition of IL-8 was not significantly different after 7min exposure to UTP in the absence of wortmannin compared to that after 150s exposure to UTP, indicating that P2Y2 receptors are still fully able to participate in crosstalk (Fig. 4.8a). However, the response to IL-8 after chronic UTP stimulation in the presence of wortmannin was significantly reduced compared to that after acute stimulation (Fig. 4.8b).

The evidence in Fig. 4.8 demonstrates that depletion of PtdIns(4,5)P_2 has a significant impact on the ability of P2Y2 receptors and CXCR2 to interact and cause enhanced Ca^{2+} signalling. However, this is on a distinctly different time scale (several minutes) to that used in previous experiments. The possibility that insufficient PtdIns(4,5)P_2 re-supply in the short term may prevent continued P2Y2 receptor Ca^{2+} signalling, and that potentiated Ca^{2+} responses may therefore be due to CXCR2-mediated activation of PtdIns(4,5)P_2 re-supply by PI 4-K was investigated. Cells were prepared as for Fig. 4.2, but treated for 20mins prior to assay with 10μM wortmannin. Changes in intracellular $[Ca^{2+}]_i$ were measured using the same store depletion protocol as in Fig. 4.1. Responses to co-addition of UTP (100μM or 3μM) and IL-8 (10nM) were not significantly affected by pre-incubation with wortmannin (Fig. 4.9.1). To

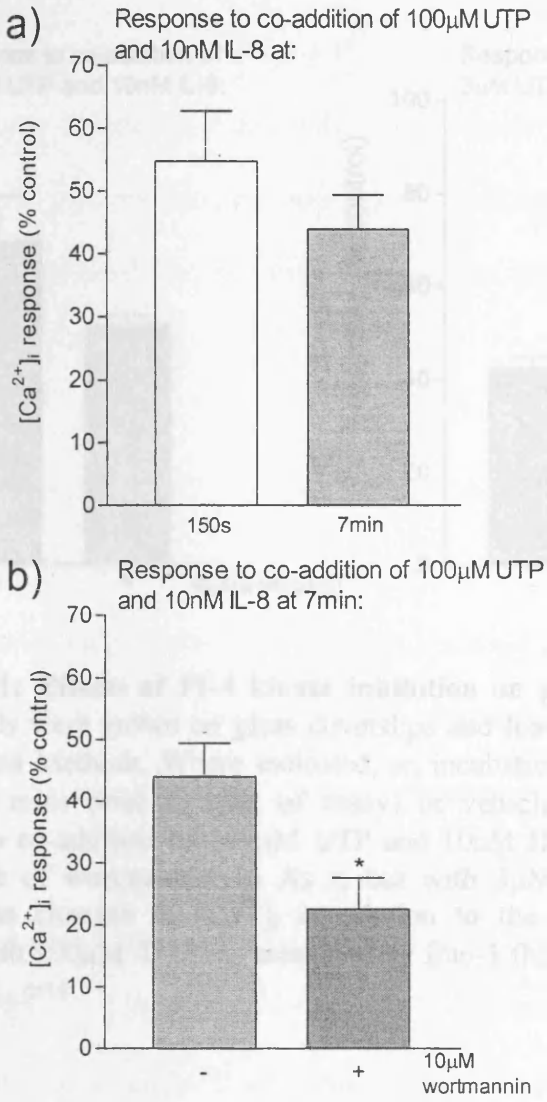


Figure 4.8: Effects of PI 4-K inhibition on potentiation induced after prolonged stimulation of P2Y2 receptors. HEK-CXCR2 cells were grown on glass coverslips and loaded as described in Materials and Methods. Cells were then stimulated using a perfusion system and fluo-3 fluorescence changes measured using Ca^{2+} imaging equipment. a) Cells were stimulated for either 150s or for 7mins with 100μM UTP before stimulating with 10nM IL-8 in the continued presence of 100μM UTP. b) Cells were stimulated for 7mins with 100μM UTP following a 20min pre-incubation with 10μM wortmannin or vehicle control. Cells were then stimulated with 10nM IL-8 in the continued presence of UTP. Values shown are responses to the co-addition, relative to the peak response to 100μM UTP. Data are mean±s.e.m, n=4. *: $P<0.05$ by unpaired Student's t-test.

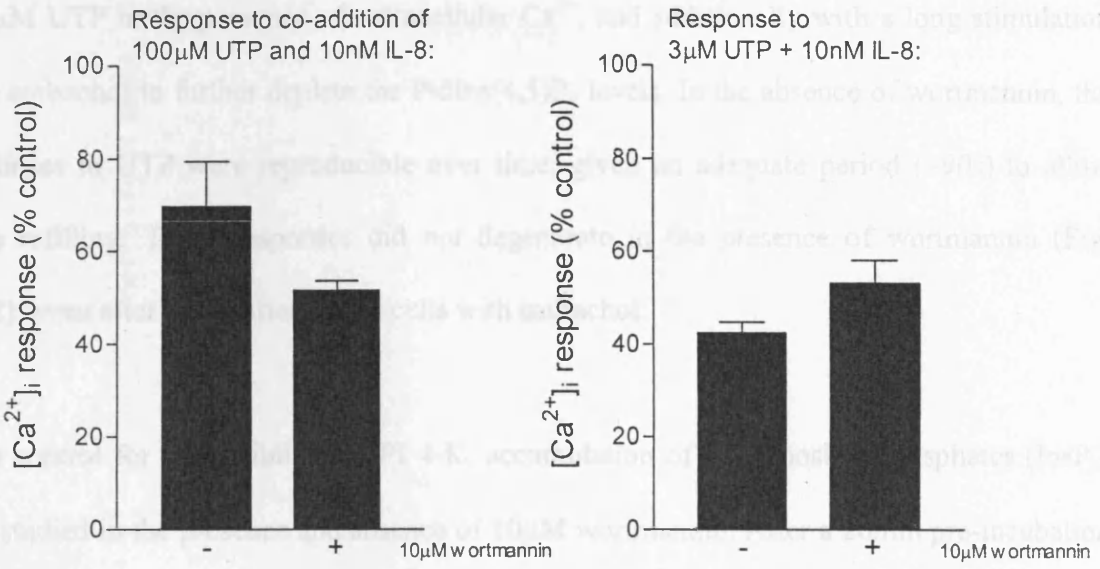


Figure 4.9.1: Effects of PI-4 kinase inhibition on potentiation. HEK-CXCR2 cells were grown on glass coverslips and loaded as described in Materials and Methods. Where indicated, an incubation with wortmannin (10μM, 20 mins prior to start of assay) or vehicle was included. a) Response to co-addition of 100μM UTP and 10nM IL-8 in the presence and absence of wortmannin. b) As a, but with 3μM UTP. Values are expressed as changes in $[Ca^{2+}]_i$ in relation to the maximal response achieved with 100μM UTP, as measured by fluo-3 fluorescence. Data are mean±s.e.m., n=4.

test whether UTP responses were indeed acutely limited by the supply of PtdIns(4,5)P_2, cells were pre-incubated as above (with 10μM wortmannin), then stimulated repeatedly with 100μM UTP in the presence of extracellular Ca^{2+}, and additionally with a long stimulation with carbachol to further deplete the PtdIns(4,5)P_2 levels. In the absence of wortmannin, the responses to UTP were reproducible over time, given an adequate period (~90s) to allow store refilling. These responses did *not* degenerate in the presence of wortmannin (Fig. 4.9.2), even after stimulation of the cells with carbachol.

As a control for the inhibition of PI 4-K, accumulation of total inositol phosphates ($InsP_x$) was studied in the presence and absence of 10μM wortmannin. After a 20min pre-incubation with 10μM wortmannin, cells loaded with [^{3}H]-*myo*-inositol were stimulated for 15mins with 1mM UTP, and [^{3}H]-$InsP_x$ gathered against a Li^+ block (see Chapter 2). UTP-stimulated $InsP_x$ generation was severely attenuated by wortmannin pre-treatment (Fig. 4.10).

Section 4.2.7: Inositol phosphate production in HEK-CXCR2 cells

Stimulation of production of $InsP_3$ by UTP and by a co-addition of UTP and IL-8 was assessed by radioligand competition binding assay using an $InsP_3$-binding protein prepared from bovine adrenal medulla. Cells stimulated with 1mM UTP elicited a small, markedly variable, increase in $InsP_3$, peaking at approximately 20s (Fig. 4.11a). The response to a co-addition of UTP and IL-8 appeared to be slightly smaller, and less variable. However, the extent of variability in these results effectively negated their worth in estimating the effects of IL-8 on $InsP_3$ production. An alternative assay for PLC activity is the quantification of $InsP_x$ (see Chapter 2). The cells were stimulated with either 100nM IL-8, 1mM UTP, or a co-addition of both, for varying durations (range: 0-30mins), and generation of [^{3}H]-$InsP_x$ determined. The results are summarised in Fig. 4.12. IL-8 alone did not elicit any significant

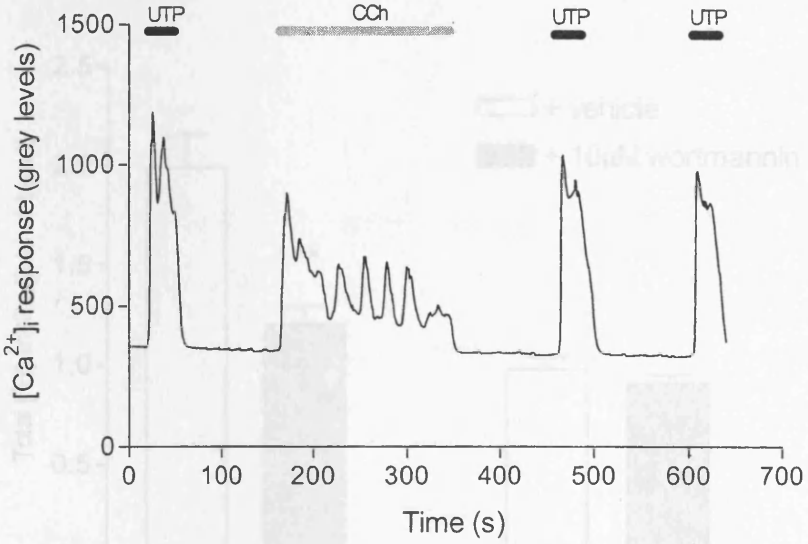


Figure 4.9.2: Lack of effect of 10μM wortmannin on short duration stimulation with agonists at Gq-coupled receptors. HEK-CXCR2 cells were grown on glass coverslips and loaded as described in Materials and Methods, incorporating a 20min pre-incubation with 10μM wortmannin. Using a Ca^{2+} imaging set-up to study changes in fluo-3 fluorescence, cells were stimulated with 20s pulses of 100μM UTP or a longer duration pulse of 100μM carbachol (CCh) in the continued presence of wortmannin and extracellular Ca^{2+}. Shown is a representative trace of four identical experiments.

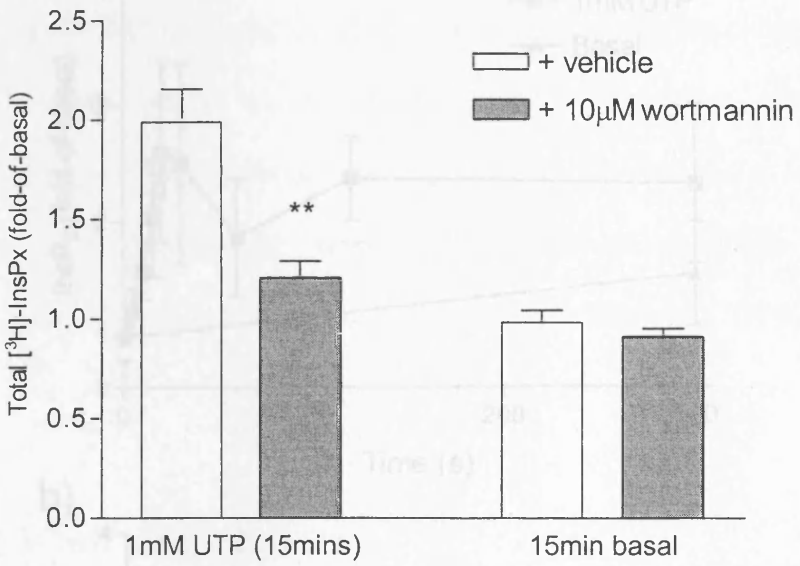


Figure 4.10: Effects of PI-4 kinase inhibition in inositol phosphate accumulation in response to UTP. Control to Figure 4.8.1. Cells were incubated for 48hrs with [^{3}H]-inositol. Cells were incubated with or without 10μM wortmannin for 20 mins prior to assay, then stimulated with 1mM UTP for 15mins in the continued presence of wortmannin where required. Total [^{3}H]-InsPx were accumulated against a 10mM Li^+ block. Data shown are mean±s.e.m, n=4. ** $P<0.01$, by unpaired Student's t-test.

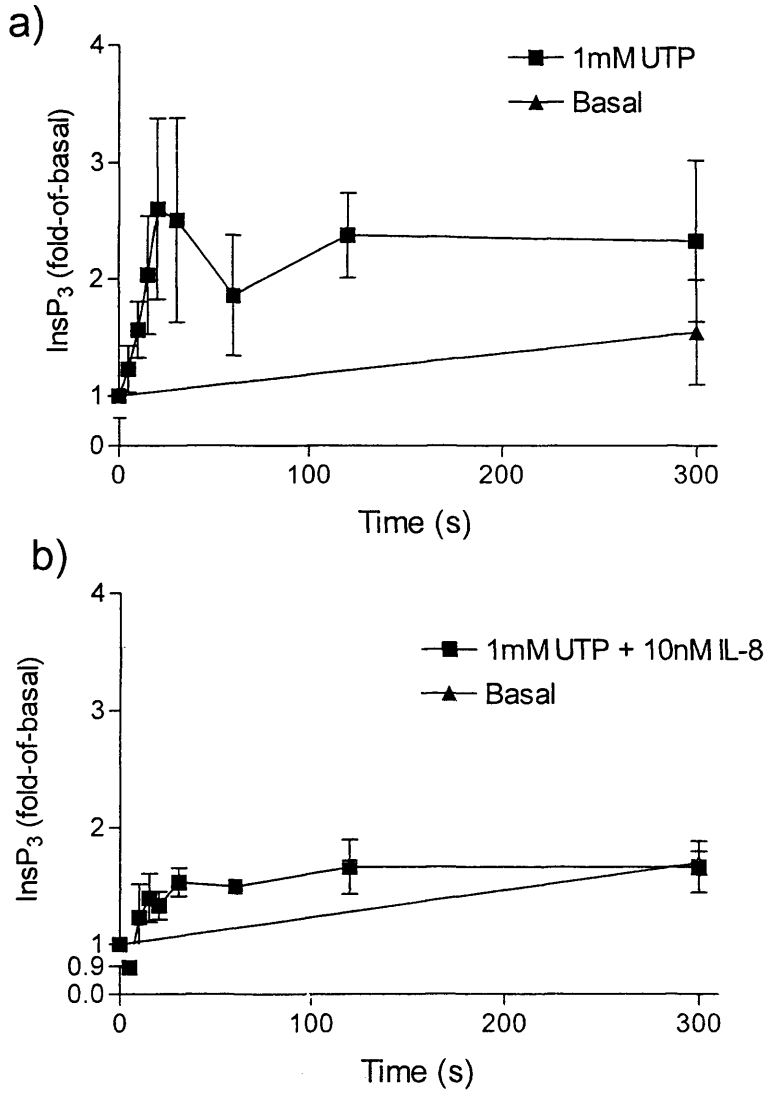


Figure 4.11: $InsP_3$ measurements for UTP and co-addition of UTP and IL-8. $InsP_3$ mass was measured by radioligand competition binding assay using a purified $InsP_3$ binding protein and [^{3}H]-$InsP_3$. Values are mean±s.e.m., n=4. Basal values used for normalisation were approximately 33±5 pmol/mg protein.

generation of [^{3}H]-InsP$_x$. In contrast, UTP alone caused accumulation of [^{3}H]-InsP$_x$ to a maximum of 1.6±0.09 fold-of-basal, while co-addition of both agonists elicited a maximum accumulation of 2.99±0.11 fold-of-basal. The [^{3}H]-InsP$_x$ production stimulated by co-addition was significantly different to that by UTP alone, and was more-than-additive. There was also a difference in the curve itself following co-stimulation, with PLC activity apparently switching off less quickly following co-addition. The point at which no further accumulation could be seen (i.e. where PLC activity is essentially zero) occurred significantly later following co-addition (15mins) than following UTP stimulation (5-10mins).

Section 4.2.8: Effects of PLC inhibition

Much of the data presented above suggests that enhanced PLC activity may underlie the elevated Ca^{2+} signalling seen when cells are stimulated with both UTP and IL-8. To test the involvement of PLC, the putative PLC inhibitor, U73122, was employed. Store-depleted cells were incubated for 5mins with either buffer, U73122 (10μM) or its negative control, U73343 (10μM) at Point A in the protocol described in Fig. 4.1. After this treatment, cells were re-stimulated sequentially (with a 20s wash period between each) with UTP (100μM) and 10nM IL-8 alone (data not shown), and finally with a co-addition of both agonists (Fig. 4.13a). The response to co-addition (64±3% of maximal response to 100μM UTP in control cells) was not significantly affected in cells treated with U73343 (64±8%), but was strongly inhibited by U73122 (9±4%). Qualitatively identical results were obtained when using an EC_{50} concentration (3μM) of UTP in the post-incubation additions (Fig. 4.13b).

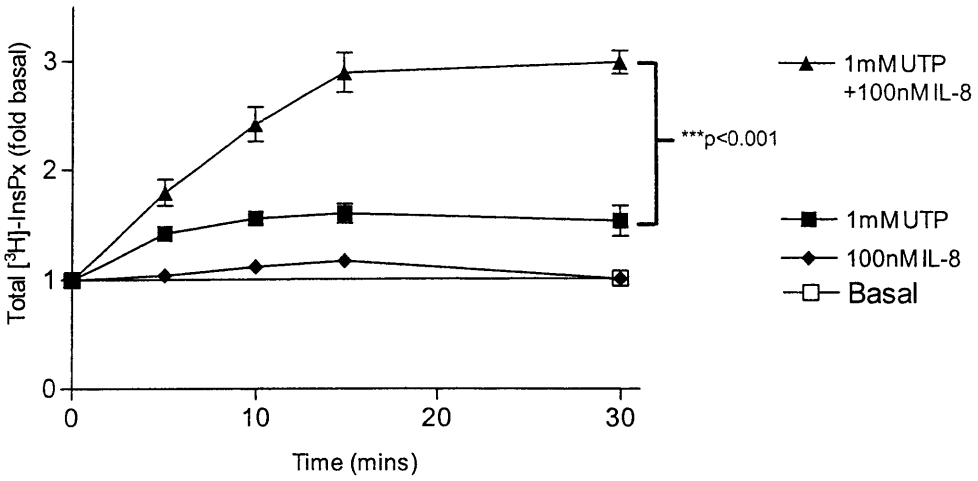


Figure 4.12: Accumulation of [^{3}H]-InsPx in HEK-CXCR2 cells. Cells were incubated for 48hrs with [^{3}H]-inositol. During varying lengths of cell stimulation, total [^{3}H]-InsPx were accumulated against a 10mM Li^+ block. Values are expressed as fold-of-basal. Basal values at t=0 were approximately 4500±200 dpm/well. Data shown are mean±s.e.m, n=4. *** $P<0.001$, by two-way analysis of variance.

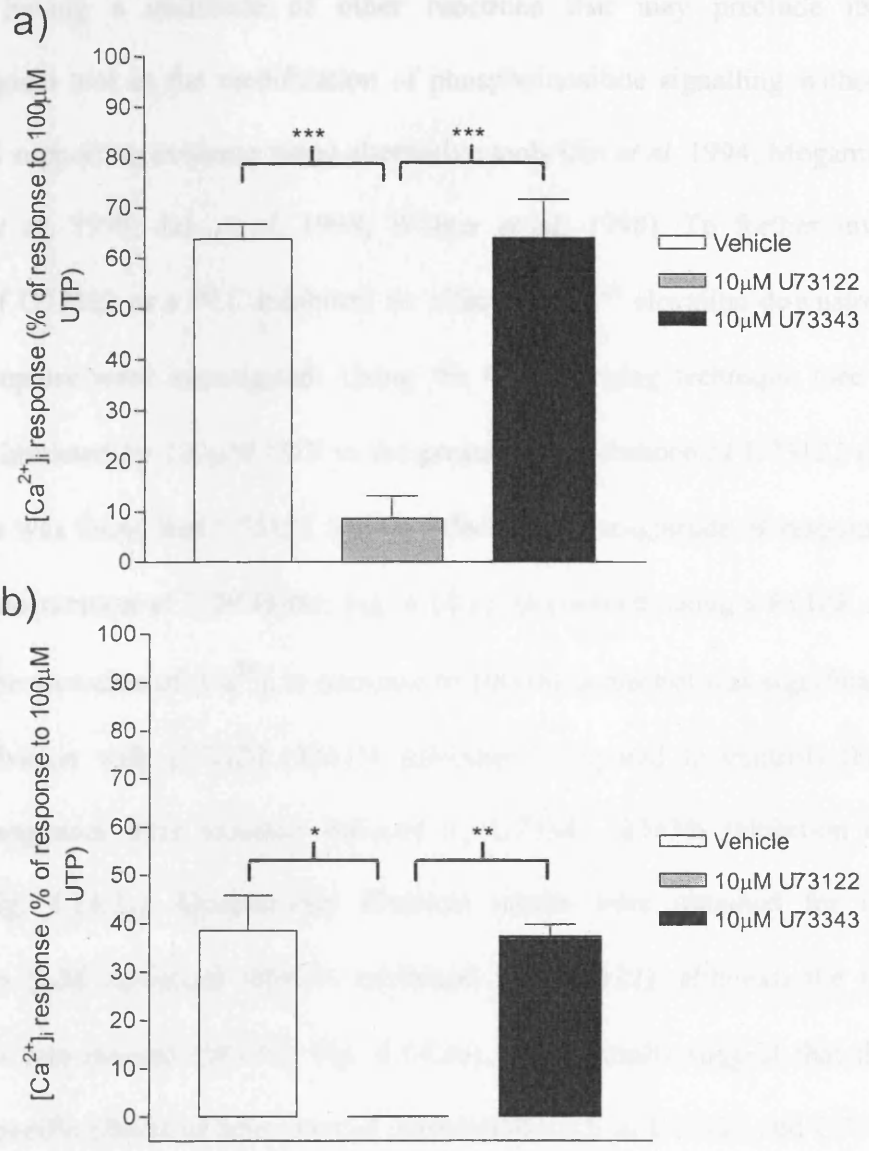


Figure 4.13: Effects of PLC inhibition on potentiation. HEK-CXCR2 cells were grown on glass coverslips and loaded with fluo-3 as described in Materials and Methods. After store drainage by repeated UTP stimulation (see Figure 4.1), cells were incubated with or without U73122 or U73343 (10µM, 5min), then stimulated with a co-addition of UTP (100µM (a) or 3µM (b)) and IL-8 (10nM). Values are expressed as changes in $[Ca^{2+}]_i$ in relation to the maximal response achieved with 100µM UTP, as measured by fluo-3 fluorescence. Data are mean±s.e.m., n=5. *: $P<0.05$; **: $P<0.01$; ***$P<0.001$, by Student's unpaired t-test, where indicated, following one-way ANOVA.

Alternative properties of U73122

There is a growing body of evidence that U73122 is not at all selective in its PLC inhibitory properties, having a multitude of other functions that may preclude its use as a pharmacological tool in the modification of phosphoinositide signalling without extensive controls and supporting evidence using alternative tools (Jin *et al*, 1994; Mogami *et al*, 1997; Pulcinelli *et al*, 1998; Jan *et al*, 1998; Walker *et al*, 1998). To further investigate the reliability of U73122 as a PLC inhibitor, its effects on Ca^{2+} elevation downstream of $G\alpha_q$-coupled receptors were investigated. Using the Ca^{2+} imaging technique (see Chapter 2), responses stimulated by 100μM UTP in the presence and absence of U73122 (10μM) were measured. It was found that U73122 had no effect on the magnitude of responses to a sub-maximal concentration of UTP (3μM; Fig. 4.14.1). In contrast, using a FLIPR assay, it was found that the elevation of $[Ca^{2+}]_i$ in response to 100μM carbachol was significantly reduced by pre-incubation with U73122 (93±1% inhibition compared to control) (Fig. 4.14.2a). However, responses were similarly reduced by U73343 (83±3% inhibition compared to control) (Fig. 4.14.2a). Qualitatively identical results were obtained for inhibition of responses to 3μM carbachol (86±1% inhibition by U73122), although the inhibition by U73343 was less marked (50±3%; Fig. 4.14.2b). These results suggest that there may be some non-specific effects of aminosteroid compounds such as U73122 and U73343 in terms of inhibition of Ca^{2+} signalling. To test whether PLC activity was directly influenced, phosphoinositide generation by 1mM UTP was assessed in the presence and absence of U73122 (Fig. 4.15). Total [^{3}H]-$InsP_x$ generation (when expressed as fold-of-basal; Fig. 4.15a) was not affected by pre-incubation with 10μM U73122, but when expressing the data as raw data (disintegrations per minute; Fig. 4.15b) it was found that U73122 stimulated a small increase in basal PLCβ activity. There was no subsequent inhibitory effect on PLCβ activity. These results were not assessed quantitatively.

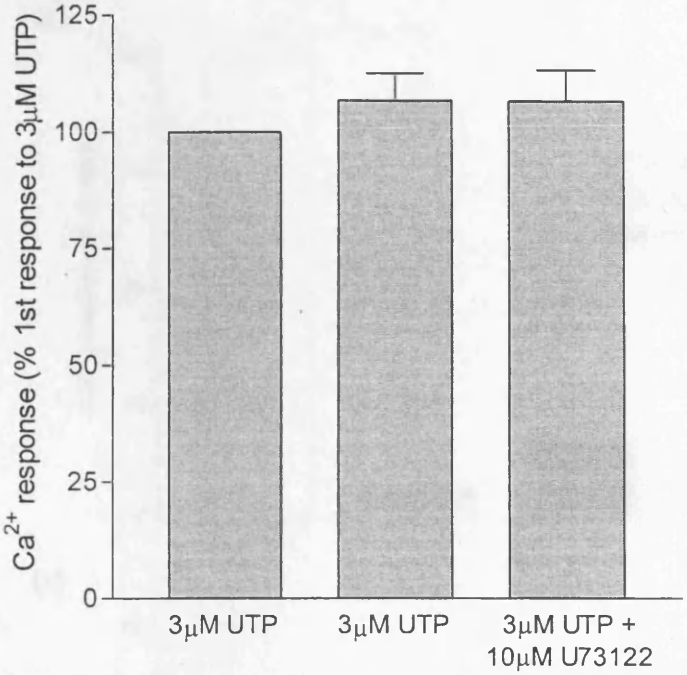


Figure 4.14.1: Effects of U73122 on responses to UTP. HEK-CXCR2 cells were grown on glass coverslips and loaded as described in Materials and Methods, then stimulated with UTP (3μM). Following a 90s perfusion of buffer, cells were re-stimulated twice with 3μM UTP, firstly in the absence of U73122 and subsequently following a 5min incubation with (and in the continued presence of) U73122 (10μM). Changes in fluo-3 fluorescence were measured as an index of $[Ca^{2+}]_i$ and expressed as a percentage of the maximal response to 3μM UTP in the absence of inhibitor. Data are mean±s.e.m., n=4.

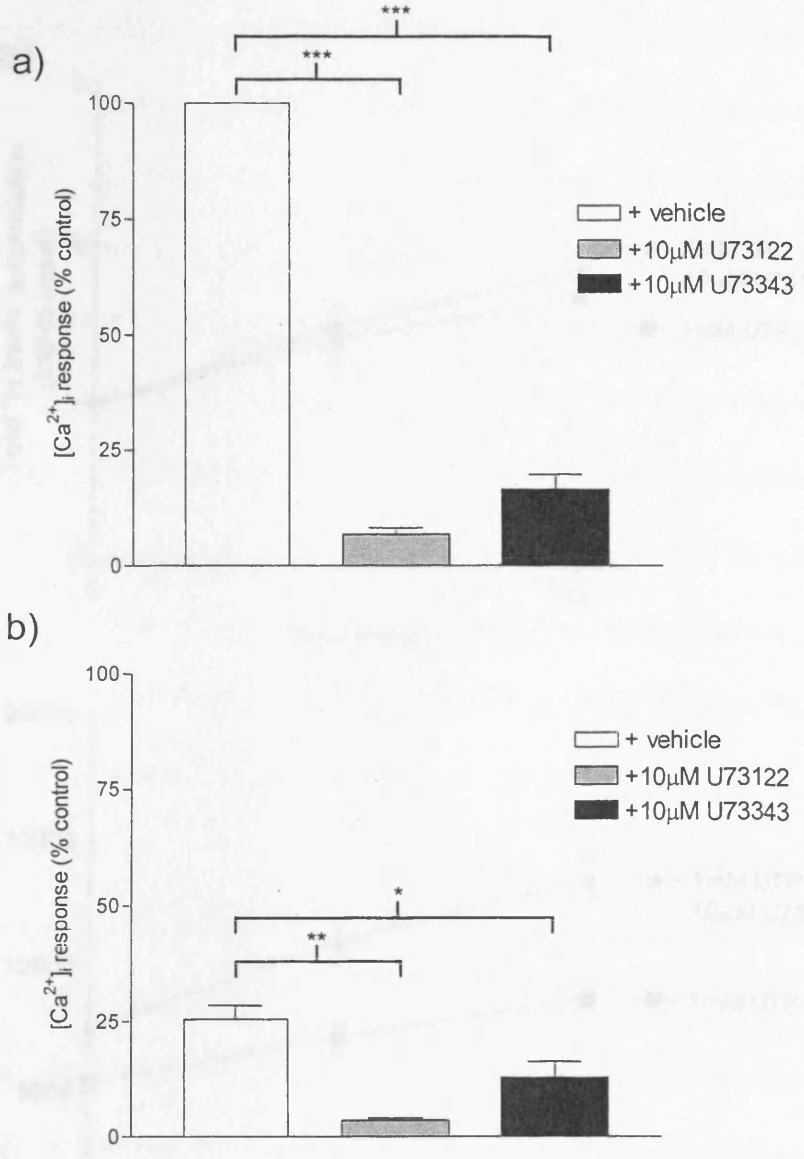


Figure 4.14.2: Effects of U73122 on responses to carbachol. Cells were prepared for FLIPR assay as described in Materials and Methods, and where indicated, incubated for 5mins prior to assay with either 10μM U73122, 10μM U73343 or vehicle (0.1% DMSO) control. Cells were then stimulated with carbachol (100μM (a) or 3μM (b)). Changes in fluo-3 fluorescence were measured as an index of $[Ca^{2+}]_i$ and expressed as a percentage of the maximal response to 100μM CCh in the absence of inhibitor. Data are mean±s.e.m., n=4. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$, by unpaired Student's t-test (where indicated) following one-way ANOVA.

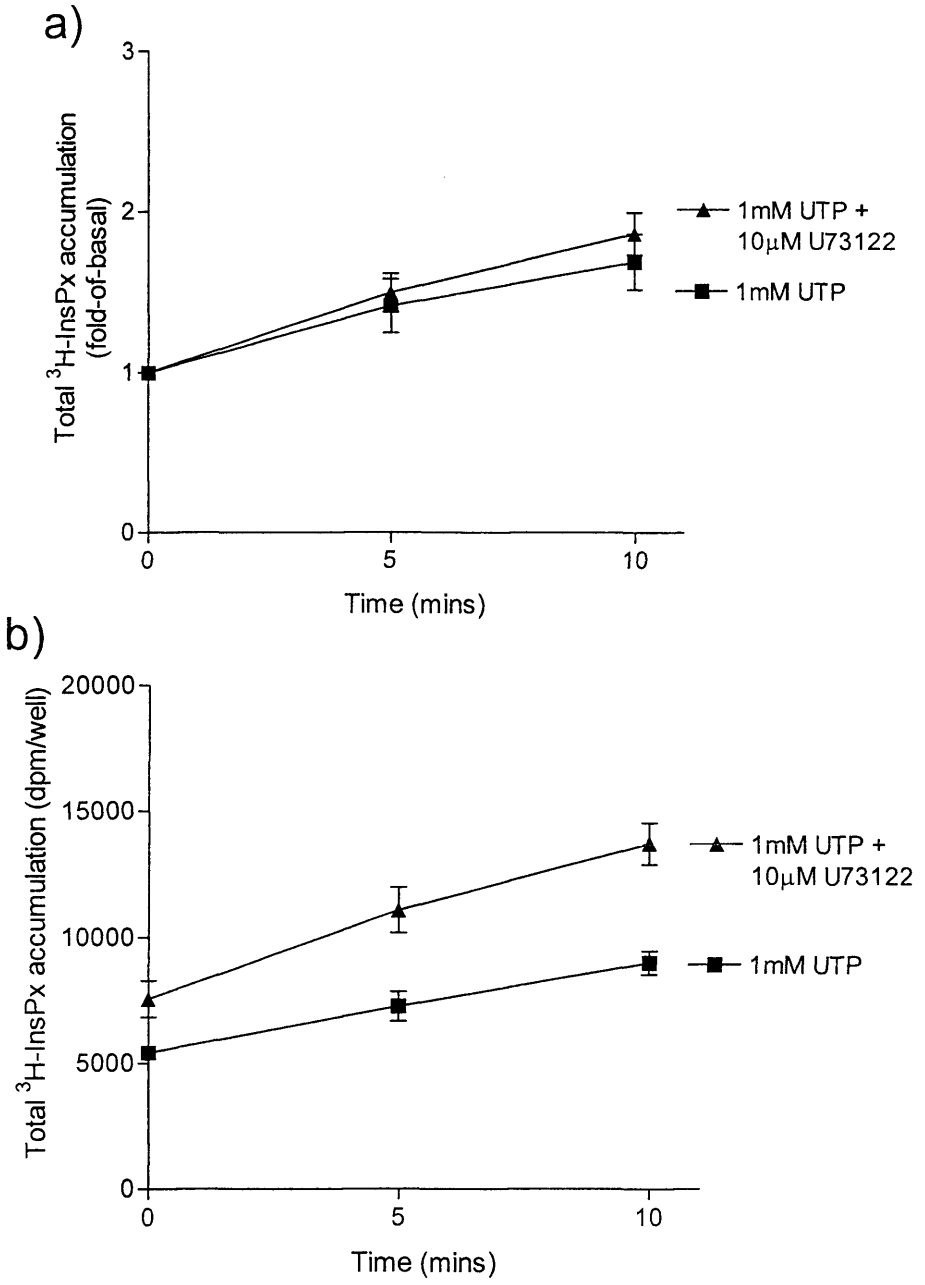


Figure 4.15: Effects of U73122 on UTP-stimulated inositol phosphate production. Cells were incubated for 48hrs with [^{3}H]-*myo*-inositol. After incubation for 5mins with or without 10μM U73122, and in the presence of 10mM Li^+,cells were stimulated with 1mM UTP for the indicated times. Data are total [^{3}H]-InsPx generation expressed as fold-of-basal (a) or as raw disintegrations per minute (b), mean±s.e.m., n=3.

Section 4.2.9: Effects of $InsP_3$ receptor sensitisation

Thimerosal was used to investigate whether the mechanism underlying the observed potentiation involved sensitisation of the $InsP_3$ receptor to stimulation by $InsP_3$. Thimerosal is commonly used to sensitise cells to the actions of $InsP_3$, causing an enhancement of $InsP_3$-mediated responses (Bootman *et al*, 1992, Vanlingen *et al*, 1999). It was used here to effectively 'pre-potentiate' the system, thus pre-empting any such effects of either UTP or IL-8. Thimerosal caused no change in the magnitude of the $[Ca^{2+}]_i$ peak in response to IL-8 alone (i.e. this remained essentially zero) (Fig. 4.16). However, thimerosal caused a more than 2-fold increase in the magnitude of the $[Ca^{2+}]_i$ elevation seen in response to 3μM UTP (140±37% *vs.* 60±7% in control cells, compared to the response to 100μM UTP in the absence of thimerosal) (Fig. 4.17a). Conversely, this agent had little effect on the size of Ca^{2+} responses to an addition of 10nM IL-8 in the presence of 3μM UTP (67±7% *vs.* 54±5% in control cells) (Fig. 4.17b) (see Discussion).

It is possible that the Ca^{2+} release by 3μM UTP in the presence of thimerosal was so substantial as to significantly deplete the Ca^{2+} store. To confirm that such a depletion of the Ca^{2+} store was not disguising any effects of thimerosal on the responses to co-addition in the sensitised system, a concentration of UTP (1μM) was used whose response, even when sensitised, would leave a substantial proportion of the Ca^{2+} store intact. The response stimulated by 1μM UTP was significantly increased by thimerosal (48±6% *vs.* 26±6% in control cells, compared to the response to 100μM UTP in the absence of thimerosal) (Fig. 4.17a). However, the response to an addition of 10nM IL-8 following pre-stimulation with 1μM UTP was still not potentiated by thimerosal (Fig. 4.17b).

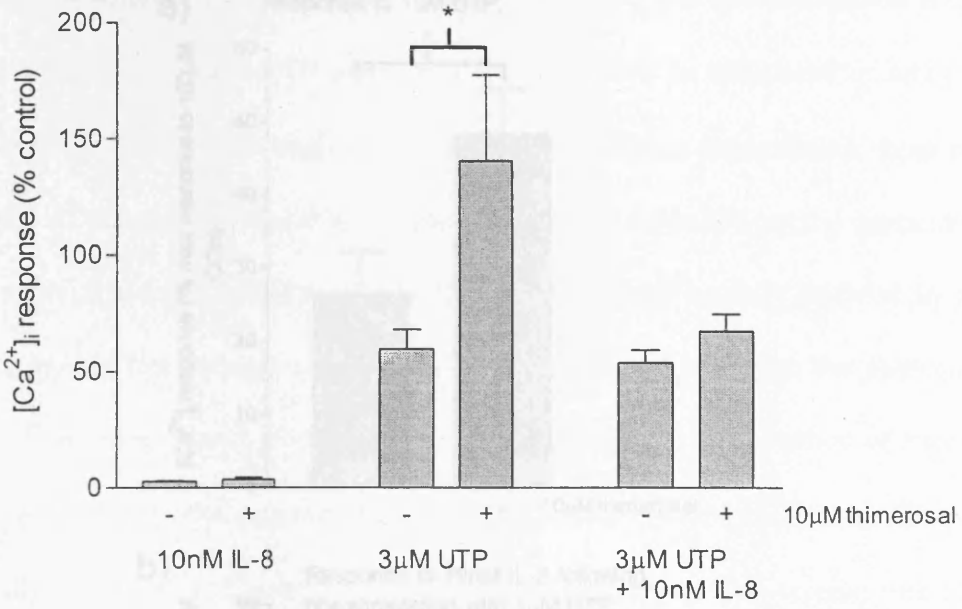


Figure 4.16: Effect of $InsP_3$ receptor sensitisation on Ca^{2+} signalling in HEK-CXCR2 cells. HEK-CXCR2 cells were grown on glass coverslips and loaded with fluo-3/AM as described in Materials and Methods. Using a Ca^{2+} imaging set-up with perfusion system, cells were stimulated with 100μM UTP as a control response, and Ca^{2+} stores allowed to refill. Where indicated, cells were then pre-incubated for 5min with 10μM thimerosal or vehicle, then stimulated either directly with 10nM IL-8, or for 150s with 3μM UTP followed by a co-addition of 3μM UTP and 10nM IL-8. Responses shown are to these secondary additions, relative to the initial response to 100μM UTP. Data are mean±s.e.m., n≥4. *: $P<0.05$, by unpaired Student's t-test).

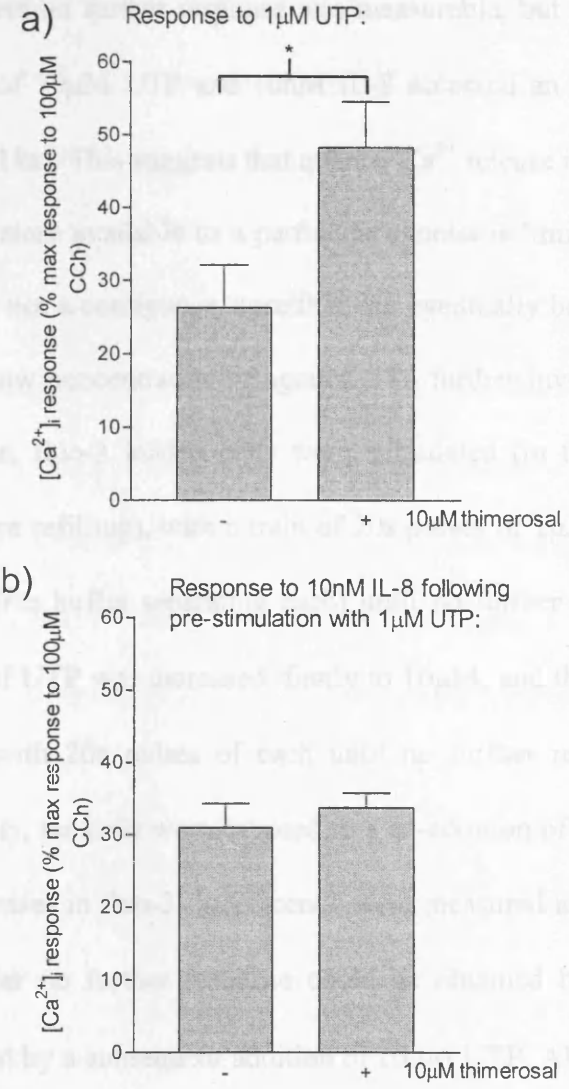


Figure 4.17: Effects of thimerosal on responses to a low concentration of UTP and co-addition of UTP and IL-8. Cells were prepared for FLIPR assay as described in Materials and Methods, and where indicated, incubated for 5mins prior to assay with 10μM thimerosal. Using the FLIPR, cells were then stimulated at t=10s with 1μM UTP and subsequently at t=150s with 10nM IL-8 (in the continued presence of UTP). Shown are responses (in the presence and absence of thimerosal) to 1μM UTP (top panel) and to 10nM IL-8, expressed as a a percentage of the maximal response to 100μM UTP. Data are mean±s.e.m, n=4. *: $P<0.05$, by unpaired Student's t-test.

Section 4.2.10: Demonstration of quantal Ca^{2+} release in HEK-CXCR2 cells

It has been shown previously that repeated stimulation with 100μM UTP depleted the Ca^{2+} store to a point where no further response was measurable, but that subsequent stimulation with a co-addition of 10μM UTP and 10nM IL-8 accessed an additional quantity of Ca^{2+} (Fig. 4.1 and Fig. 4.18a). This suggests that quantal Ca^{2+} release is possible in these cells (i.e. that the size of the store available to a particular agonist is limited by the concentration of agonist used, and is not a contiguous store that can eventually be fully depleted by repeated stimulation with a low concentration of agonist). To further investigate the phenomenon of quantal Ca^{2+} release, fluo-3 loaded cells were stimulated (in the absence of extracellular Ca^{2+}, to prevent store refilling), with a train of 20s pulses of 1μM UTP (with 20s perfusion of nominally Ca^{2+}-free buffer separating each) until no further Ca^{2+} release was observed. The concentration of UTP was increased, firstly to 10μM, and then to 100μM, and the cells further stimulated with 20s pulses of each until no further response was seen to either concentration. Finally, the cells were exposed to a co-addition of 100μM UTP and 10nM IL-8 (Fig. 4.18b). Increases in fluo-3 fluorescence were measured as an index of $[Ca^{2+}]_i$. It was found that even after no further response could be obtained by 1μM UTP, a substantial response was elicited by a subsequent addition of 10μM UTP. Although there appeared to be little Ca^{2+} available for release by 100μM UTP after drainage by 10μM UTP, there was a further release of Ca^{2+} in response to co-addition of UTP and IL-8 after complete depletion of the store available to 100μM UTP. This suggests that the intracellular Ca^{2+} stores are limited by the concentration of agonist accessing them, and that simply increasing agonist concentration (and thus $InsP_3$ production) was sufficient to broaden the range of Ca^{2+} stores accessed.

In addition to stores being limited by the *concentration* of agonist releasing them, the *identity* of the agonist or receptor also may restrict the system to accessing only a highly localised

fraction of the global stores. To test whether portions of the stores accessible by certain agonists are discrete from one another, two different agonists (2MeSADP and carbachol, acting at P2Y1 nucleotide and muscarinic M3 receptors, respectively) were used to stimulate cells that had been repeatedly stimulated with 100μM UTP to deplete the "UTP-sensitive" stores (Fig. 4.19 top and bottom, respectively). Following store depletion with UTP, stimulation with 10μM 2MeSADP caused a Ca^{2+} response with a magnitude of 36±6% compared to the initial response to 100μM UTP (response to final stimulation with 100μM UTP: 8±1%). Similarly, 100μM carbachol also caused a substantial Ca^{2+} elevation following depletion of the UTP-sensitive store (88±12% of initial response to 100μM UTP; response to final stimulation with 100μM UTP: 6±3%). Likewise, following depletion of the carbachol-sensitive store, 100μM UTP was also capable of accessing a substantial additional store, causing a response comparable (98±31%) to the maximal response to carbachol.

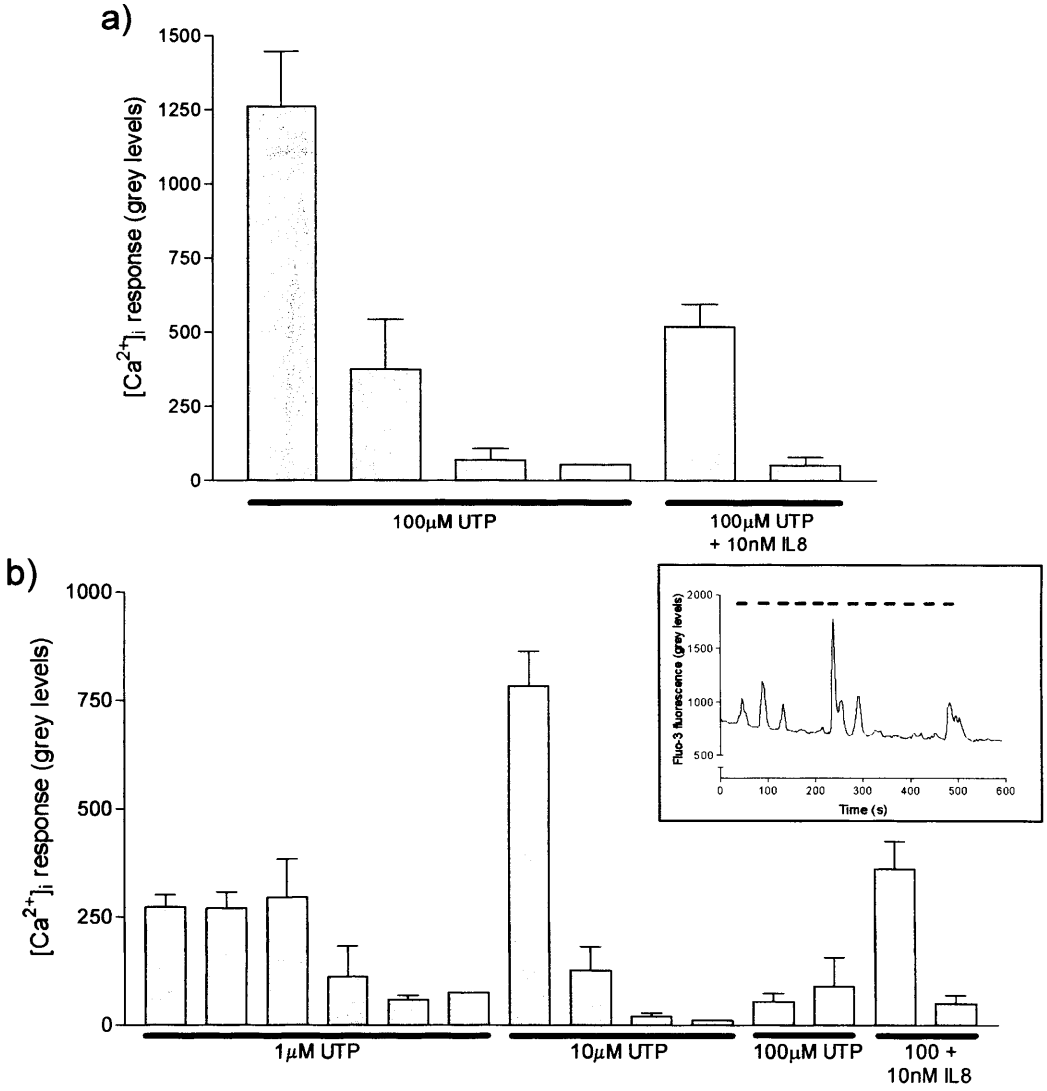


Figure 4.18: Demonstration of quantal Ca^{2+} release in HEK-CXCR2 cells. Cells were grown on poly-D-lysine-coated glass coverslips, and loaded with fluo-3/AM. Fluo-3 was excited using light from a xenon lamp filtered to 488nm using a monochromator, and its fluorescence was used as a direct measure of $[Ca^{2+}]_i$. a) Cells were stimulated repeatedly with 20s pulses of 100μM UTP in the nominal absence of extracellular Ca^{2+}. When the response to 100μM UTP was reduced to zero, cells were stimulated with a co-addition of 100μM UTP and 10nM IL-8 (again in the absence of extracellular Ca^{2+}). b) Cells were prepared as in a), but were initially stimulated with repeated 20s pulses of 1μM UTP until no further Ca^{2+} response was observed. UTP concentration was then increased to 10μM and cells were again stimulated until there was no further Ca^{2+} response, and then likewise with 100μM UTP, and a co-addition of 100μM UTP and 10nM IL-8. Values shown are changes in fluo-3 fluorescence (arbitrary units). Data are mean±s.e.m., n≥3. Inset) Sample trace from 4.18b. Bars represent drug additions: black: 1μM UTP; red: 10μM; blue: 100μM UTP; green: 100μM UTP and 10nM IL-8.

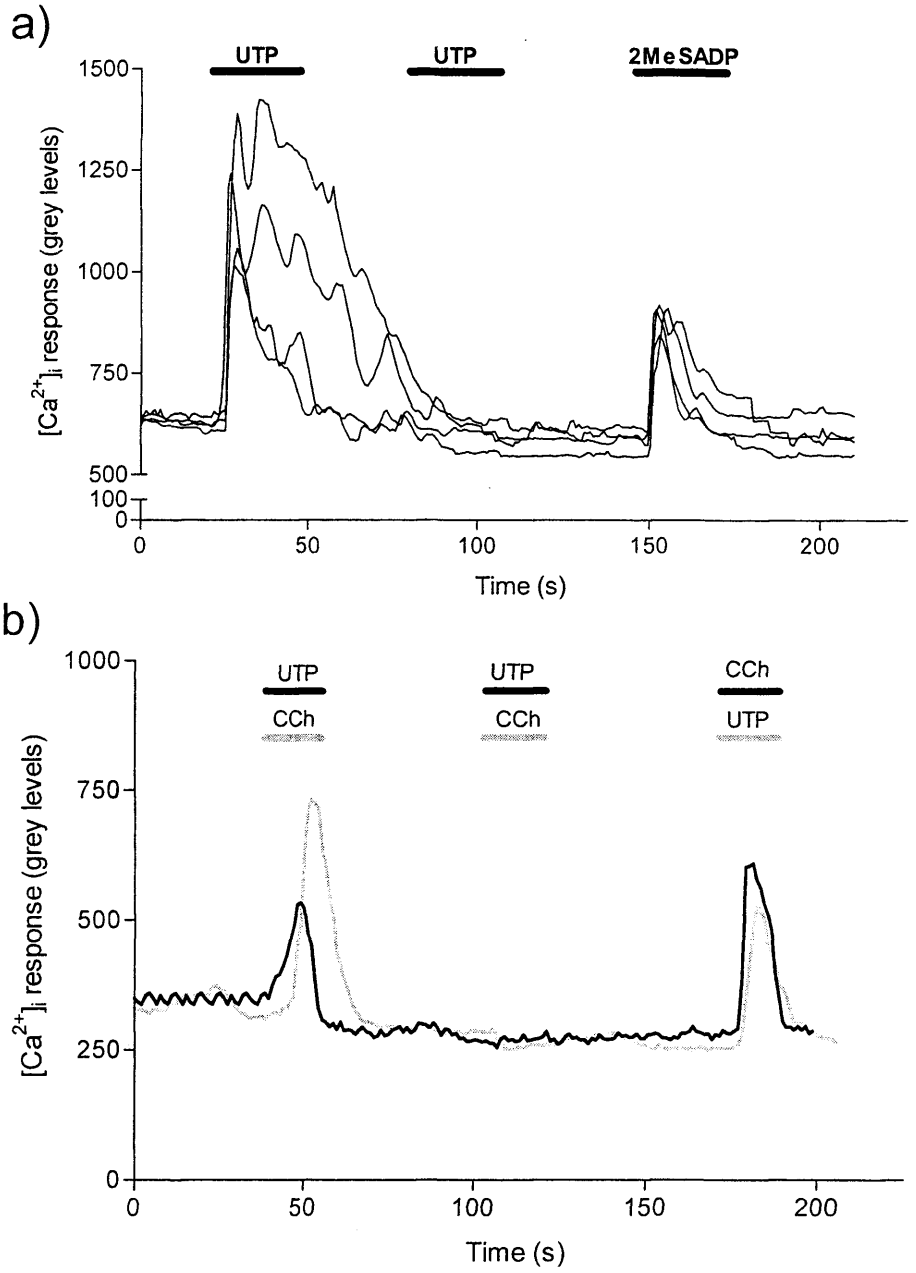


Figure 4.19: Existence of 'agonist-specific' intracellular Ca^{2+} stores. a) Using a Ca^{2+} imaging system, fluo-3-loaded HEK-WT cells were stimulated with 100μM UTP until no further response was observed, and subsequently with 10μM 2MeSADP. Shown are four traces representative of more than 20 individual cells. b) Using a similar protocol, cells were stimulated with either 100μM UTP or 100μM CCh until no further response was observed, and subsequently with 100μM CCh and 100μM UTP, respectively. Shown is one trace representative of more than 20 individual cells.

Section 4.3: Discussion

Section 4.3.1: Summary of data

The aim of this chapter was to use a range of pharmacological inhibitors in an attempt to identify some of the critical components of the potentiation mechanism. These studies have shown that the potentiation is not dependent on PKC or tyrosine kinase activation, nor on any activity of PI 3-K. In addition, alternative Ca^{2+}-signalling pathways including cADPR- and sphingosine-1-phosphate-mediated Ca^{2+} release are not involved in this crosstalk. It is now evident that the PLC/$InsP_3$ pathway is central to the potentiated Ca^{2+} signalling, given that an inhibitor of $InsP_3$ receptors blocked the response to IL-8 in the presence of UTP. In addition, it is shown that potentiation is seen in the generation of inositol phosphates, and requires PtdIns(4,5)P_2 (although is not acutely dependent on the *rate* of supply of this phospholipid by the actions of PI 4-kinase). These data suggest that up-regulation in the activity of the PLC/$InsP_3$ pathway underlies the crosstalk between P2Y2 receptors and CXCR2.

Section 4.3.2: Independence from PKC

Potentiation could arise from inhibition of the protein kinases that cause protein phosphorylation, preventing de-activation either of the receptor or of some downstream element such as PLCβ. Alternatively, protein kinase activation may cause phosphorylation of some downstream factor that facilitates CXCR2 coupling to Ca^{2+} release. PKA was deemed unlikely to be relevant in terms of the crosstalk between P2Y2 receptors and CXCR2. Given the normal coupling of these receptors, the requirement of cAMP elevation for activation of this enzyme is unlikely to be satisfied by either of these receptors (indeed, the coupling of CXCR2 to $G\alpha_i$ is likely to inhibit it). Inhibition of PKC would be expected to decrease the magnitude of potentiation, regardless of which of two possible models was followed. Firstly,

if PKC was negatively regulating P2Y2-mediated Ca^{2+} release, and CXCR2 somehow lifted this regulation, pre-inhibition of PKC would be expected to reduce potentiation, since the role of CXCR2 would have been pre-empted. Alternatively, if P2Y2-activated PKC activates some downstream factor that is permissive to CXCR2-mediated Ca^{2+} signalling, inhibition of PKC would prevent this from occurring and potentiation would be reduced. However, inhibition of PKC using the broad specificity PKC inhibitor, staurosporine, had no effect on the magnitude of potentiation (Fig. 4.2), indicating that PKC phosphorylation is neither permissive nor prohibitive to this communication, and that neither of these models can be used to describe this crosstalk. In addition, the use of staurosporine has the added advantage that, at the concentration used, it also inhibits tyrosine kinase activation (Secrist *et al*, 1990; Ohmichi *et al*, 1992), thus ruling out any actions of these enzymes.

Section 4.3.3: Possible involvement of PI 3-K

There is some evidence for a possible function of Gβγ subunits in regulating PLCβ membrane association *via* PI 3-K. Studies on PLCβ1 have shown that it associates in the membrane with the PI 3-K product, phosphatidylinositol-3-phosphate (PtdIns(3)P) (Razzini *et al*, 2000). Given that PI 3-K isoforms containing certain catalytic subunits (p110β or p110γ) have heightened responsiveness to Gβγ stimulation (Stephens *et al*, 1994 and 1997), the potentiation of Ca^{2+} signalling could be achieved by causing Gβγ-stimulated PI 3-K activity and subsequent promotion of PLCβ localisation in the membrane. The lack of effect of the PI 3-K inhibitor, wortmannin (Fig. 4.3.1) in blocking the potentiated response to IL-8 suggests that this mechanism is not at play here. The use of the ERK activation assay as a positive control for PI 3-K inhibition by 300nM wortmannin (Fig. 4.3.2) was not ideal, given that factors other than PI 3-K activate ERK in these cells (Gao *et al*, 1999; Vindis *et al*, 2000; Turner *et al*, 2001). In addition, the stimulation of ERK activity by serum in quiescent cells was surprisingly small. An assay such as the inhibition of PKB/Akt activation (which is

entirely dependent on PI 3-K activity (Burgering & Coffer, 1995; Franke *et al*, 1995; Meier *et al*, 1997)) may have been preferable. However, it has been shown previously that an incubation similar to that used here (20mins, 300nM wortmannin) abolishes PI 3-K activity in these and other cell types (Meier *et al*, 1997; van der Kaay *et al*, 1997; Sweeney *et al*, 2001; Hunyady *et al*, 2002; Fujino *et al*, 2002). Therefore, the reduction of ERK activation to nearly basal levels is probably an acceptable positive control for the activity of wortmannin.

Section 4.3.4: Alternative Ca^{2+}-releasing messengers

The potentiation of signalling downstream of P2Y2 receptors and CXCR2 was visible not only at the level of Ca^{2+} release, but also at the level of phosphoinositide generation (Fig. 4.12), albeit on a different time-scale. The demonstration that simply increasing $InsP_3$ concentration is sufficient to induce potentiation is strong evidence against the involvement of other, non-$InsP_3$-sensitive stores such as those accessed *via* ryanodine receptors. Nevertheless, there is convincing evidence for co-operativity between $InsP_3$ receptors and ryanodine receptors that demanded some form of investigation into the potential involvement of these stores. There is considerable interplay between $InsP_3$, cADPR and the novel Ca^{2+}-releasing mediator, nicotinic acid-adenine dinucleotide phosphate (NAADP; Genazzani *et al*, 1997) in terms of mediating the strength of Ca^{2+} signalling in some cell types (Cancela *et al*, 1999 and 2002; Churchill and Galione, 2001; Santella *et al*, 2000). The thapsigargin insensitivity of NAADP-sensitive stores (Genazzani and Galione, 1996) perhaps rules out this store in terms of the crosstalk between P2Y2 receptors and CXCR2, which was shown in Chapter 3 to be thapsigargin-sensitive. It is interesting to note, however, that NAADP production is often stimulated by increases in cAMP (Wilson & Galione, 1998), suggesting a possible role in instances of crosstalk involving $G\alpha_s$-coupled receptors such as the β2 adrenoceptor, studied here for its interaction with the muscarinic M3 receptor. Unlike the

P2Y2 receptor-CXCR2 crosstalk, this interaction is not manifested as a potentiation of $InsP_3$ generation (see Chapter 5), and thus a mechanism involving synergy between $InsP_3$ and NAADP could have a role in the potentiation of Ca^{2+} signalling by these receptors.

More relevant to the P2Y2 receptor-CXCR2 crosstalk is the interplay between $InsP_3$ and the ryanodine receptor agonist, cADPR. In pancreatic acinar cells, $InsP_3$, NAADP and cADPR appear to act in a co-operative manner to perpetuate Ca^{2+} oscillations (Cancela *et al*, 1999 and 2002), apparently by facilitating the spread of CICR from the apical pole into the basolateral region, across the mitochondrial barrier that exists in these cells (Tinel *et al*, 1999). Similar priming of CICR seen in sea urchin eggs appears to be due to over-loading of these $InsP_3$- and cADPR-sensitive stores (Churchill and Galione, 2001), a process which is known to decrease the EC_{50} for Ca^{2+} release (Nunn and Taylor, 1992). However, the ramifications of the failure of ryanodine (an antagonist of ryanodine receptors) to interfere with the crosstalk between P2Y2 receptors and CXCR2 (Fig. 4.5) are two-fold. Firstly, it suggests that ryanodine receptor–gated stores are not directly responsible for the additional Ca^{2+} released by the simultaneous presence of UTP and IL-8, although there is still the possibility that Ca^{2+} from these stores may be moved into the $InsP_3$-sensitive store for release from there. Secondly, these data also rule out the possibility that ryanodine receptors are mediating the facilitation of a CICR that may account for the secondary response seen following administration of IL-8 in the presence of UTP.

The use of DMS to block sphingosine kinase was intended to rule out any involvement of the sphingosine-1-phosphate Ca^{2+} release pathway, an involvement that, even theoretically, was difficult to reconcile with a feasible model of communication between two GPCRs that could result in enhanced Ca^{2+} signalling. More likely is that coupling to sphingosine kinase may serve to bolster an existing Ca^{2+} response, since there is no precedent for a receptor being

encouraged to couple to sphingosine kinase by another GPCR. Therefore, the lack of effect of DMS (Fig. 4.4.2) was unsurprising. The reason for the small but significant potentiation of the Ca^{2+} response to a co-addition of 3μM UTP and 10nM IL-8 in the presence of DMS (Fig. 4.4.2b) is unknown, but may be due to some non-specific effect of DMS.

Section 4.3.5: PLCβ substrate supply

The removal of some rate-limiting step in the PLCβ/$InsP_3$ Ca^{2+} release pathway would be a reasonable mode of signal potentiation. A possible locus for such a function would be the rate of re-supply of the PLC substrate, PtdIns(4,5)P_2 or its precursor PtdIns(4)P. There is some evidence that cells are able to preserve PtdIns(4,5)P_2 levels at the expense of PtdIns(4)P (Willars *et al*, 1998) and that the generation of PtdIns(4)P from PtdIns *via* the PI 4-kinase activity may be rate limiting in the generation of PtdIns(4,5)P_2 (Willars *et al*, 1998). Thus, an increase in the rate of PtdIns(4,5)P_2 supply, through activation of PtdIns 4-kinase (and/or possibly PtdIns(4)P 5-kinase (PtdIns(4)P 5-K)) could enhance phospholipid and Ca^{2+} signalling. There is evidence that these kinases are regulated by agonist stimulation, through either direct (i.e. not through a secondary mediator) GPCR stimulation of PI 4-K (Huang *et al*, 2002) and PtdIns(4)P 5-K(Stephens *et al*, 1993), or indirect mechanisms *via* various factors including PKC (Conway *et al*, 1993), GTP (Smith & Chang, 1989), phosphatidic acid (Moritz *et al*, 1992) and Rho proteins (Chong *et al*, 1994; Zhang *et al*, 1996). Indeed, it has been argued that agonist-mediated increases in PtdIns(4,5)P_2 are responsible for potentiation between $G\alpha_q$-coupled M_1 and M_3 muscarinic receptors (Offermans *et al*, 1994; Schmidt *et al*, 1996a and b) (Fig. 4.20). Roles have been proposed in this regulation for Rho GTPases (Chong *et al*, 1994; Chikumi *et al*, 2002) and small GPCR-activated tyrosine kinases of the Src family (Liu & Sturek, 1996; Semenchuk & Di Salvo, 1995; Babnigg *et al*, 1997; Rümenapp et al, 1998; Tolloczko *et al*, 2000). Src family proteins are activated by Gβγ

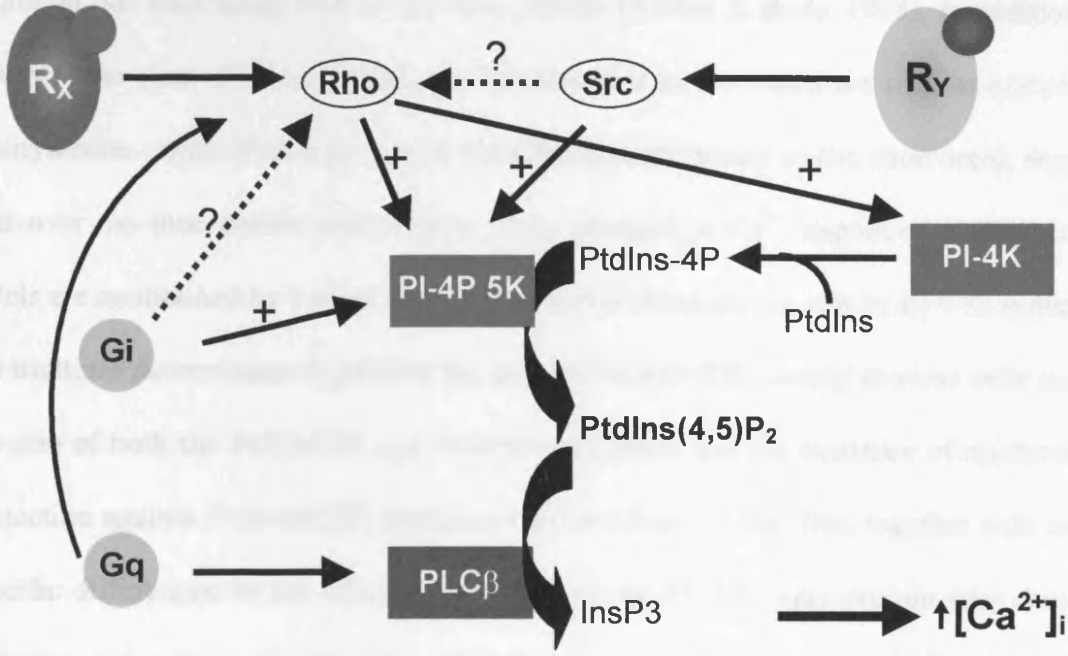


Figure 4.20: Schematic showing the PLCβ substrate replenishment model. A $G\alpha_{q/11}$-coupled GPCR causes elevated intracellular $[Ca^{2+}]$, but does so within limits maintained by the supply of PLCβ substrate, PtdIns(4,5)P_2. Gi proteins derived from stimulation of an accessory receptor (or from infidelity within the same receptor population; see text), activate PtdIns(4)P 5-K, accelerating PtdIns(4)P conversion into PtdIns(4,5)P_2, lifting the restraints on PLCβ signalling and potentiating $InsP_3/[Ca^{2+}]_i$ responses. Alternatively, Rho proteins, activated downstream of a GPCR (R_X; e.g. PGDF- or thrombin receptors), or Src tyrosine kinases (activated by e.g. serotonergic receptors (R_Y)) are also capable of regulating PI 4-kinase and PtdIns(4)P 5-K activity.

subunits downstream of many GPCRs (Igishi & Gutkind, 1998), and as such may be considered good candidates for potentiating the signalling of a co-stimulated, $G\alpha_q$-mediated pathway. For these reasons, the effects of inhibition of one of these enzymes, PI 4-K, by 10μM wortmannin was investigated. It was found here that PI 4-K inhibition by wortmannin had no effect on responses to the co-addition of UTP and IL-8 following store depletion (Fig. 4.9.1), suggesting that PI 4-K activity is not a crucial determinant of this response. It was also noted that substrate supply did not appear to be at all limiting to responses to UTP (Fig. 4.9.2). It has been demonstrated previously that wortmannin blockade of PI 4-K is not alone sufficient to affect PtdIns(4,5)P_2 levels in the plasma membrane on a short term basis,

although this does occur over longer time periods (Várnai & Balla, 1998). In addition, these authors also show that less specific phosphoinositide kinase inhibitors such as quercetin and phenylarsine oxide (PAO) do inhibit phosphoinositide levels in the short term, suggesting that over the time course used here to study changes in Ca^{2+} responses, phosphoinositide levels are maintained by a panel of phosphoinositide kinases, not just by PI 4-K. Indeed there are multiple determinants regulating the rate of PtdIns(4,5)P_2 supply in some cells including the size of both the PtdIns(4)P and PtdIns(4,5)P_2 pools and the existence of mechanisms of protection against PtdIns(4,5)P_2 depletion (Willars *et al*, 1998). This, together with any cell-specific differences in the effects of wortmannin on PI 4-K, may explain why there is no apparent acute effect of wortmannin on $InsP_3$-mediated agonist induced Ca^{2+} responses in the HEK-CXCR2 cells, and also why the effects of wortmannin are only seen in a more extended time course. It also suggests that PI 4-K activation is unlikely to have a significant effect on phosphoinositide supply since cellular measures are clearly in place to ensure that PI 4-K activity is not limiting to the magnitude of Ca^{2+} responses seen in these cells. Therefore, the activation of PI 4-K as a potential mechanism underlying the crosstalk demonstrated here is concluded to be highly unlikely.

Section 4.3.6: Phospholipase C

Given adequate substrate supply, one of the more convincing models for Ca^{2+}-potentiating crosstalk is a convergence of signals at PLC, causing up-regulation of PLC activity and enhanced Ca^{2+} release as a simple result of increased second messenger mass. This model fits well with the data presented here. The involvement of $InsP_3$ is confirmed by both the involvement of $InsP_3$ receptors (Fig. 4.7) and the requirement for the PLC substrate, PtdIns(4,5)P_2 (Fig. 4.8), and more directly by the demonstration of enhanced phosphoinositide generation in the combined presence of UTP and IL-8 (Fig. 4.12). In addition, blockade of PLC activity with the putative PLC inhibitor U73122 ablated the

potentiation response. However, following work in other laboratories, questions have been raised over the specificity of some of these pharmacological tools, predominantly 2-APB and U73122. There have been reports that 2-APB, in addition to its activity as an $InsP_3$ receptor antagonist, is also an activator of these same channels at similar concentrations to those studied here (Ma *et al*, 2002). This compound was suggested to completely empty $InsP_3$-gated intracellular stores *via* an action at $InsP_3$ receptors. However, no Ca^{2+} elevation was seen in the HEK-CXCR2 cells upon administration of 2-APB, suggesting that this was not occurring in these cells. However, given that the stores released in the combined presence of UTP and IL-8 are $InsP_3$-sensitive, it is conceded that emptying of such stores would inevitably impact upon any response dependent on them, and the effectiveness of 2-APB in blocking the potentiation response could be due to some inconspicuous effect on the filling state of the Ca^{2+} stores. This is, nonetheless, good evidence that the stores accessed are $InsP_3$-sensitive. It seems unlikely anyway that 2-APB could, by store depletion, cause a 75% inhibition of the UTP-mediated Ca^{2+} release from $InsP_3$-sensitive stores (Fig. 4.6) without that depletion being clearly apparent. It was concluded that, with some minor reservations, 2-APB was acting largely as intended in blocking $InsP_3$ receptors, and that even its potential alternative effects also indirectly implied the same conclusions as those drawn under the assumption that 2-APB is acting solely to inhibit these receptors. A further activity of 2-APB is the blockade of plasma membrane store-operated Ca^{2+} channels (SOCs) (Bootman *et al*, 2002; Wu *et al*, 2002; Ma *et al*, 2002). This occurs at concentrations of 2-APB around ten-fold lower than those used here to block $InsP_3$ receptors. These effects will be of little consequence within the protocol used here given that the experiments were performed in the absence of extracellular Ca^{2+}, and thus any activation of SOCs will not result in Ca^{2+} influx.

The specificity of the PLC inhibitor, U73122, has also been questioned elsewhere. Among the alternative effects of U73122 are modulation of ion channels, both plasmalemmal and

intracellular (i.e. $InsP_3$ receptor channels) (Jin *et al*, 1994; Mogami *et al*, 1997; Pulcinelli *et al*, 1998; Jan *et al*, 1998), potentiation of $InsP_3$ signals (Mogami *et al*, 1997), and interference with Gi/o protein signalling (Walker *et al*, 1998). Controls were included in this study for alternative effects of U73122 and to test its specificity of action.

The report that U73122 inhibits signalling downstream of $G\alpha_i$-coupled GPCRs (Walker *et al*, 1998) would adequately explain its activity in ablating the potentiation response seen following stimulation of the $G\alpha_i$-coupled CXCR2. Furthermore, the effects of U73122 and U73343 were highly variable between even $G\alpha_q$-coupled receptors. The Ca^{2+} responses to UTP were not affected by U73122 (Fig. 4.14.1). In contrast, the responses to either maximal or half-maximal concentrations of carbachol were abolished by the presence of U73122 (Fig. 4.14.2). However, they were also severely reduced by the presence of U73343, suggesting that this action may have been independent of any specific action on PLC. Importantly, this is supported by the failure of U73122 to inhibit UTP-stimulated phosphoinositide production (Fig. 4.15), suggesting that this agent was indeed not having any significant effect on PLC activity. In fact, U73122 actually appeared to elevate basal PLC activity. U73122 has been reported previously to release intracellular Ca^{2+} (Jin *et al*, 1994; Mogami *et al*, 1997; Pulcinelli *et al*, 1998), thought to be through an action at $InsP_3$ receptors. The pre-release of intracellular Ca^{2+} by U73122-stimulated production of phosphoinositides may be the cause of its apparent inhibitory effects on PLC-mediated Ca^{2+} release. However, this does not account for previously reported inhibitory effects of U73122 on phosphoinositide accumulation (Jin *et al*, 1994), unless there is some level of negative feedback on PLC activity by either Ca^{2+} release or PKC stimulation downstream of it. It is accepted here that the use of U73122 as a 'selective' PLC inhibitor has many caveats, and these data would not alone be sufficient to suggest the involvement of PLC enzymes. However, the other data presented here (in particular, the potentiation of phosphoinositide turnover, which is

especially compelling), constitutes a reasonable body of evidence for the convergence of this crosstalk pathway at the level of PLC.

Section 4.3.7: $InsP_3$ receptors

While the potentiation of phosphoinositide generation would adequately explain the enhanced Ca^{2+} signalling seen in these cells, there are other models that cannot be discounted. For instance, certain examples of crosstalk have been shown to be apparently independent of $InsP_3$ generation (Short & Taylor, 2001). This may be because crosstalk was resulting in local and/or transient enhancement of $InsP_3$ generation that was not detectable by the methods used. However, the interaction may genuinely be $InsP_3$-independent, occurring, for example, by a mechanism that involves an increase in the sensitivity of the $InsP_3$ receptors thereby allowing further Ca^{2+} release without the absolute requirement for a concomitant increase in $InsP_3$ levels. PKA is thought to sensitise $InsP_3$ receptors through phosphorylation (Burgess *et al*, 1991; Hajnóczky *et al*, 1993; Wojcikiewicz & Luo, 1998; Bruce *et al*, 2002) and this provides a mechanism through which receptors that elevate cAMP could participate in crosstalk. However, while this may be relevant to the communication demonstrated here between the $G\alpha_s$-coupled β2 adrenoceptors and either muscarinic M3 receptors or P2Y2 nucleotide receptors (discussed further in Chapter 5), it seems unlikely to be involved in the crosstalk between nucleotide receptors and the $G\alpha_i$-coupled CXCR2. Nevertheless, the effects of the $InsP_3$ receptor sensitising agent, thimerosal, were tested in the HEK-CXCR2 cells. It was found that although thimerosal potentiated UTP-stimulated Ca^{2+} signalling, it had little effect on the potentiation response (Figs. 2.17 and 2.18). The interpretation of these data is complex. Thimerosal should in theory enhance the observed Ca^{2+} response to a given quantity of $InsP_3$, regardless of what agonist(s) generates it. This is clearly not the case (Fig. 4.16 and Fig. 4.17). That the response to IL-8 following UTP pre-stimulation is not greater than it is in the absence of thimerosal may

suggest a limitation imposed by the size of the Ca^{2+} store – the thimerosal-potentiated UTP response may have drained the store such that no further Ca^{2+} is available for a thimerosal-potentiated response to IL-8. However, this is shown not to be the case using a low concentration of UTP as the pre-stimulant (Fig. 4.17). Even in the presence of thimerosal, the response to 1μM UTP is only ~50% of the maximal response achievable by 100μM UTP, indicating that ample Ca^{2+} remains in the store. However, thimerosal still does not potentiate the subsequent response to IL-8, showing that the limited size of the Ca^{2+} store is not attenuating the response to IL-8 in the presence of thimerosal. Alternatively, if CXCR2 somehow sensitises the $InsP_3$ receptor, accounting for the further release of Ca^{2+} by increasing the accessibility of Ca^{2+} stores to P2Y2-stimulated $InsP_3$, then thimerosal pre-sensitisation of the $InsP_3$ receptor should negate any effect of CXCR2 stimulation. However, IL-8 clearly does still stimulate a response in the presence of thimerosal. In short, unless the Ca^{2+} response to UTP and IL-8 co-addition is not $InsP_3$-mediated, thimerosal would be expected to either increase or decrease the response to IL-8 in the presence of UTP, but in fact does neither. It is concluded from these data that CXCR2 does not mediate the sensitisation of $InsP_3$ receptors such that UTP-generated $InsP_3$ releases a further amount of Ca^{2+}, given that the presence of thimerosal does not supplant this action.

A further test of this $InsP_3$ receptor sensitisation hypothesis was the proposed use of the cell-permeant $InsP_3$ ester, $InsP_3$/BM (Li *et al*, 1997). Perfusion of $InsP_3$/BM should allow identical rates of entry into cells irrespective of agonist treatments. Therefore, sensitisation of $InsP_3$ receptors by stimulation of CXCR2 could be assessed by analysing the latency of a response to $InsP_3$/BM. The response should occur more rapidly if the system if 'primed' by IL-8 as the threshold for an $InsP_3$-mediated response will be exceeded in a shorter period of $InsP_3$/BM perfusion. However, unavailability of this analogue precluded against performing this experiment.

Section 4.3.7: Quantal Ca^{2+} release

The utilisation of an alternative experimental protocol using perfusion systems allowed more flexibility in terms of the number of additions that could be made, and isolated the potentiation response from any concomitant UTP-mediated release. The design of the protocol achieves maximum unloading of the UTP-sensitive store with minimum exposure to agonist, therefore minimising the extent of P2Y2 receptor desensitization, which would complicate interpretation of data generated using this protocol. Use of this method demonstrates the involvement of a UTP-insensitive, yet still $InsP_3$-sensitive, Ca^{2+} store, which suggests a level of organisational complexity within this store. It appears to be exquisitely regulated, being apparently sub-divided into minute fractions, with specific agonists only able to release a certain proportion of these fractions. This is supported by the demonstration that the store accessed by UTP is at least partially discrete from those accessed by IL-8 (in the presence of UTP), 2MeSADP, and carbachol (Figs. 2.19 and 2.20), and by the evidence of similar phenomena reported elsewhere (Short *et al*, 2000).

The inability of UTP to release the entire Ca^{2+} store by repeated stimulation suggests that this ER store does not act as a singular contiguous reservoir, more as a series of connected but discrete cisternae (reviewed by Blaustein and Golovina, 2001). This concept has been supported by work showing that during the elevation of $InsP_3$, the ER $[Ca^{2+}]$ ($[Ca^{2+}]_{ER}$) can become heterogeneous, suggesting that Ca^{2+} in these sub-divisions of the ER does not automatically compensate for loss of Ca^{2+} from adjacent regions (Arnaudeau *et al*, 2001; Montero *et al*, 1997). This spatial regulation may point to a need for close apposition of components of a signalling pathway, including the receptor, G-protein and the effector (PLC), and that only those regions of ER in close juxtaposition to these complexes are accessible to them, leaving more distant stores untouched. How this may be regulated is

unclear, but evidence that P2Y receptors are restricted in their coupling to certain PLC isoforms may suggest that the localisation of specific PLCβ isoforms may dictate which regions of ER are accessible (Strassheim & Williams, 2000; Arthur *et al*, 2001). The implication here is that each signalling complex considered in isolation has a limited radius of activity determining the 'range' within which $InsP_3$ can exert its effects. This depends on how much $InsP_3$ generation the receptor/PLC complex can mediate, a situation also dependent on agonist concentration. For an increase in $InsP_3$ generation to be effective as a mode of Ca^{2+} response potentiation, the boundaries between these ranges must be clear i.e. there must be little or no exchange of Ca^{2+} across these boundaries. This property allows UTP to drain only within a particular range (the "UTP-sensitive" store), while the co-addition of UTP and IL-8 enhances $InsP_3$ production, extends the range of $InsP_3$ action, and accesses a Ca^{2+} store up to a more distant boundary (the "UTP-IL-8 sensitive" store). In the absence of quantal Ca^{2+} release, repeated stimulation with a Ca^{2+}-releasing agonist (e.g. UTP) in the absence of store refilling would eventually drain the entire cellular Ca^{2+} pool, and any further response to the addition of a secondary agonist (e.g. IL-8) would not be possible. Therefore, the Ca^{2+} released in response to a given concentration of an agonist is characterised by the total drainage of a section of the store within a very localised range, rather than by fractional drainage of the store on a more global scale. It is concluded that quantal Ca^{2+} release is crucial for a cell to mediate Ca^{2+}-potentiating GPCR crosstalk *via* enhancement of $InsP_3$ production.

This chapter aimed to elucidate some of the crucial components of the crosstalk pathway that mediates Ca^{2+} response potentiation downstream of co-stimulated CXCR2 and P2Y2 receptors. The finding that P2Y2 receptors and CXCR2 interact at the level of phosphoinositide generation as well as at the level of Ca^{2+} release suggests that the mechanism underlying the interaction involves the PLCβ/$InsP_3$ pathway of Ca^{2+} release. This

is confirmed by the demonstration that 2-APB, an inhibitor of $InsP_3$ receptors, blocks the response to IL-8 in the presence of UTP, as does the depletion of the PLCβ substrate phospholipid, $PtdIns(4,5)P_2$. Evidence that inhibition of other signalling factors such as PKC, PI 3-K, and alternative Ca^{2+} signalling second messengers does not impinge upon IL-8 responses following UTP stimulation allows the elimination of several potential mechanisms, and narrows the field of candidate mechanisms to a more manageable number. The following chapter will directly address the issue of the mechanism causing the observed potentiation, attempting to directly test the favoured hypothesis. It will then go on to identify why P2Y1 receptors are deficient in their ability to participate in a similar interaction, and to determine whether crosstalk between muscarinic M3 receptors and β2 adrenoceptors utilises the same pathway in potentiating Ca^{2+} release

Chapter 5: Evidence for convergence of GPCR signalling at phospholipase C, but also for potential alternative mechanisms of crosstalk in HEK cells

Section 5.1: Introduction

The work in the previous chapter was aimed at identifying some of the main components of the mechanism underlying the potentiation between CXCR2 and P2Y receptors, and found that it involved the PLCβ/$InsP_3$ mode of Ca^{2+} release. There are a number of points at which signal convergence could lead to enhancement of $InsP_3$-mediated Ca^{2+} signalling. However, the fact that phosphoinositide generation was also enhanced in the presence of both UTP and IL-8 suggested that the interaction could involve enhanced activation of PLCβ by a direct action at this enzyme or at some point upstream of it. The data presented in the previous chapter correlate well with a limited number of mechanistic models. Experiments in this chapter aim to gain a clearer understanding of the mechanism underlying this crosstalk by addressing these specific models of interaction.

There is a substantial amount of published evidence that PLCβ activity can be modulated by crosstalk between GPCRs, and the ability of this enzyme to respond to varying extents to stimulation by both $G\alpha_{q/11}$ and Gβγ subunits is key to this. From studies on purified PLCβ and in whole cell preparations, it has become apparent that PLCβ can be regulated by $G\alpha_q$ and Gβγ in a co-operative manner (Smrcka & Sternweis, 1993; Schmidt *et al*, 1996c and 1998) although the mechanisms underlying this are not absolutely clear. Binding of $G\alpha_q$ to a PLCβ (possibly PLCβ3) in intact cells has been proposed to prime the enzyme to subsequent activation by Gβγ subunits derived from activated δ-opioid receptors (Yoon *et al*, 1999).

This type of mechanism has also been proposed from studies on the muscarinic M_2 receptor co-transfected with $G\alpha_q$ or $G\alpha_{16}$ (Zhu & Birnbaumer, 1996), where the muscarinic receptor stimulated a PTX-insensitive inositol phosphate response that was synergistically enhanced by the additional presence of excess $G\beta\gamma$ subunits. Exactly how $G\alpha_q$ sensitises PLCβ to $G\beta\gamma$ subunits is not clear but it could involve a conformational change in PLCβ that relieves a steric hindrance to $G\beta\gamma$ binding. Thus, in the presence of $G\alpha_q$, $G\beta\gamma$ could have easier access to its binding site and activate PLCβ more strongly (see Fig. 5.1, A→B). This could explain a number of crosstalks between $G\alpha_q$-coupled receptors and various $G\alpha_s$- or $G\alpha_i$-coupled receptors, where Ca^{2+} responses to $G\alpha_s$- and $G\alpha_i$-coupled receptors are enhanced by prior or concomitant stimulation of a co-expressed $G\alpha_q$-GPCR (Dickenson *et al*, 1993; Dickenson & Hill, 1996, 1998a and b; Connor et al, 1996, 1997a and b; Short & Taylor, 2001). However, it is also known that certain crosstalks do not involve this mechanism (Tomura *et al*, 1997). The interaction between adenosine A1 receptors and thyrotropin receptors in COS-7 cells could not be mimicked by over-expressed Gβ and Gγ subunits following pre-stimulation of PLC with a constitutively-active $G\alpha_{11}$ subunit. This was concluded to rule out the possibility of any synergy between $G\alpha_{q/11}$ and $G\beta\gamma$ at PLCβ. The adenosine-thyrotropin receptor crosstalk was instead attributed to an increase in the number of $G\alpha_{q/11}$ activation cycles downstream of the thyrotropin receptor, with adenosine A1 receptor-derived $G\beta\gamma$ subunits delimiting the rate at which inactive GDP-$G\alpha_q$ was re-associated with the active receptor as part of the GDP-$G\alpha\beta\gamma$ heterotrimer. However, the $G\alpha_q$-mediated priming of PLCβ to $G\beta\gamma$ derived from $G\alpha_i$-coupled GPCRs (Yoon *et al*, 1999; see Fig. 5.1, A→B) is strongly supported by experimental evidence. Thus, the coupling of certain receptors to stimulation of PLCβ is potentiated by either co-activation of $G\alpha_q$-coupled GPCRs or by co-expression of constitutively active $G\alpha_q$ subunits (Schmidt *et al*, 1996a; Zhu *et al*, 1996; Chan *et al*, 2000). Part of this chapter examines the involvement of $G\alpha_q$ and $G\beta\gamma$ subunits in the crosstalk

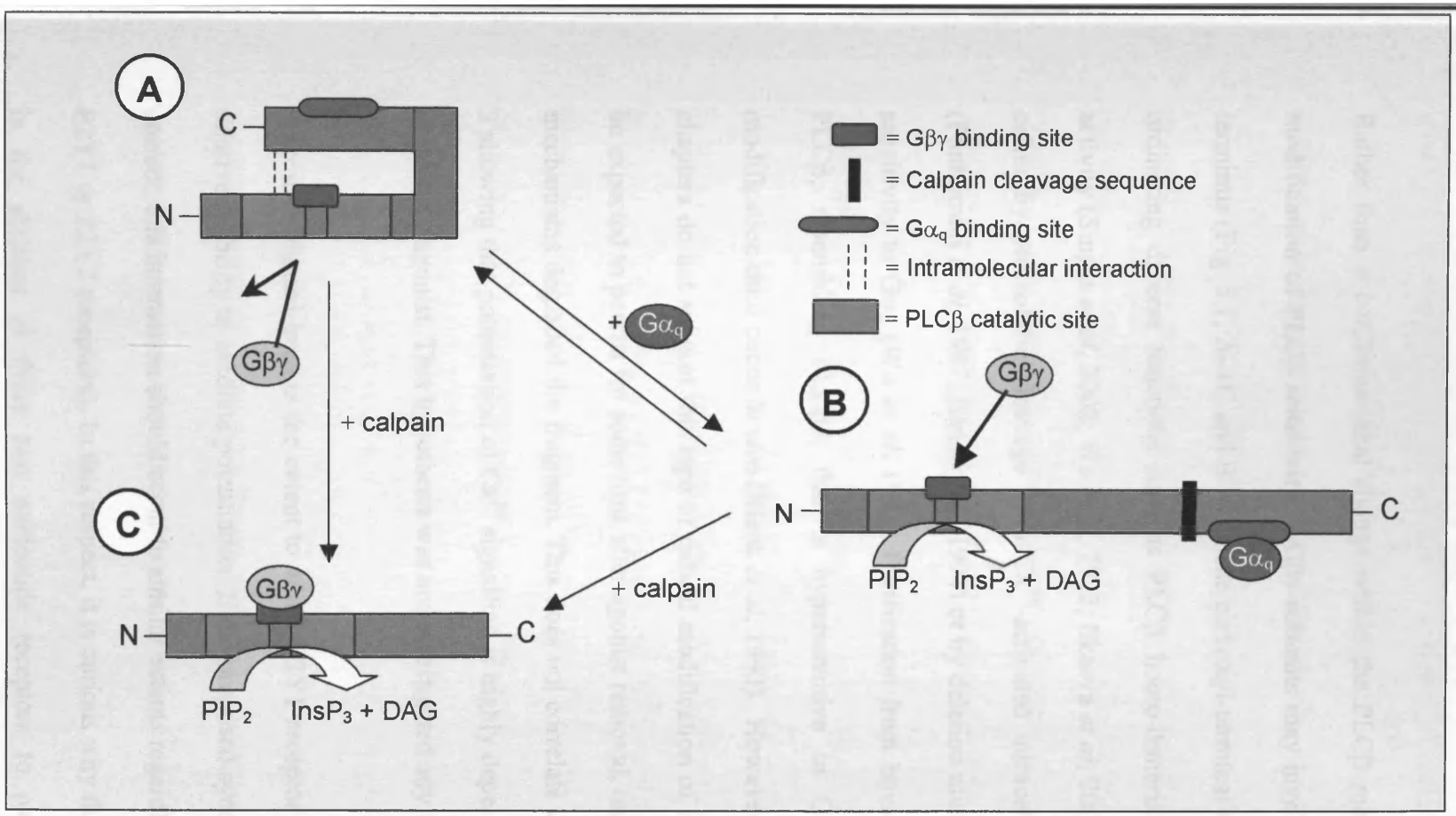


Fig. 5.1: Schematic showing proposed interactions between PLCβ, calpain and the $G\alpha_q$ and Gβγ subunits of GPCRs. In the unstimulated PLCβ enzyme (A), Gβγ has restricted access to Gβγ-binding site. However, in B, binding of $G\alpha_q$ causes both PLCβ activation and alteration of protein tertiary structure such that the Gβγ binding site becomes accessible, allowing Gβγ to also stimulate PLCβ activity. It is not clear whether unfolding is required for PLCβ activation by $G\alpha_q$. The Gβγ binding site may also be revealed by C-terminal truncation of either the folded or unfolded forms (C), possibly by calpain activated, for example, downstream of a GPCR.

between CXCR2 and P2Y2 receptors, with the intention of testing this hypothesis as the most likely method of interaction between these receptors.

Rather than a conformational change within the PLCβ molecule, the mechanism for the modification of PLCβ sensitivity to Gβγ-subunits may involve the removal of its carboxyl terminus (Fig. 5.1, A→C and B→C). The carboxyl-terminal tail is crucial to the enzyme, co-ordinating diverse functions such as PLCβ homo-dimerization, $G\alpha_q$ binding, and GAP activity (Singer *et al*, 2002; Wu *et al*, 1992; Ilkaeva *et al*, 2002). Removal of the C-terminus, either by proteolytic cleavage by a Ca^{2+}-activated intracellular protease such as calpain (Pontremoli *et al*, 1987; Banno *et al* 1994) or by deletion mutation (Lee *et al* 1993) abolishes sensitivity to $G\alpha_q$ (Wu *et al*, 1992). Purification from bovine brain cytosol of a truncated PLCβ, resembling PLCβ3, that is hypersensitive to Gβγ, indicates that C-terminal modification does occur *in vivo* (Blank *et al*, 1993). However, the data presented in previous chapters do not support this type of radical modification of PLCβ. A cleaved protein would be expected to persist for some time after agonist removal, until the normal cellular recycling mechanisms degraded the fragment. This does not correlate well with the data from Chapter 3 showing that potentiation of Ca^{2+} signalling is highly dependent on the continued presence of primary agonist. This hypothesis was not investigated any further.

Also investigated here is the extent to which P2Y1 receptor desensitisation accounts for its relative inability to mediate potentiation. If the proposed synergy between $G\alpha_{q/11}$ and Gβγ is correct, this interaction should occur to similar extents regardless of the source of $G\alpha_{q/11}$ (e.g. P2Y1 or P2Y2 receptors). In this respect, it is curious why there is such a marked difference in the abilities of these two nucleotide receptors to participate in potentiation. The demonstration that P2Y1 does substantially desensitise, allied with the previous evidence that continued receptor activation is absolutely required for potentiation, would adequately

explain the failure of P2Y1 receptors to act in the same way as P2Y2 receptors. While a role for PKC was ruled out in the previous chapter, there is a potential role for other cellular kinases such as the GRKs. The possibility that desensitisation of P2Y1 receptors following receptor phosphorylation (leading to e.g. receptor-G-protein uncoupling) may explain its inability to mediate potentiation was addressed directly with two protocols designed to minimise the amount of P2Y1 receptor stimulation prior to the addition of IL-8 such that this receptor should not be substantially desensitised at this point. In addition, phosphatase inhibitors were used to investigate whether a particular phosphorylation state of the P2Y2 receptor or of some downstream factor was necessary for potentiation i.e. whether inhibition of the removal of phosphate attenuated or enhanced the observed potentiation response.

There may also be a role for PKA in the regulation of PLCβ activity. Inhibitory PKA actions against $G\alpha_q$-mediated Ca^{2+} responses are reasonably commonplace (Laglia *et al*, 1996; Hoiting *et al*, 1996; Liu & Simon, 1996; Yue *et al*, 1998 and 2000; Dodge & Sanborn, 1998), and can be due either to direct influence on PLCβ activity (Yue *et al*, 1998) or at an alternative locus (Hoiting *et al*, 1996). However, there are instances where PKA increases the activity of PLCβ (Blackmore & Exton, 1986; Pittner & Fain, 1989). Nevertheless, PKA activation would not be expected to occur downstream of CXCR2 given its coupling to adenylyl cyclase-inhibiting $G\alpha_i$ proteins, so this is highly unlikely to account for the crosstalk between CXCR2 and P2Y2. It may have a role in the crosstalk between the muscarinic M3 receptor and β2 adrenoceptor, but any activity of PKA could be argued to be occurring at a number of diverse loci other than PLCβ. This issue is also dealt with further herein.

This chapter aims to address specific mechanisms of GPCR crosstalk to determine the way in which P2Y2 receptors and CXCR2 interact to induce Ca^{2+} signal potentiation. The

involvement of $G\alpha_q$ and $G\beta\gamma$ will be tested to provide evidence as to whether the model of synergy between $G\alpha_q$ and $G\beta\gamma$ subunits described above is valid. In addition, this chapter will also attempt to define whether P2Y1 receptor desensitisation underlies the relative inability of these receptors to participate in potentiation. Finally, the interaction between muscarinic M3 receptors and β2 adrenoceptors will be investigated to assess whether it utilises the same mode of interaction as P2Y2 receptors and CXCR2.

Section 5.2: Results

Section 5.2.1: Potentiation cannot be explained by simple additivity of $InsP_3$ responses

It is possible that similar examples of potentiation could be due to the addition of two sub-threshold concentrations of $InsP_3$ that together exceed threshold and stimulate Ca^{2+} release from intracellular stores. It seems unlikely that either IL-8 or isoproterenol could contribute enough $InsP_3$ to initiate a secondary response of the magnitude seen here without breaching threshold itself. Nevertheless, the theory was tested that these two agonists could be combined to breach threshold. Cells were stimulated with each agonist alone (10nM IL-8, 10μM isoproterenol), and then with a co-addition of both together. These treatments did not stimulate an observable intracellular Ca^{2+} response (Fig. 5.2).

Section 5.2.2: Quantification of G-protein activation is not possible when using nucleotide agonists

To assess whether the potentiation described in this study was due to enhanced activation of G-proteins, stimulations were performed in HEK-CXCR2 membrane preparations in the presence of a radio-labelled, non-hydrolysable GTP analogue, [^{35}S]-GTPγS (Fig. 5.3). This GTP analogue binds relatively irreversibly to activated G protein α-subunits, and quantification of membrane-bound radioactivity can therefore be used as a measurement of G-protein activation. Based on studies using a similar protocol, membranes were stimulated for 40mins (Clark *et al*, 1996; Walker *et al*, 1998; Rouleau *et al*, 2002) with either IL-8, UTP, or a combination of both. IL-8 alone at 100nM or 10nM stimulated an appreciable increase in [^{35}S]-GTPγS binding (approximately 196% and 167% of basal, respectively). However, UTP at 1mM or 100μM caused a substantially reduced [^{35}S]-GTPγS binding (17% and 30% of basal, respectively) (Fig. 5.3a). Furthermore, both co-additions (1mM UTP + 100nM IL-8, and 100μM UTP + 10nM IL-8) exhibited reduced [^{35}S]-GTPγS binding

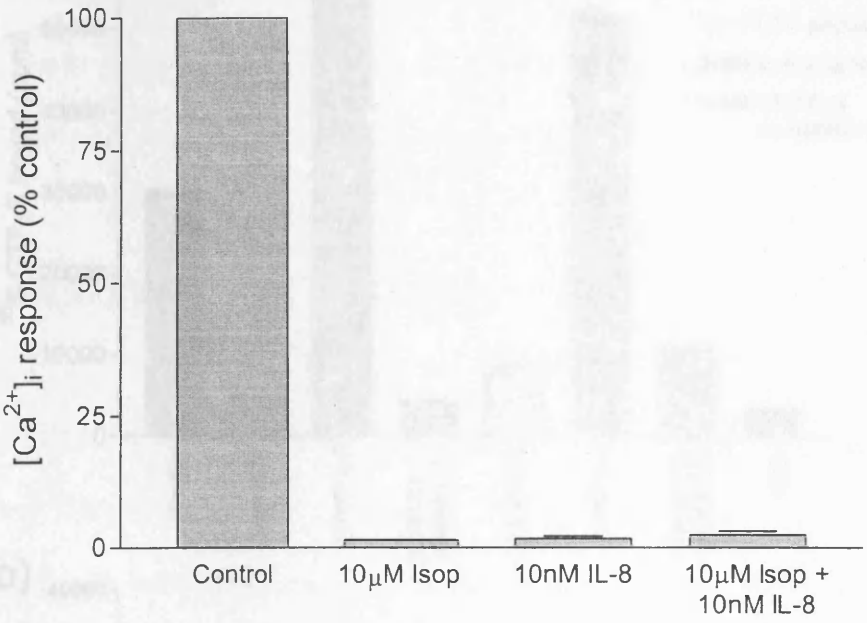


Figure 5.2: Response additivity does not underlie potentiation. HEK-CXCR2 cells were grown on glass coverslips and loaded with fluo-3/AM as described in Materials and Methods. Ca^{2+} stores were drained with repeated UTP stimulation in the absence of extracellular Ca^{2+} as in Fig. 4.1. Following this pre-treatment, and after UTP was removed, cells were stimulated with either 10μM isoproterenol (Isop) or 10nM IL-8, or a combination of both. Fluo-3 fluorescence changes were measured using a Ca^{2+} imaging system as an index of $[Ca^{2+}]_i$ elevation. Values shown are the responses to additions made after store drainage compared to maximal response to 100μM UTP ("Control"). Data are mean±s.e.m., n=4.

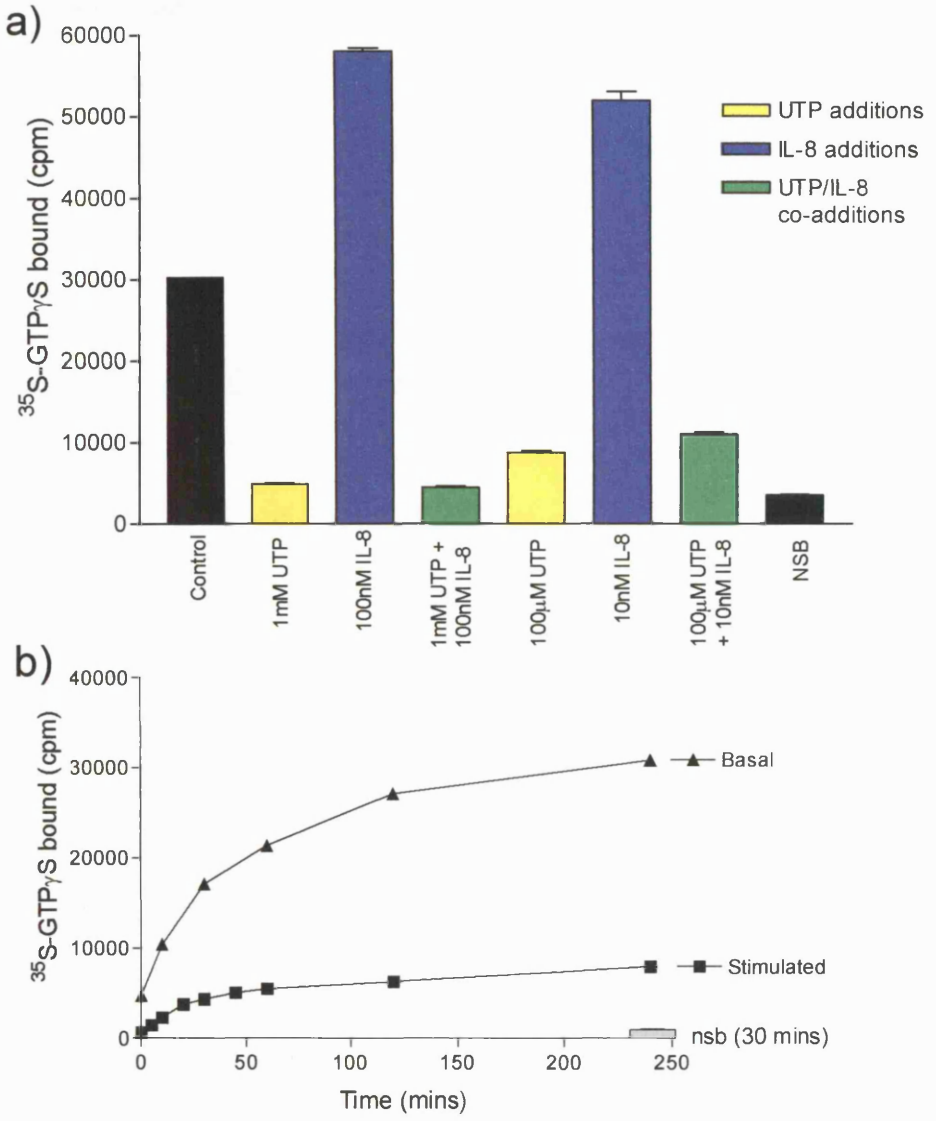


Figure 5.3: UTP paradoxically inhibits [^{35}S]-GTPγS binding. Total [^{35}S]-GTPγS binding was performed on HEK-CXCR2 cell membranes as a measure of G-protein activation. Membranes were incubated at 30°C with agonist, 10μM GDP and 100pM [^{35}S]-GTPγS, then captured on GF-B filters. Bound radioactivity was quantified by liquid scintillation counting. Values are bound radioactivity, expressed as counts per minute (cpm) per sample. Data are a single data set (n=1), performed in triplicate. a) 40min agonist stimulation with indicated agonist combinations or buffer (Control). NSB = non-specific binding, determined by [^{35}S]-GTPγS binding in the presence of 10μM unlabelled GTPγS. b) Time course of stimulation of [^{35}S]-GTPγS binding by 1mM UTP (Stimulated) compared to basal. Non-specific binding was determined at 30mins.

(approximately 15% and 36% of basal, respectively). [^{35}S]-GTPγS binding following any drug additions containing UTP was only marginally greater than non-specific binding (12% of basal). In addition, a time course of G-protein activation following variable durations of stimulation with 1mM UTP showed that, at all time points studied between 10mins and 240mins, UTP inhibited [^{35}S]-GTPγS binding (Fig. 5.3b).

Section 5.2.3: Effects of phosphatase inhibition

To test whether a dephosphorylation event was enabling the potentiated Ca^{2+} signalling seen following addition of IL-8 in the presence of UTP, the effects of two phosphatase inhibitors were studied. Okadaic acid is an inhibitor of protein phosphatases 1 and 2A (Holmes & Boland, 1993). A 5min incubation with okadaic acid was included in the Ca^{2+} imager protocol described in Fig. 4.1. The subsequent response to a co-addition of UTP (either 100μM or 3μM) with 10nM IL-8 was not significantly affected by okadaic acid (Fig. 5.4). An inhibitor of PP2B, cyclosporin A (CsA; Groblewski *et al*, 1994), was also investigated, using the FLIPR protocol illustrated in Fig 1.2. A 20min incubation with CsA (10μM) caused a significant increase in the response to 100μM UTP alone (142±7% relative to control; $P<0.01$; Fig. 5.5a), but did not affect the response to 3μM UTP (Fig. 5.5b). CsA had no significant effect on the response to co-addition of UTP (100μM or 3μM) and IL-8 (Fig. 5.5c and Fig. 5.5d).

Section 5.2.4: Effects of RGS2-mediated increase in $G\alpha_q$ GTPase activity

Co-operativity between $G\alpha_q$ and $G\beta\gamma$ subunits in causing PLCβ activation seems to be one of the more likely explanations for the potentiation of both phosphoinositide turnover and Ca^{2+} responses demonstrated here. In order to assess the participation of $G\alpha_q$ subunits, HEK-

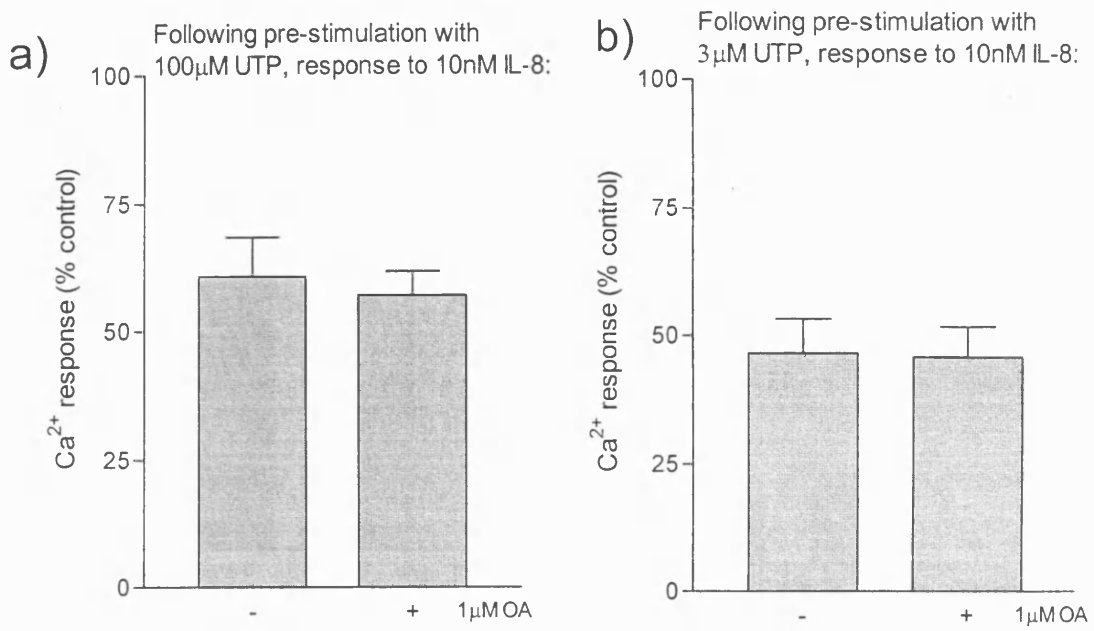


Figure 5.4: Effects of phosphatase inhibition on potentiation. HEK-CXCR2 cells were grown on glass coverslips and loaded with fluo-3/AM as described in Materials and Methods. An incubation with okadaic acid (OA; 1μM, 5 mins) was included at Point A (Fig. 4.1). Changes in $[Ca^{2+}]_i$ were assessed using a Ca^{2+} imaging system. a) Response to co-addition of 100μM UTP and 10nM IL-8 in the presence and absence of okadaic acid. b) As a), but with 3μM UTP. Values are expressed as measured changes in $[Ca^{2+}]_i$ in relation to the maximal response achieved with 100μM UTP, as measured by fluo-3 fluorescence. Data are mean±s.e.m., n=4.

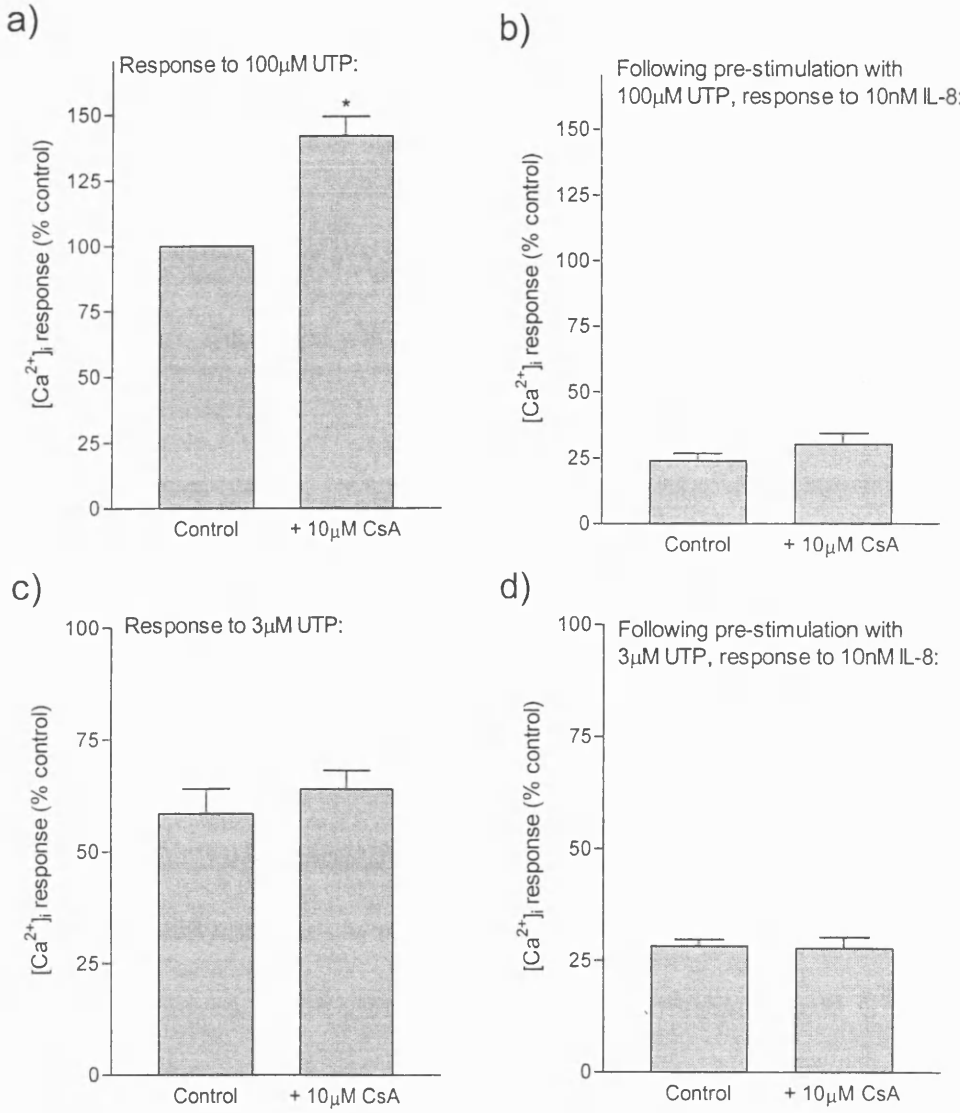


Figure 5.5: Effects of phosphatase inhibition on potentiation. HEK-CXCR2 cells were prepared for FLIPR assay as described in Materials and Methods. Using the FLIPR, cells were incubated for 20mins prior to assay with the PP2B phosphatase inhibitor, cyclosporin A (CsA; 10μM) before stimulating, firstly with 100μM UTP and subsequently with 10nM IL-8 in the continued presence of UTP. a) and c) Response to 100μM and 3μM UTP, respectively, in the presence and absence of CsA. b) and d) Responses to co-addition of 100μM UTP and 10nM IL-8 (b) or 3μM UTP and 10nM IL-8 (d) in the presence and absence of CsA. Values are expressed as changes in $[Ca^{2+}]_i$ in relation to the maximal response achieved with 100μM UTP, as measured by fluo-3 fluorescence. Data are mean±s.e.m., n=4. * $P<0.05$, by unpaired Student's t-test.

CXCR2 cells were transfected with RGS2, a GAP that attenuates $G\alpha_q$ signalling by accelerating specifically $G\alpha_q$ intrinsic GTPase activity[§] (Heximer *et al*, 1997), while having little apparent effect on $G\alpha_i$ signalling (Kammermeier & Ikeda, 1999). HEK-CXCR2 cells were transfected with RGS2-containing vector (or 'empty' vector containing no DNA insert) and grown under blasticidin selection to create a non-clonally selected, semi-stable culture of RGS2-expressing cells. HEK-CXCR2/RGS2 cells (or 'empty' pTracer-transfected HEK-CXCR2 cells) were stimulated with 100μM UTP followed, 150s later, with 10nM IL-8 (in the continued presence of UTP). In cells expressing RGS2, the response to 10nM IL-8 in the presence of UTP was reduced by approximately 65% compared to control cells (Fig. 5.6). It was not possible to demonstrate here the effects of RGS2 on the responses to UTP. However, inhibitory effects on P2Y2 receptor signalling have been recorded previously elsewhere (Mark *et al*, 2000; Nlend *et al*, 2002).

Section 5.2.5: Effects of sequestering Gβγ subunits

Sequestration of Gβγ subunits was investigated to further test the hypothesis that these subunits were involved in the crosstalk between P2Y2 receptors and CXCR2. Using the same technique as above for the transfection, selection and study of the effects of RGS2, the HEK-CXCR2 cells were transfected with the transducin $G\alpha_{t1}$ sub-unit, which is known to act as a 'sponge' for Gβγ sub-units (Heximer *et al*, 2001). $G\alpha_{t1}$ expression was confirmed by Western blot analysis (Fig. 5.7). Some cross-reactivity, probably with $G\alpha_i$ subunits, was observed. Compared to cells transfected with 'empty' vector, expression of $G\alpha_{t1}$ caused a significant (approx. 60%; $P<0.05$ by Student's t-test) reduction in the magnitude of the response to IL-8 following UTP pre-stimulation (Fig. 5.7).

[§] It has been shown that RGS2 also has some activity against $G\alpha_s$ signalling, although this appears to be more due to inhibition of adenylyl cyclase isoforms than to $G\alpha_s$-specific RGS2 GAP activity (Kehrl and Sinnarajah, 2002). For the purposes of this experiment, the absence of any $G\alpha_s$ involvement in this interaction (Fig. 3.12b) allows RGS2 to be considered to act specifically at $G\alpha_q$ (i.e. it has no activity against $G\alpha_i$ etc).

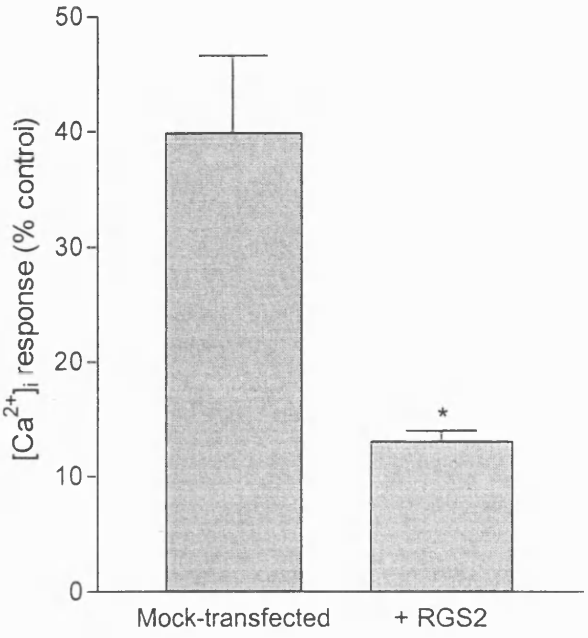


Figure 5.6: Effects of RGS2 on potentiation response. Cells expressing RGS2 (or mock transfected cells) were loaded with fura-2/AM, then stimulated at t=10s with 100μM UTP and at 150s with 10nM IL-8 (in the continued presence of UTP). Changes in $[Ca^{2+}]_i$ were measured using a Ca^{2+} imaging system. Shown are Ca^{2+} responses to IL-8, expressed as a percentage of the maximal response to 100μM UTP. Data are mean±s.e.m., n=4. * $P<0.05$, by unpaired Student's t-test.

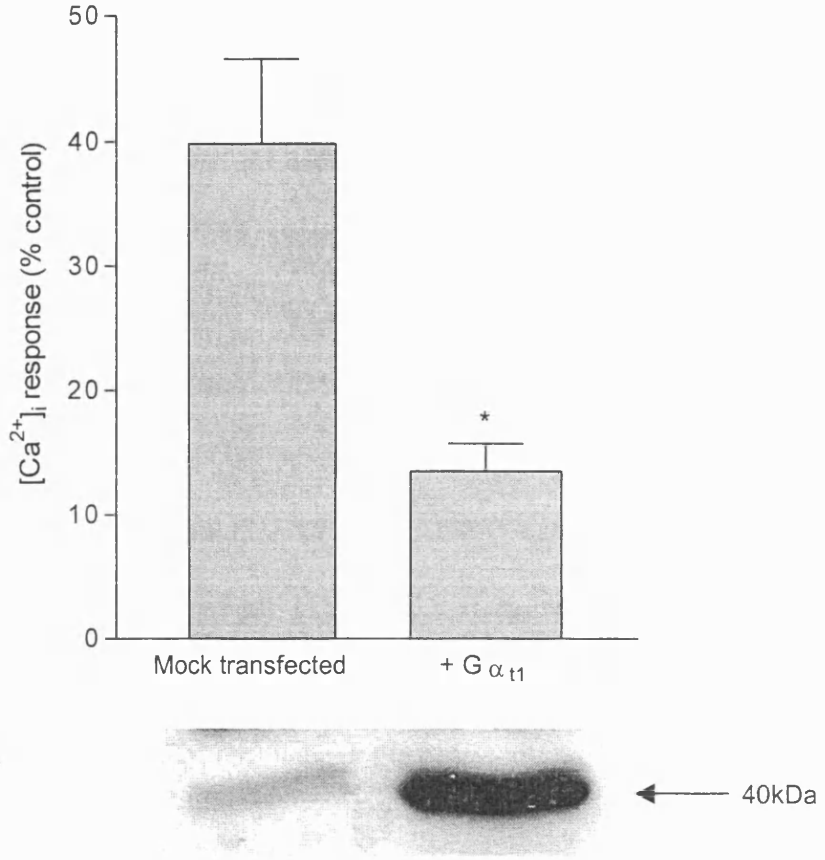


Figure 5.7: Effects of the Gβγ scavenger, $G\alpha_{t1}$, on potentiation of IL-8 signalling. Using confocal microscopy, cells expressing the Gβγ scavenger, $G\alpha_{t1}$ (or mock transfected cells) were stimulated at t=10s with 100μM UTP and at 150s with 10nM IL-8 (in the continued presence of UTP). Shown are responses to IL-8, expressed as a percentage of the maximal response to 100μM UTP. Data are mean±s.e.m., n=4. * $P<0.05$, by unpaired Student's t-test. Shown below the chart is Western blot analysis of $G\alpha_{t1}$ expression in each cell population.

Section 5.2.6: Investigation into whether the desensitisation of the P2Y1 receptor underlies its relative inability to mediate potentiation

One hypothesis for the failure of the P2Y1 nucleotide receptor to mediate potentiation of CXCR2 Ca^{2+} signalling was that the P2Y1 receptor fully desensitised before IL-8 addition, and that the absolute requirement for continued receptor stimulation was therefore not met. To test the activity of the P2Y1 receptor over time, accumulation of [^{3}H]-$InsP_x$ was measured as an index of PLCβ activity. In [^{3}H]-*myo*-inositol-loaded cells, [^{3}H]-$InsP_x$ generated during stimulation with 1mM UTP continued to accumulate up to around 5-10mins (Fig. 5.8), at which point production ceased and no further accumulation was observed. In contrast, a concentration of 2MeSADP (10μM) that gave an equivalent peak Ca^{2+} response to that induced by 1mM UTP (Fig. 3.5), caused a generation of [^{3}H]-$InsP_x$ that was short-lived, with no further accumulation occurring after approximately 2mins (i.e. 30s before the addition of IL-8 would be made in the potentiation experiments).

It was shown previously that P2Y1 nucleotide receptors had a lower capacity to potentiate CXCR2 Ca^{2+} signalling than did the P2Y2 receptor (Figs. 1.8 and 1.9). This is shown graphically in Fig. 5.9a. While P2Y1 and P2Y2 receptor agonists (2MeSADP (1μM) and UTP (100μM), respectively) elicit roughly equivalent peak increases in $[Ca^{2+}]_i$, the Ca^{2+} response to 10nM IL-8 is markedly different following each pre-stimulation. It was suggested in Chapter 3 that this may be due to differences in the extent of P2Y receptor desensitization such that the P2Y1 receptors did not, at the time of IL-8 addition, fulfil the requirement for ongoing activation of the pre-stimulating receptor. To test whether the state of activity of the P2Y1 receptors is different in the presence of IL-8, a time course of [^{3}H]-$InsP_x$ generation was constructed. Cells were stimulated with either 10μM 2MeSADP, or a co-addition of 10μM 2MeSADP and 100nM IL-8 (Fig. 5.9b). Stimulation with 2MeSADP alone resulted in a maximal [^{3}H]-$InsP_x$ generation of 1.23±0.1 fold-of-basal, with

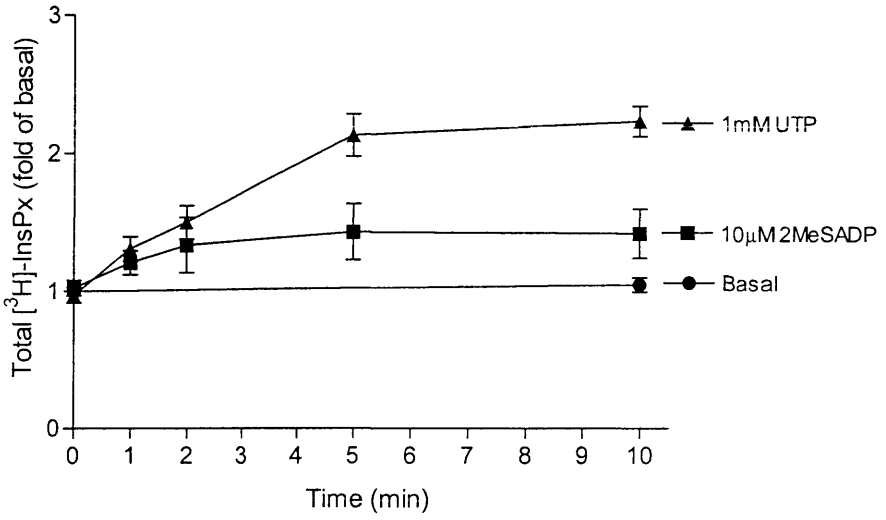


Figure 5.8. P2Y1- and P2Y2 nucleotide receptors have contrasting desensitisation rates. [^{3}H]-*myo*-inositol-loaded HEK-CXCR2 cells were stimulated (in the presence of Li^+ to block inositol monophosphatase activity) with either 1mM UTP (triangles), 10μM 2MeSADP (squares) or buffer (circles). Values are expressed as fold-of-basal. Basal values were 4188±392 dpm/well. Data are mean±s.e.m., n=4.

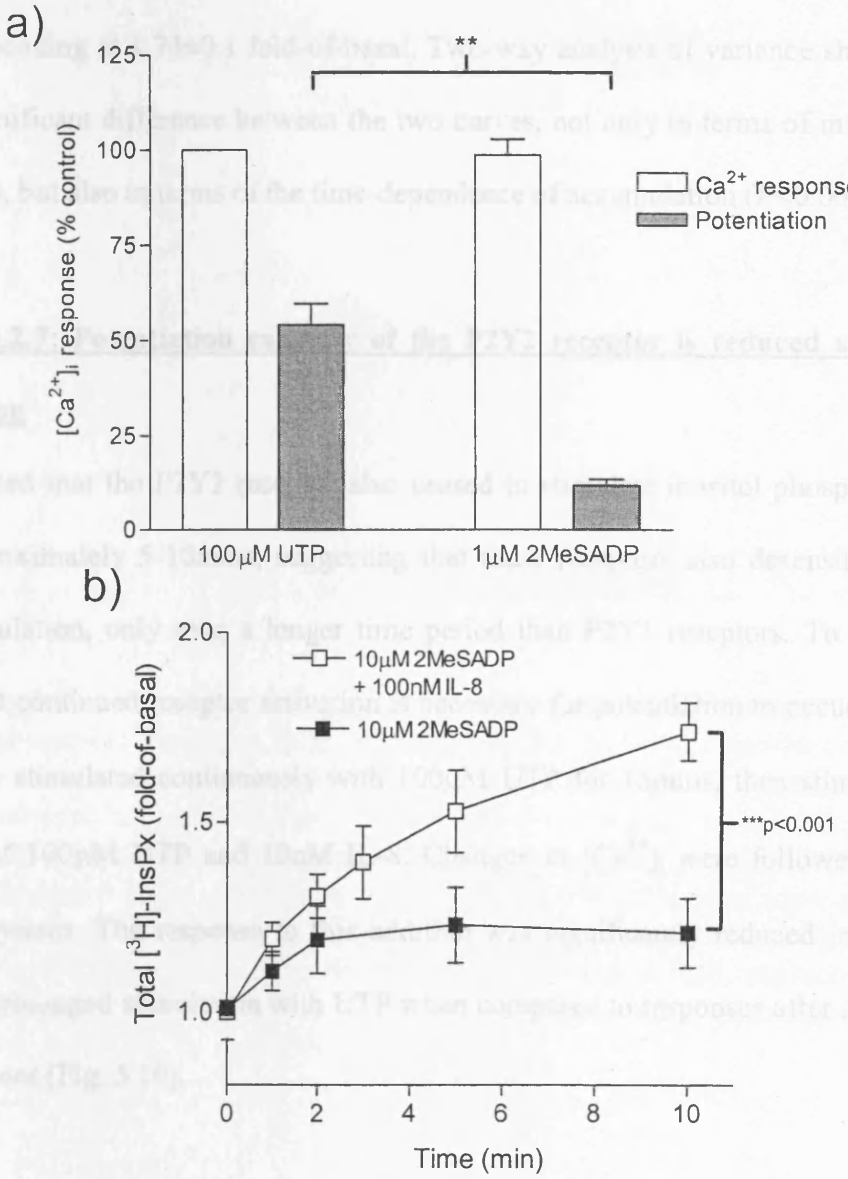


Figure 5.9: P2Y receptor subtypes display different abilities to potentiate IL-8 signals. a) Using a Ca^{2+} imaging system, cells were pre-stimulated with nucleotide agonists before stimulating at t=150s with 10nM IL-8 (in the continued presence of nucleotide). Shown are the maximal Ca^{2+} responses elicited by the nucleotide agonists themselves (white bars) and by the subsequent addition of IL-8 (grey bars), expressed as a percentage of the maximal response to 100μM UTP. Data are mean±s.e.m., n=4. ** $P<0.01$, by unpaired Student's t-test. b) Total [^{3}H]-InsPx generation was measured as described in Materials and Methods. Cells were stimulated with either 10μM 2MeSADP or a co-addition of 10μM 2MeSADP and 100nM IL-8. Data are mean±s.e.m., n=4. *** $P<0.001$, by two-way ANOVA.

accumulation ceasing at approximately 2mins. Stimulation with a co-addition of 2MeSADP and IL-8 caused $InsP_x$ generation that continued until the termination of the experiment at 10mins, peaking at 1.74±0.1 fold-of-basal. Two-way analysis of variance showed that there was a significant difference between the two curves, not only in terms of maximal response ($P<0.001$), but also in terms of the time-dependence of accumulation ($P<0.001$).

Section 5.2.7: Potentiation capacity of the P2Y2 receptor is reduced after prolonged stimulation

It was noted that the P2Y2 receptor also ceased to stimulate inositol phosphate generation after approximately 5-10mins, suggesting that these receptors also desensitise in terms of PLC stimulation, only over a longer time period than P2Y1 receptors. To further test the theory that continued receptor activation is necessary for potentiation to occur, fluo-3-loaded cells were stimulated continuously with 100μM UTP for 15mins, then stimulated with an addition of 100μM UTP and 10nM IL-8. Changes in $[Ca^{2+}]_i$ were followed using a Ca^{2+} imaging system. The response to this addition was significantly reduced in cells that had received prolonged stimulation with UTP when compared to responses after a shorter (150s) pre-treatment (Fig. 5.10).

If desensitisation is an issue in the failure of P2Y1 receptors to stimulate a robust potentiation of CXCR2 signalling, then measures to minimise desensitisation should improve the ability of these receptors to stimulate this potentiation. This was tested by two separate methods. The first was to achieve store drainage using 100μM UTP (leaving P2Y1 receptors unaffected by agonist-induced receptor desensitisation) and a single addition of 10μM 2MeSADP to drain any remaining 2MeSADP-accessible stores. This was followed by co-addition of 10μM 2MeSADP and 10nM IL-8. The second protocol was the pre-stimulation

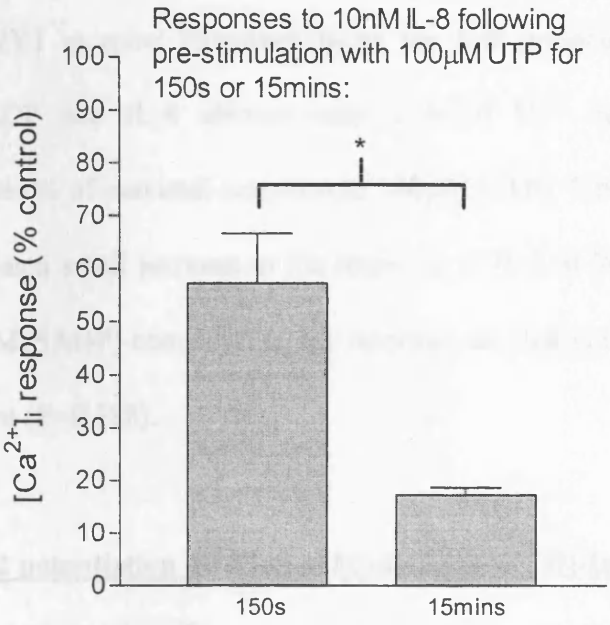


Figure 5.10. Extended pre-stimulation with UTP attenuates the subsequent response to IL-8. HEK-CXCR2 cells were grown on glass coverslips and loaded with fluo-3/AM as described in Materials and Methods. Changes in fluo-3 fluorescence corresponding to changes in $[Ca^{2+}]_i$ were monitored using a single cell imaging system. Cells were stimulated using constant perfusion with 100μM UTP, then subsequently with 10nM IL-8, either at t=150s or at t=15mins, in the continued presence of UTP. Data shown are responses to this addition of IL-8, expressed as a percentage of the response to 100μM UTP alone. Data are mean±s.e.m., n=4. * $P<0.05$, by Student's unpaired t-test.

of the P2Y1 receptor with 10μM 2MeSADP for only 30s before addition of 10nM IL-8 (in the continued presence of 2MeSADP). Both methods minimise the duration of agonist occupation of the P2Y1 receptor. However, using the first protocol (Fig. 5.11a), the co-addition of 2MeSADP and IL-8 elicited only a small Ca^{2+} response, barely above background levels (6±1% of maximal response to 100μM UTP). Using the second protocol (Fig. 5.11b), there was a small increase in the response to IL-8 at 30s (18±4% of maximal response to 10μM 2MeSADP) compared to the response at 150s (11±1%), but this was not statistically significant (P=0.188).

Section 5.2.8: M3-β2 potentiation does not exhibit elevated [^{3}H]-InsP$_x$ production

The demonstration of elevated Ca^{2+} responses following co-stimulation of muscarinic M3 and β2 adrenoceptors was used in Chapter 3 as an additional example of potentiating crosstalk in HEK cells. However, to assess whether the two examples of crosstalk were comparable, phosphoinositide generation stimulated by various combinations of carbachol, isoproterenol and the adenylyl cyclase-activating agent, forskolin, was tested. Cells stimulated with either carbachol (100μM), carbachol and isoproterenol (10μM), or carbachol and forskolin (10μM) all produced similar total accumulations of [^{3}H]-InsP$_x$ (Fig. 5.12).

Section 5.2.9: Crosstalk between muscarinic M3 and β2 adrenoceptors cannot be mimicked by artificially elevating cAMP

It was shown in Chapter 3 that a crosstalk between P2Y2 nucleotide receptors and β2 adrenoceptors involved Gs proteins (Fig. 3.12b). While the CTX sensitivity of the muscarinic M3 receptor-β2 adrenoceptor crosstalk was not assessed, it seems reasonable to predict that $G\alpha_s$ may be involved here also. Given that this would be predicted to lead to the stimulation of cAMP generation by adenylyl cyclase, one of the more obvious candidate hypotheses describing Ca^{2+}-potentiating crosstalk is the modulation of some signalling

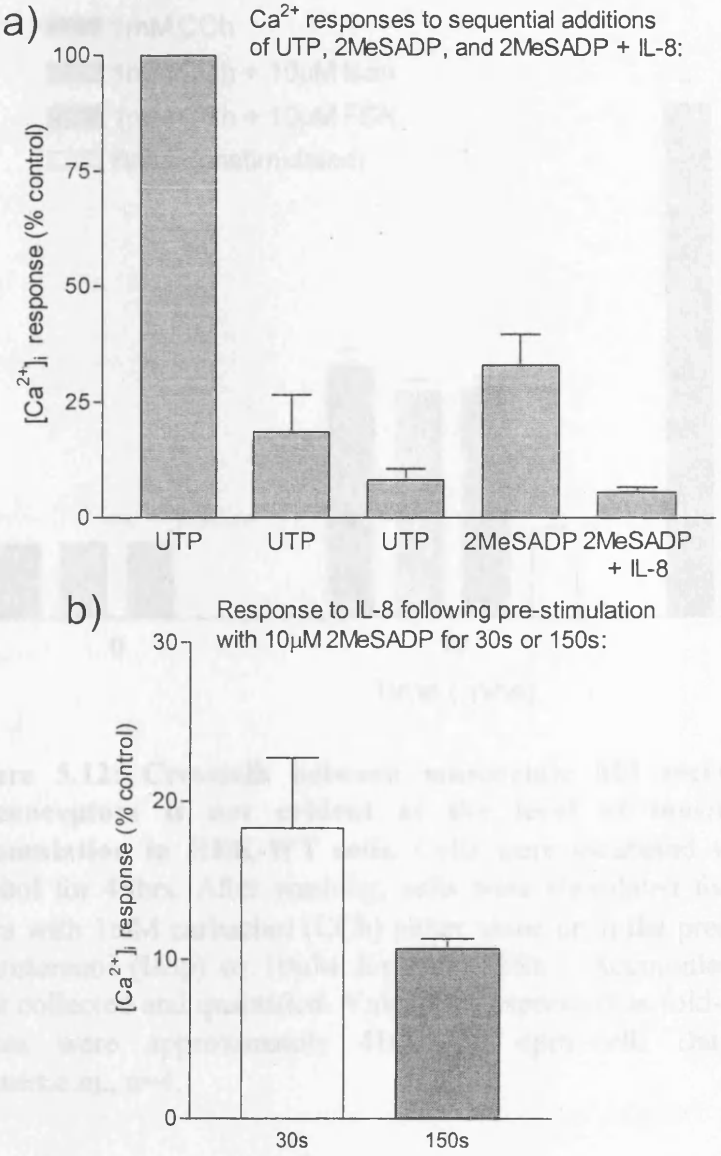


Figure 5.11: Two protocols to minimise P2Y1 receptor exposure to agonist have little effect on the ability of this receptor to potentiate IL-8-induced Ca^{2+} signals. a) Using a Ca^{2+} imaging system, cells were stimulated with pulses of 100μM UTP in the absence of extracellular Ca^{2+} until no further response was observed, and subsequently with 10μM 2MeSADP. After a short period of washout, store-depleted cells were exposed to a simultaneous addition of 10μM 2MeSADP and 10nM IL-8. Shown are responses to each of the sequential additions, expressed as a percentage of the maximal response to 100μM UTP, mean±s.e.m., n=4. b) Cells were pre-stimulated with 10μM 2MeSADP for either 30s or 150s, and subsequently with 10nM IL-8 (in the continued presence of 2MeSADP). Shown are responses to IL-8, expressed as a percentage of the maximal response to 10μM 2MeSADP, mean±s.e.m., n=4. The response to IL-8 following 30s or 150s of 2MeSADP stimulation were not statistically different.

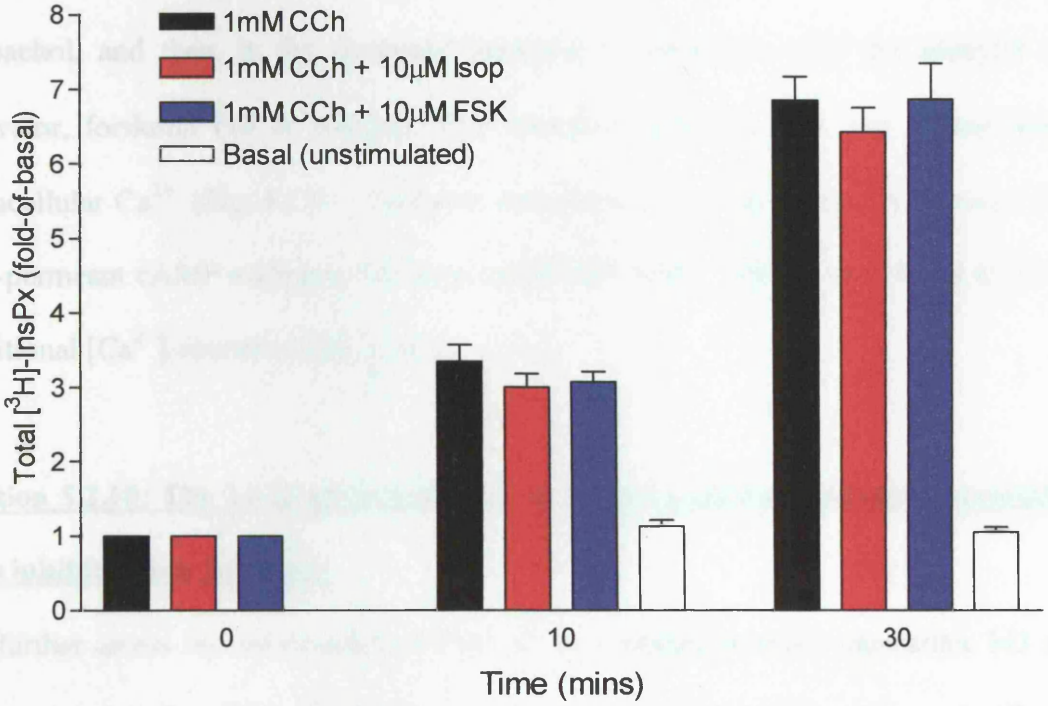


Figure 5.12: Crosstalk between muscarinic M3 receptors and β2 adrenoceptors is not evident at the level of inositol phosphate accumulation in HEK-WT cells. Cells were incubated with [^{3}H]-*myo*-inositol for 48hrs. After washing, cells were stimulated for the indicated times with 1mM carbachol (CCh) either alone or in the presence of 10μM isoproterenol (Isop) or 10μM forskolin (FSK). Accumulated [^{3}H]-InsPx were collected and quantified. Values are expressed as fold-of-basal. Basal values were approximately 4100±150 dpm/well. Data shown are mean±s.e.m., n=4.

component by the cAMP-dependent protein kinase, PKA. To test whether PKA activation was able to substitute for β2 adrenoceptor stimulation, cells were pre-stimulated with 100μM carbachol, and then, in the continued presence of carbachol, with the adenylyl cyclase activator, forskolin (10 or 100μM). This treatment failed to elicit any further release of intracellular Ca^{2+} (Fig. 5.13). Likewise, substitution of forskolin in this protocol with the cell-permeant cAMP analogue, dibutyryl cAMP (dbcAMP; 10mM), also failed to cause any additional $[Ca^{2+}]_i$ elevation (Fig. 5.14).

Section 5.2.10: The 14-22 myristoylated amide PKA inhibitor attenuates crosstalk, but also inhibits other responses

To further assess the involvement of PKA in the crosstalk between muscarinic M3 and β2 adrenoceptors, the PKA inhibitor, myristoylated amide 14-22, was used. The more commonly used PKA inhibitor, H-89, was not used as this has been shown to have antagonist activity at the β2 adrenoceptors (Penn *et al*, 1999; Budd *et al*, 2000) in addition to its effects on PKA. Cells were pre-incubated for 20mins with 20μM 14-22 amide (Graziani *et al*, 2002). Using a FLIPR assay, cells were then stimulated at t=10s with 100μM carbachol and again at t=150s with 10μM isoproterenol (in the continued presence of carbachol). Responses to isoproterenol following carbachol pre-stimulation were significantly reduced by 14-22 amide (54±4% inhibition *vs.* control cells) (Fig. 5.15b). However, responses to carbachol alone were also significantly reduced by the presence of 14-22 amide (by 59±3%) (Fig. 5.15a). Furthermore, in controls testing the effects of PKA inhibition on the P2Y2 receptor-CXCR2 potentiation mechanism, 14-22 amide inhibited responses to both 100μM UTP alone (by 61±2%) and to a co-addition of 100μM UTP and 10nM IL-8 (by 60±2%) (Fig. 5.16).

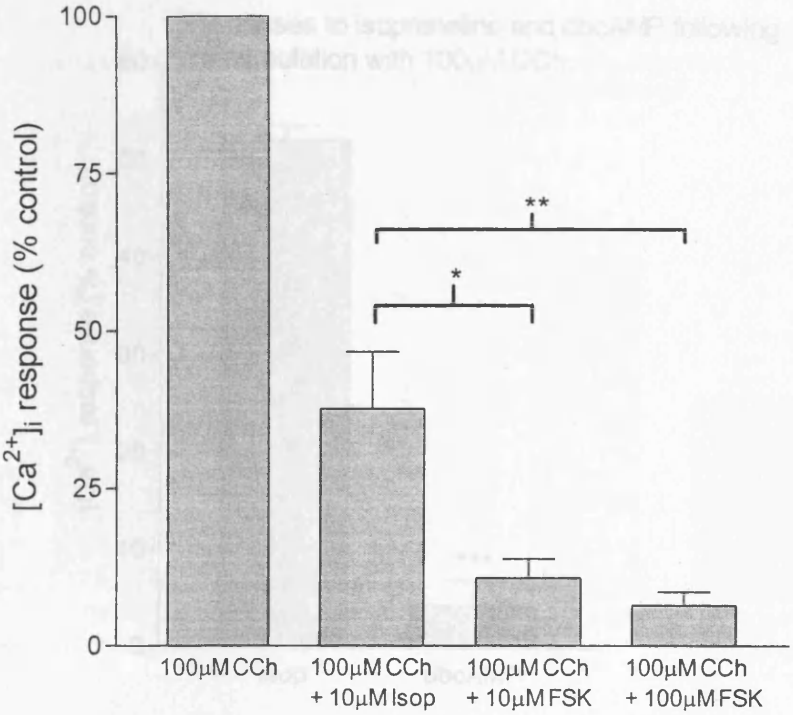


Figure 5.13: Crosstalk between muscarinic M3 receptors and β2 adrenoceptors in wild-type HEK cells is not mimicked by cAMP elevation by forskolin. HEK-WT cells were grown on glass coverslips and loaded with fluo-3/AM as described in Materials and Methods. Cells were stimulated with 100μM carbachol (CCh) and then, 150s later, with either 10μM isoproterenol (Isop), or forskolin (FSK) at 10μM or 100μM, each in the continued presence of CCh. Changes in fluo-3 fluorescence were measured as an index of $[Ca^{2+}]_i$ elevation using a Ca^{2+} imaging system. Values shown are the control response to 100μM CCh, and the responses to each secondary addition relative to this control response. Data are mean±s.e.m., n≥4. Responses to these secondary additions were compared statistically by one way analysis of variance ($p<0.05$), and then by unpaired Student's t-test. * denotes statistical significance according to t-test (*: $P<0.05$; **: $P<0.01$)

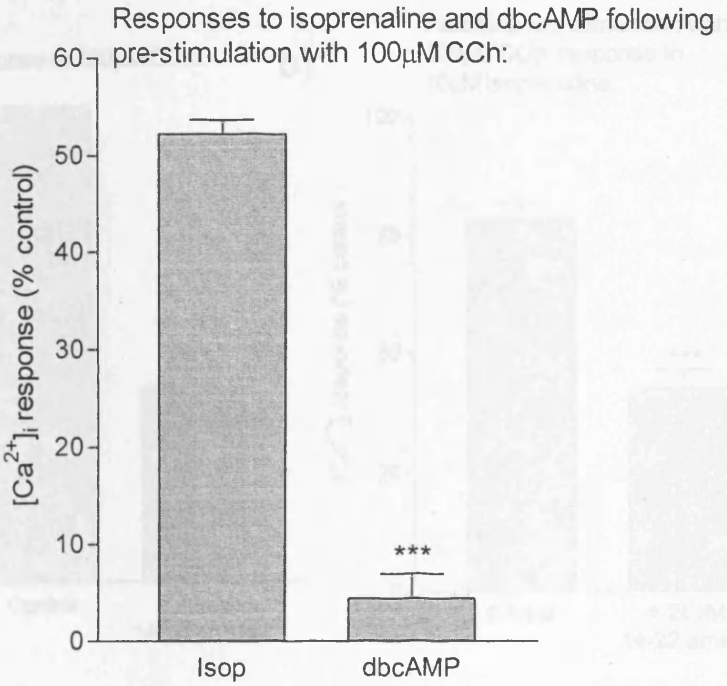


Figure 5.14: Crosstalk phenomenon between muscarinic M3 receptors and β2 adrenoceptors in wild-type HEK cells is not mimicked by cAMP elevation by a cell-permeable analogue of cAMP. HEK-WT cells were plated into 96-well FLIPR plates and loaded with fluo-3 dye as in Fig. 3.2. Cells were stimulated with 100μM carbachol and then, 150s later and in the continued presence of CCh, with either 10μM isoproterenol (Isop), or 10mM dibutyryl cAMP (dbcAMP). Changes in fluo-3 fluorescence were measured as an index of $[Ca^{2+}]_i$. Values shown are responses to each secondary addition relative to the control response (100μM CCh, not shown). Data are mean±s.e.m., n≥4. *** $P<0.001$, by unpaired Student's t-test.

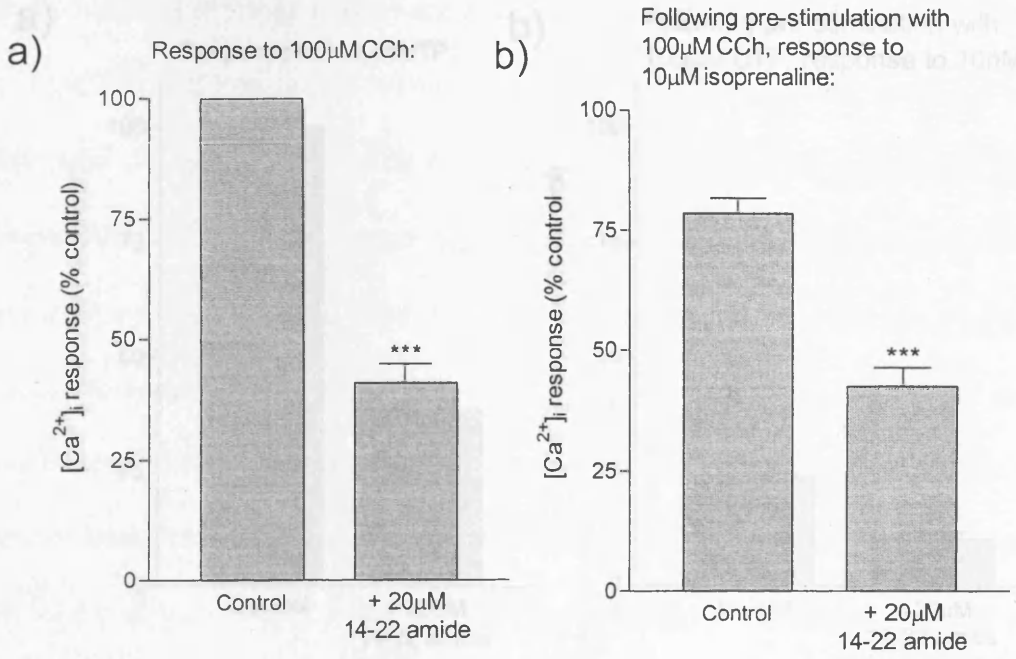


Figure 5.15: Effects of PKA inhibition on responses to M3 muscarinic receptor stimulation and on potentiation. HEK-WT cells were grown on glass coverslips and loaded with fluo-3/AM as described in Materials and Methods. Cells were pre-incubated for 20mins with 20μM 14-22 myristoylated amide. Using the FLIPR, and in the continued presence or absence of 14-22 amide, cells were stimulated at t=10s with 100μM carbachol (CCh) and at t=150s with 10μM isoproterenol, in the continued presence of CCh. Shown are responses to CCh (a) and to the subsequent addition of isoproterenol (b), expressed as a percentage of the maximal response to 100μM CCh in the absence of 14-22 amide. Data are mean±s.e.m., n=4. *** $P<0.001$, by Student's unpaired t-test.

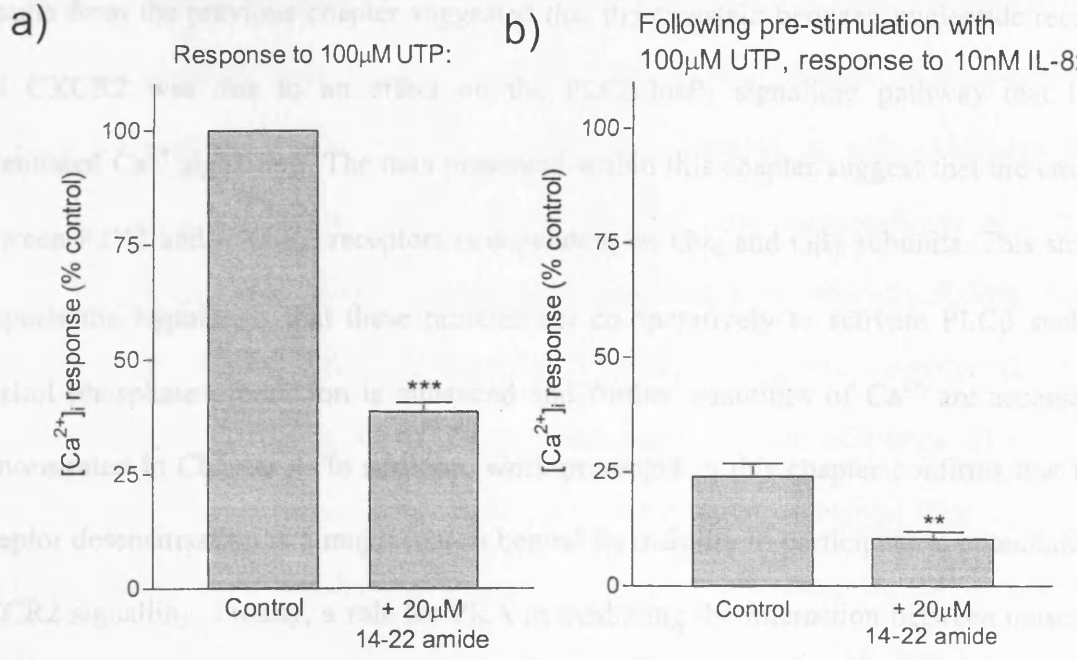


Figure 5.16: Effects of PKA inhibition on responses to P2Y2 receptor stimulation and on potentiation. HEK-CXCR2 cells were grown on glass coverslips and loaded with fluo-3/AM as described in Materials and Methods. Cells were pre-incubated for 20mins with 20μM 14-22 myristoylated amide. Using the FLIPR, and in the continued presence or absence of 14-22 amide, cells were stimulated at t=10s with 100μM UTP and at t=150s with 10nM IL-8, in the continued presence of UTP. Shown are responses to UTP (a) and to the subsequent addition of IL-8 (b), expressed as a percentage of the maximal response to 100μM UTP in the absence of 14-22 amide. Data are mean±s.e.m., n=4. **: $P<0.01$; ***: $P<0.001$, by Student's unpaired t-test.

Section 5.3: Discussion

Section 5.3.1: Summary of data

Results from the previous chapter suggested that the crosstalk between nucleotide receptors and CXCR2 was due to an effect on the PLCβ/$InsP_3$ signalling pathway that led to potentiated Ca^{2+} signalling. The data presented within this chapter suggest that the crosstalk between P2Y2 and CXCR2 receptors is dependent on $G\alpha_q$ and $G\beta\gamma$ subunits. This strongly supports the hypothesis that these proteins act co-operatively to activate PLCβ such that inositol phosphate production is enhanced and further quantities of Ca^{2+} are accessed, as demonstrated in Chapter 4. In addition, work presented in this chapter confirms that P2Y1 receptor desensitisation is a major reason behind its inability to participate in potentiation of CXCR2 signalling. Finally, a role for PKA in mediating the interaction between muscarinic M3 receptors and β2 adrenoceptors has been ruled out.

Section 5.3.2: PLCβ modification, but not by truncation

Calpain activation and subsequent truncation of PLCβ was proposed in Section 5.1 as a potential mechanism for PLCβ hypersensitivity to $G\beta\gamma$ subunits. There is evidence for the activation of calpain *in vivo* (Banno *et al*, 1995; Li & Iyengar, 2002), and also for the truncation of PLCβ and the generation of a $G\beta\gamma$-hypersensitive PLCβ fragment (Banno *et al*, 1994). For example, calpain activation has been shown to occur downstream of agonist-stimulated $G\alpha_q$-coupled GPCRs, such as those for thrombin and collagen in platelets (Banno *et al*, 1995) and the muscarinic M3 receptor (Li & Iyengar, 2002). The activation of calpain requires substantial increases in cytosolic $[Ca^{2+}]$ (Suzuki *et al*, 1992) and it is possible that if cleavage does indeed occur in response to $G\alpha_q$-activating agonists that it does so in high $[Ca^{2+}]$ microdomains such as in the vicinity of $InsP_3$ receptors and plasma membrane Ca^{2+} channels. However, in SH-SY5Y neuroblastoma cells, while activation of an endogenous

$G\alpha_q$-coupled muscarinic receptor reveals Ca^{2+} signalling by a $G\alpha_i$-coupled δ-opioid receptor (Connor *et al*, 1997a), elevation of $[Ca^{2+}]_i$ by either thapsigargin or maitotoxin was unable to substitute for muscarinic receptor activation. This suggests either that elevation of $[Ca^{2+}]_i$ alone is insufficient for calpain activation, or that the elevation induced by these agents may not adequately reflect the kinetics or localised concentration of agonist-induced $[Ca^{2+}]_i$ elevation. Data presented in Chapter 3 showed that even concentrations of UTP and 2MeSADP that give equivalent Ca^{2+} responses themselves have markedly different abilities to cause potentiation of IL-8 signalling (Fig. 3.9), supporting the conclusion that simply elevating Ca^{2+} is not sufficient to induce potentiation by the activation of calpain or otherwise. In addition, the requirement for continued receptor activation (Fig. 3.11) argues against the involvement of any radical cleavage events, since these would be expected to persist for some time after removal of the primary agonist. While this may explain certain instances of crosstalk that occur for some time after removal of the pre-stimulating agonist (for example, Short & Taylor, 2000), it is difficult to reconcile with the data presented here.

Section 5.3.3: Arguments against additivity of Gβγ or $InsP_3$

There is evidence to suggest that the concentration of free Gβγ subunits may need to reach a critical level before they are able to stimulate effectors, particularly βγ-sensitive isoforms of PLCβ. Indeed, several receptor types exhibit PTX-sensitive (i.e. Gi-dependent) Gβγ-mediated PLCβ responses whose magnitude correlates with receptor number (Peralta *et al*, 1988; Cotecchia *et al*, 1990; Boddeke *et al*, 1992; Biber *et al*, 1997). Thus, gain-of-function crosstalk between two receptors could arise from the pooling of Gβγ subunits from two receptor populations. The lack of specificity of Gβγ subunits observed by some groups (Zhu *et al*, 1996; Quitterer & Lohse, 1999; Ruiz-Velasco & Ikeda, 2000; Wellner-Wienitz *et al*, 2001) would facilitate this possibility. However, this simplistic view should be set against the observations that increasing receptor number does not automatically lead to an ability to

stimulate PLCβ (Gudermann *et al*, 1992), and that others have argued that Gβγ subunits can be inherently selective for the effectors they activate (Maier *et al*, 2000; Jiang *et al*, 1996). Nonetheless, Gβγ appears to have a role in several examples of crosstalk between $G\alpha_i$- and $G\alpha_q$-coupled receptors, since they can be attenuated by Gβγ scavengers such as the C-terminal fragment of βARK or $G\alpha_t$ (Selbie *et al*, 1997; Dickenson & Hill, 1998b; Chan *et al*, 2000). In addition, the over-expression of Gβγ facilitates PLC stimulation by a variety of Gs- and Gi-coupled GPCRs (Zhu & Birnbaumer, 1996). Data presented here shows that Gβγ plays a role in the interaction between P2Y2 receptors and CXCR2, given the inhibition of this crosstalk in the presence of the transducin α subunit, $G\alpha_{t1}$ (Fig. 5.7). This is not due to simple pooling of Gβγ subunits so as to exceed some threshold for PLCβ activation. Thus, both IL-8 and the β2 adrenoceptor agonists, (±)-arterenol and isoproterenol, were shown to stimulate substantial Ca^{2+} responses following pre-stimulation of cells with agonists at $G\alpha_q$-coupled receptors, but not alone, indicating that the release of Gβγ in the absence of pre-stimulation is not sufficient to breach a threshold. Indeed, the absence of any Ca^{2+} response following co-stimulation of CXCR2 and β2 adrenoceptors (Fig. 5.2) suggests that neither of these receptors is releasing even half of the Gβγ required to exceed threshold. It seems highly unlikely that such robust Ca^{2+} responses (up to 70% of that in response to a maximal stimulation of a $G\alpha_q$-coupled receptor) could be achieved by pooling of two sub-threshold concentrations of Gβγ.

This argument applies also to elevations of $InsP_3$ by these agonists (Gβγ-mediated or otherwise). Thus, it is unlikely that such large Ca^{2+} responses could be attained by combining two sub-threshold concentrations of $InsP_3$. However, with respect to $InsP_3$ levels, it could be argued that UTP pre-stimulation raises the level of $InsP_3$ in the cytosol to a plateau, as has been shown previously for $G\alpha_q$-mediated $InsP_3$ increases in many systems, and that any

further $InsP_3$ generation from this platform would result in a second Ca^{2+} peak as $InsP_3$ levels re-crossed threshold (Fig. 5.17b). In opposition to this is that while the basal [$InsP_3$] may be perceived to have been re-set by the plateau, it is probable that the threshold level for further Ca^{2+} release would have also been increased. Therefore, the requirement for $InsP_3$ above this level for any further Ca^{2+} peaks would still be unachievable by either CXCR2 or β2 adrenoceptors (Fig. 5.17c). Furthermore, having argued for the existence of quantal Ca^{2+} release in the previous chapter, the new threshold level would have to be at or above the peak $InsP_3$ level seen in response to UTP, since for quantal Ca^{2+} release to take place, $InsP_3$ generation should be greater than that which has already occurred (Fig. 5.17d). To achieve this, the $InsP_3$ input downstream of CXCR2 would need to be so substantial that it is highly improbable that it would not exceed threshold in its own right in the absence of UTP pre-stimulation. Additionally, it is unlikely that it could not be combined with the $InsP_3$ generation stimulated by activation of β2 adrenoceptors to initiate a subsequent Ca^{2+} response. It is thus unlikely that pooling of either Gβγ subunits or $InsP_3$ could account for the Ca^{2+} responses revealed here following P2Y2 receptor pre-stimulation.

Section 5.3.4: Receptor oligomerisation as a mode of crosstalk

Improved coupling of a Gq-coupled receptor to $G\alpha_q$, or the introduction of $G\alpha_q$-coupling to a normally Gi/s-coupled receptor, is a feasible means of facilitating Ca^{2+} signalling. There is evidence that this phenomenon can occur following receptor dimerisation. A variety of receptor combinations that form dimeric assemblies have been shown to have enhanced $[Ca^{2+}]_i$ responses when co-expressed (not necessarily dependent on any existing Ca^{2+} signalling capability in the monomers), including metabotropic glutamate receptors (mGluRs) with adenosine A1 receptors (Ciruela *et al*, 2001), CCR2 with CCR5 chemokine receptors (Rodríguez-Frade *et al*, 2001; Mellado *et al*, 2001b), angiotensin AT1 receptor

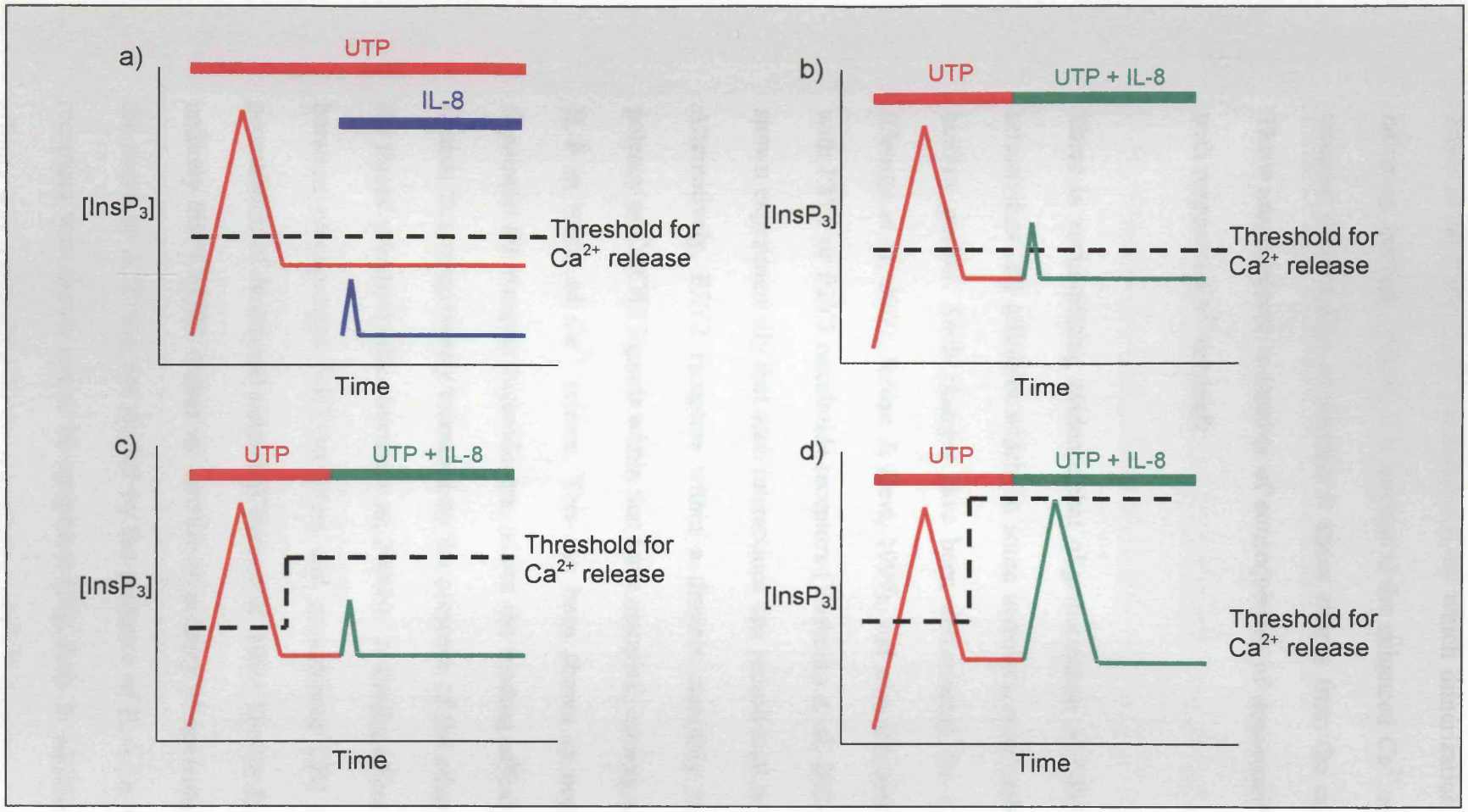


Fig. 5.17: Schematic describing three possible outcomes of co-stimulation with UTP and IL-8 in terms of $InsP_3$ generation. a) Stimulation with UTP produces a peak of $InsP_3$ generation followed by a sustained plateau phase above basal levels. In contrast, IL-8 alone may produce a much smaller amount of $InsP_3$ following stimulation of CXCR2, which is unable to breach some threshold level required for initiation of an intracellular Ca^{2+} event. b) However, if the basal level of $InsP_3$ is raised by pre-stimulation (i.e. the plateau level becomes the new 'basal' level), the small amount of $InsP_3$ produced by CXCR2 stimulation may now be able to exceed threshold and cause a Ca^{2+} response. c) It should also be considered, though, that while the basal level of $InsP_3$ may rise following UTP pre-stimulation, the threshold level required for a Ca^{2+} release event may also rise, such that even superimposing the IL-8-stimulated $InsP_3$ response on top of the UTP-stimulated plateau will not exceed this level. d) It is likely that if quantal Ca^{2+} release is occurring that the new threshold will be at the peak level of $InsP_3$ generated by UTP (since any further release requires more $InsP_3$ to access a wider Ca^{2+} store). It is probable that the IL-8-stimulated $InsP_3$ generation would have to be substantial, making it highly unlikely that, in the absence of UTP stimulation, it would not exceed threshold in these naïve cells.

with bradykinin B2 receptors (AbdAlla *et al*, 2000) and CCR2 homodimers (Rodríguez-Frade *et al*, 1999). The mechanism(s) by which dimerization enhances Ca^{2+} signalling is, however, unclear. Indeed, it is unclear if the enhanced Ca^{2+} signalling is directly linked to receptor dimerisation or whether it arises simply from the co-expression of the receptors. There are, however, a number of consequences of dimerisation that should be considered with respect to Ca^{2+} crosstalk.

There is accumulating evidence that oligomerization of GPCRs can alter ligand binding selectivities and affinities which, in some instances, may reflect the formation of a novel binding pocket. Such changes have been documented for opioid receptor heterodimers (George *et al*, 2000; Jordan & Devi, 1999), and also for adenosine receptor heterodimers with P2Y1 or P2Y2 nucelotide receptors (Yoshioka *et al*, 2001). However, it has yet to be shown experimentally that such interactions can indeed lead to potentiation of Ca^{2+} signals. Alternatively, P2Y2 receptors within a dimeric assembly may positively influence the potency of CXCR2 ligands within that same complex, causing a large shift in the potency of IL-8 in terms of Ca^{2+} release. This has been shown to occur in somatostatin SSTR5-dopamine D1 receptor heterodimers, where the binding affinity of each receptor for their ligands is synergistically increased by the presence of the other receptor, without changing the ligand selectivity (Rocheville *et al*, 2000b). A similar effect has also been demonstrated between co-expressed 5-HT receptors and cannabinoid CB1 receptors with concomitant potentiation of functional outputs (Cheer *et al*, 1999; Devlin & Christopoulos, 2002). It is unlikely that CXCR2 causes an increase in potency of nucleotides at P2Y2 receptors since the curve to ATP was not shifted by the presence of IL-8 i.e. the crosstalk between these receptors was shown not to be reciprocal (Fig. 3.6). In addition, if an increase in agonist affinity was responsible for the potentiation of Ca^{2+} signalling, the predicted response would be a curve shift without any effect on maximal response (unless receptor reserve was limiting

the maximal achievable response to UTP). Studies on binding affinities of CXCR2 would be a sensible extension of these studies in order to assess the relative binding of IL-8 in the absence and presence of UTP if this model was considered to be relevant. However, these same receptors in leukocytes bind IL-8 and GROα with similar affinity to that in the HEK-CXCR2 cell line, yet cause substantial Ca^{2+} responses only in leukocytes (Dr. I. Dainty, AstraZeneca, personal communication). It therefore seems unlikely that poor binding affinity is the limiting factor in the failure of CXCR2 to cause Ca^{2+} responses in the HEK-CXCR2 cell line. Increasing the efficacy of ligand-induced responses, or re-directing the pathway of signal transduction are likely to be more feasible modes of causing potentiation.

While the alteration of binding characteristics concerns the properties of the extracellular portion of the GPCR, dimerisation may also involve modification of the intracellular face, altering G-protein selection. The theory of surrogate transduction works around a sharing principle, where two receptors can effectively 'borrow' the transduction machinery of a dimeric partner. The resultant response is typical of one receptor partner, but occurs in response to a ligand at the other, as demonstrated elegantly using somatostatin receptors and dopamine receptors (Rocheville *et al*, 2000a and b). It was shown that a GPCR heterodimer can 'mix-and-match' its binding and signalling components to form a novel signalling profile. Similarly, adenosine A1 receptor and P2Y1 nucleotide receptor heterodimers, display a novel binding pocket that binds P2Y1 nucleotide receptor agonists, but is blocked by adenosine A1 receptor antagonists, and signal in an A1 receptor-like manner (PTX-sensitive inhibition of cAMP) (Yoshioka *et al*, 2001). However, in this example, there was no reciprocation (i.e. adenosine receptor agonists could not signal in a P2Y1-like manner), suggesting that there may be some selectivity to how transduction machinery can be shared.

One mechanism through which surrogate transduction could be achieved is the 'domain swapping' model (Gouldson *et al*, 1998) in which two receptors in a putative dimer can 'swap' the domains responsible for signal transduction. This was demonstrated in α2 adrenoceptor-muscarinic M_3 receptor chimeras (Maggio *et al*, 1993 and 1999) where transmembrane domains (TMDs) VI and VII of each receptor were fused to TMDs I-V of the other. Phosphoinositide hydrolysis was seen only in cells co-expressing these constructs. Whether this is possible simply by having the two halves of a transduction unit (binding and transduction components) in close proximity in the putative dimer, or whether a physical transfer of components occurs (Fig. 5.18) is unclear. Either way, this mode of signal potentiation can be viewed as the result of a clustering of GPCRs that assembles components of two or more receptors (e.g. the high binding affinity of one receptor and the potent signal transduction of another) to form a more active/efficacious complex. A variant of this model has been proposed to account for potentiation of Ca^{2+} signalling in cells co-expressing angiotensin AT1 and bradykinin B2 receptors, where the bradykinin B2 receptors are thought to 'present' G-proteins to the AT1 receptors within a dimeric complex to facilitate Ca^{2+} signalling in response to AT1 receptor agonists (AbdAlla et al, 2000). However, in terms of the P2Y2 receptor-CXCR2 crosstalk, the sharing of $G\alpha_q$ does not explain the potentiation of phosphoinositide signalling, since presumably only one receptor at a time can use the $G\alpha_q$ subunit. Thus, PLCβ activity would be no greater than if only UTP was present. Similarly, by 'hijacking' the $G\alpha_q$ subunits from P2Y2 receptors, CXCR2 would limit itself to the UTP-accessible Ca^{2+} store, unless CXCR2 simply pulled P2Y2 receptors out of their normal membrane localisation by dimerisation without disrupting their coupling, bringing them into contact with previously inaccessible Ca^{2+} stores. Monomeric P2Y2 receptors may therefore access the normal "UTP-sensitive" store, while dimerised P2Y2 receptors, while still acting normally through UTP-mediated activation of $G\alpha_q$, access the normally "UTP-

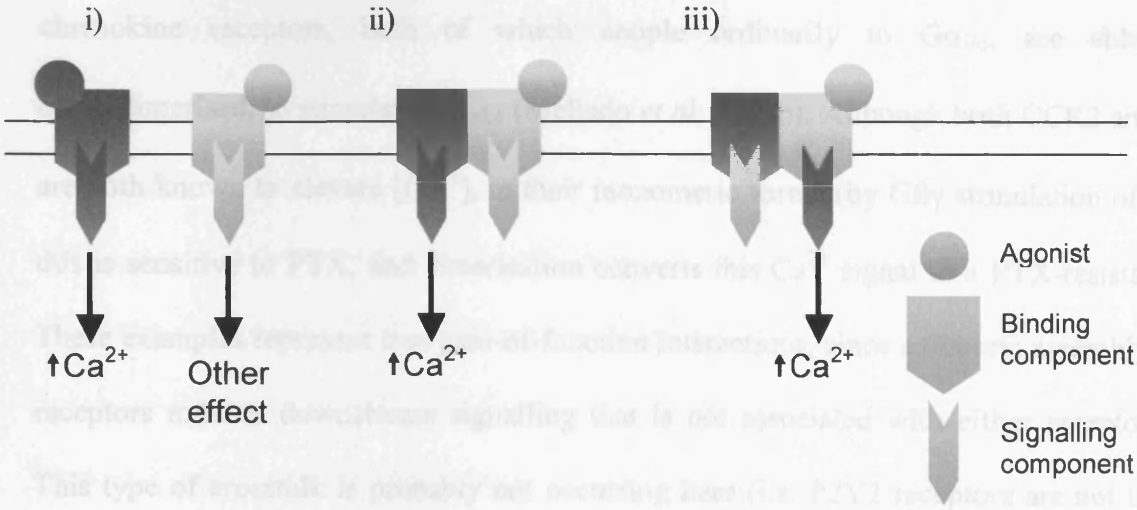


Figure 5.18: Models for surrogate transduction/domain swapping. i) Monomeric signalling – receptor A and receptor B have distinct binding and signalling characteristics. ii) Domain sharing model - the two receptors in a dimeric conformation bind receptor B-specific agonists but transduce a receptor A-type signalling event. This model requires only the proximity of a second receptor-transduction pathway and uses both types of binding components and signalling components in a 'communal' machinery. iii) Domain swapping model – dimerisation induces a physical exchange of signalling components, forming a new 'chimeric' receptor that again binds receptor B-specific ligands and transduces a receptor A-type response.

insensitive" store by virtue of their association with CXCR2. However, this still fails to explain the potentiation of phosphoinositide generation. This would not be expected to change, since the stoichiometry between PLCβ and $G\alpha_q$ would not have changed, and the rate of PLCβ activity would also have remained constant. It would be most interesting, though, to investigate the dimerisation state of muscarinic M3 receptors and β2 adrenoceptors following co-stimulation, since these receptors appear to access an extended Ca^{2+} store without any increase in phosphoinositide generation.

A variation of the surrogate transduction model is the G-protein switching model. For instance, μ- and δ-opioid receptors have been shown to switch their G-protein coupling specificity following heterodimerisation (George *et al*, 2000). In addition, CCR2- and CCR5

chemokine receptors, both of which couple ordinarily to $G\alpha_{i/o}$, are able, when heterodimerised, to stimulate $G\alpha_{q/11}$ (Mellado *et al*, 2001b). Although both CCR2 and CCR5 are both known to elevate $[Ca^{2+}]_i$ in their monomeric forms (by $G\beta\gamma$ stimulation of PLCβ), this is sensitive to PTX, and dimerisation converts this Ca^{2+} signal to a PTX-resistant type. These examples represent true gain-of-function interactions, since a dimeric assembly of two receptors induces downstream signalling that is not associated with either receptor alone. This type of crosstalk is probably not occurring here (i.e. P2Y2 receptors are not inducing CXCR2 to couple to $G\alpha_q$) since the potentiation seen is still PTX-sensitive (Fig. 3.13). However, there remains the possibility that the trigger for CXCR2 coupling to $G\alpha_q$ is some action of G_i, for example, a Gi-directed association of active receptors, with recruitment of $G\alpha_q$ by CXCR2 occurring as a consequence of this. Direct investigation of any changes in G-protein coupling (by binding of [^{35}S]-GTPγS to activated Gα subunits in a cell membrane preparation) was precluded by the finding that 100μM UTP reduces [^{35}S]-GTPγS binding, presumably by competition between UTP and GTPγS (Fig. 5.3). This also, therefore, ruled out any potential extension of [^{35}S]-GTPγS binding assays to study changes in recruitment of individual G-protein types by including a G-protein-specific immuno-precipitation step into the protocol, which would have enabled the demonstration of enhanced coupling specifically to $G\alpha_q$ in the co-stimulated system.

Increased $InsP_3$ production would be expected if an increase in coupling of either receptor to $G\alpha_q$ was solely responsible for potentiation of Ca^{2+} signalling, and the fact that phosphoinositide generation in the presence of both carbachol and isoprenaline is no different from that to carbachol alone (Fig. 5.12) suggests that additional $G\alpha_q$-coupling cannot underlie this crosstalk. Therefore, given that the interaction between muscarinic M3 receptors and β2 adrenoceptors did not result in enhanced $InsP_x$ generation, the [^{35}S]-GTPγS

binding technique was considered perhaps unnecessary in the investigation of this crosstalk, although it would have been a utilizable assay in this instance.

Whilst it would have been extremely interesting to test the physical interaction between P2Y2 receptors and CXCR2 by co-immunoprecipitation, this was not possible due to the lack of a suitable, commercially available antibody for either receptor for use in either Western blotting or in immuno-precipitations. This approach was therefore not a feasible option for the study of heterodimerisation between these receptors.

Section 5.3.5: Further investigation of differential potentiation capabilities of P2Y receptors

The demonstration of $G\alpha_q$ involvement in the P2Y2 receptor-CXCR2 crosstalk, shown by the inhibition of potentiation using the $G\alpha_q$-specific RGS protein, RGS2 (Fig. 5.6), strongly suggests that active $G\alpha_q$ subunits are also necessary for crosstalk. This again raises the issue of why P2Y1 receptors are less able to mediate potentiation. The similarity in maximal Ca^{2+} responses following stimulation of each of these receptors suggests that P2Y1 receptors activate $G\alpha_q$ to an extent comparable to that by P2Y2 receptors, at least in the initial stages after agonist exposure. This is perhaps further evidence that desensitisation is central to this phenomenon, such that $G\alpha_q$ stimulation is minimal in the protocols used to study P2Y1 receptors thus far. This issue was directly addressed by a number of methods to evaluate the extent to which P2Y1 receptors are desensitised at the point at which IL-8 was added.

Stimulation of inositol phosphate accumulation by P2Y1 receptors appeared to cease after as little as 120s of agonist presence (Fig. 5.8), unlike during stimulation of P2Y2 receptors, where accumulation continued up to around 5-10mins (Figs. 2.13 and 3.3). If this reflects receptor desensitisation (rather than desensitisation of PLCβ or some other factor), it shows

that the P2Y1 receptor would be largely inactivated before the addition of IL-8 following 150s of continuous P2Y1 receptor stimulation. This relative lack of activated $G\alpha_q$ would clarify why potentiation is less apparent downstream of these receptors (Figs. in Chapter 3, and Fig. 5.9a). It is noted that potentiation of inositol phosphate generation does occur in the presence of IL-8 compared to application of 2MeSADP alone (Fig. 5.9b), but that this is small relative to that seen following stimulation with UTP and IL-8, consistent with the smaller effect of this nucleotide on IL-8-mediated Ca^{2+} signalling. It is possible, therefore, that the reduction in active $G\alpha_q$ subunits is responsible for the limitation of potentiation shown here. This was strongly supported by the demonstration that the ability of P2Y2 receptors to mediate potentiation was reduced by extending the duration of UTP stimulation of these receptors to 15mins, a point at which P2Y2 receptors would themselves be largely desensitised (at least in terms of phosphoinositide generation) (Fig. 5.10). This being the case, it was decided to investigate whether the ability of P2Y1 to mediate crosstalk was increased if the pre-stimulation protocol was modified so as to minimise the extent of receptor desensitisation. Two methods were used, both designed to minimise the extent of P2Y1 receptor desensitisation at the point of IL-8 addition. Results were inconclusive. Reduction of P2Y1 receptor exposure to 2MeSADP to only 30s appeared to cause a small (but non-significant) increase in the ability of this receptor to participate in potentiation. However, the same could not be said when store drainage was performed by UTP, following which the response to co-addition of 2MeSADP and IL-8 was still very limited compared to that in response to co-addition of UTP and IL-8 following the same drainage protocol. It seems that there may be more than one reason for the relative inability of P2Y1 to participate in potentiation. There may be some relevance to the fact that the "2MeSADP-sensitive" Ca^{2+} store is at least partially distinct from the "UTP-sensitive" store (Fig. 4.18). It is possible that the P2Y1 receptors and CXCR2 are partitioned into separate membrane localisations, and cannot interact due to the physical distance between them. Similarly, if dimerisation is

significant to the potentiation, the inactivity of P2Y1 receptors may result from some intrinsic inability to form dimers with CXCR2. The possibility that certain receptors may have a fundamental inability to interact with certain other receptors in a given cellular environment is discussed in the general discussion at the end of this thesis.

Section 5.3.6: Relevance of phosphorylation state

The role of a PKC-mediated phosphorylation event in potentiation was ruled out in the previous chapter by the demonstration that a broad spectrum PKC inhibitor had no effect on the magnitude of the Ca^{2+} response to IL-8 following UTP pre-stimulation. However, the demonstration that PLCβ desensitisation appeared to be delayed in the combined presence of UTP and IL-8 (Fig. 4.12), suggested that one of these agonists may be exerting some modulatory control over the activity of PLCβ. It is known that CXCR2 phosphorylates and inactivates PLCβ3 (Richardson *et al*, 1998), so a role for the P2Y2 receptor in activating a phosphatase that helps PLCβ3 to resist this modulation may be postulated. However, the use of inhibitors of protein phosphatase 1 (PP1)/PP2A (okadaic acid) and PP2B (cyclosporin A), demonstrated the lack of involvement of these phosphatases in the potentiation of Ca^{2+} signalling (Figs. 3.4 and 3.5). This ruled out mechanisms such as the de-phosphorylation and re-activation of either a Ca^{2+}-signalling GPCR or an effector such as PLCβ.

The inhibition of adenylyl cyclase activity by $G\alpha_i$-coupled CXCR2 is well known (Hall *et al*, 1999). It is unclear to what extent P2Y2 receptors are able to elevate cAMP. Reported positive effects on basal and forskolin-stimulated cAMP levels (Post *et al*, 1998; Hopfner *et al*, 2001; Suh *et al*, 2001) appear to be somewhat cell-type specific (van der Weyden *et al*, 2000b), and differ from effects on receptor-induced cAMP formation (Suh *et al*, 2001). The effects of P2Y2 receptor stimulation on cAMP levels in HEK cells have not been documented, and are not studied here. While it was considered unlikely that P2Y2 receptor-

induced cAMP generation would underlie the interaction between this receptor and CXCR2, it could not be ruled out. Conversely, even in the absence of any P2Y2 receptor effects on cAMP, it should be considered that PKA may impose some form of tonic inhibition on $G\alpha_q$ signalling, perhaps by negative regulation of PLCβ (Laglia *et al*, 1996; Hoiting *et al*, 1996; Yue *et al*, 1998 and 2000; Dodge and Sanborn, 1998). Thus, subsequent removal of this inhibition by a $G\alpha_i$-coupled receptor (e.g. CXCR2) could potentially underlie the secondary response seen following IL-8 addition. The results of the administration of the myristoylated amide 14-22 PKA inhibitor are somewhat ambiguous. While pre-inhibition of PKA with 14-22 amide would be expected to negate the effects of CXCR2 (Fig. 5.16), it would also be expected to enhance signalling by P2Y2 receptors by removing this tonic inhibitory control. Even if no increase in peak magnitude was seen following this treatment, the response would, at the very least, stay the same. It is difficult to create a model of PKA inhibition that adequately explains the reduction of both P2Y2 receptor-mediated and potentiation responses. It is somewhat easier to interpret these results if PKA actually *facilitates* P2Y2 receptor-mediated responses. Thus, the reduction of the potentiation response by 14-22 amide would occur secondary to a reduction in P2Y2 receptor signalling. This justifies the rejection of PKA as a possible factor in the positive interaction between P2Y2 receptors and CXCR2.

A model involving PKA is more applicable to the interaction between muscarinic M3 receptors and $G\alpha_s$-coupled β2 adrenoceptors. There are several potential points where PKA could cause phosphorylation to modulate signalling capacity. There are instances where PKA increases the activity of PLCβ (Blackmore and Exton, 1986; Pittner and Fain, 1989), although there are rather more examples of PKA exerting negative regulatory control over PLCβ (Laglia *et al*, 1996; Hoiting *et al*, 1996; Yue *et al*, 1998 and 2000; Dodge and Sanborn, 1998).

PKA may sensitise $InsP_3$ receptors through phosphorylation (Burgess *et al*, 1991; Hajnóczky *et al*, 1993; Wojcikiewicz and Luo, 1998; Bruce *et al*, 2002). The phosphorylation of $InsP_3$ receptors has been shown to shape Ca^{2+} signals in pancreatic acinar cells in response to peptide agonists (Straub *et al*, 2002). Potentiation of Ca^{2+} release by $G\alpha_q$-coupled GPCRs through stimulation of either $G\alpha_s$-coupled GPCRs and/or forskolin has been well documented in numerous cell types, including brown fat cells (Leaver & Papone, 2002), articular chondrocytes (D'Andrea *et al*, 1996), hepatocytes (Burgess *et al*, 1991; Hajnóczky *et al*, 1993), pancreatic β-cells (Liu *et al*, 1996b) and parotid cells (Rubin & Adolf, 1994). PKA has either been strongly implicated or shown directly to be involved in all these examples of potentiation, although there is some division as to the mechanism. There is some opposition to the idea that the $InsP_3$ receptor itself is the relevant PKA target, since there are conflicting reports on the effect of PKA phosphorylation on the $InsP_3$ receptor (Supattapone *et al*, 1988; Volpe & Alderson-Lang, 1990). An alternative mechanism is that a PKA-mediated phosphorylation event enhances the Ca^{2+} loading state of the intracellular stores. Indeed, increased Ca^{2+} loading reduces the EC_{50} for $InsP_3$-mediated Ca^{2+} release (Nunn and Taylor, 1992) and several studies have demonstrated a PKA-induced increase in the $InsP_3$-releasable Ca^{2+} pool (Hajnóczky *et al*, 1993; Burgess *et al*, 1991; Enouf *et al*, 1987). Of the evidence supporting the direct phosphorylation of the $InsP_3$ receptor, most examples fall into one of two categories. There is evidence that PKA-mediated phosphorylation of the $InsP_3$ receptor enhances channel open-time (Hajnóczky *et al*, 1993; Volpe & Alderson-Lang, 1990) and/or an increase in sensitivity of the $InsP_3$ receptor (Wojiciewicz & Luo, 1998). The effects of cAMP/PKA can occur in the absence of changes in $InsP_3$ levels, implying that altered production or metabolism of $InsP_3$ is not required.

In light of these data, it is interesting that the co-stimulation of HEK-WT cells with carbachol and isoproterenol did not cause phosphoinositide production above that produced by carbachol stimulation alone (Fig. 5.12). This alone rules out the application of any model that would predict an increase in $InsP_3$ production, such as the co-operativity between $G\alpha_q$ and $G\beta\gamma$, or the gain of $G\alpha_q$ coupling following dimerisation. Signal enhancement downstream of PLCβ and phosphoinositide production strongly implicates the $InsP_3$ receptor and/or the Ca^{2+} store, and there is much evidence supporting the roles of PKA in both of these processes, as shown above. However, it is shown here that PKA is not involved in the crosstalk between muscarinic M3 receptors and β2 adrenoceptors. Neither forskolin nor dbcAMP were able to substitute for $G\alpha_s$ stimulation (Fig. 5.13 and Fig. 5.14). An identical situation occurs in neocortical and hippocampal neuronal primary cultures, where D1/D5 dopamine receptors elicit Ca^{2+} responses only following priming with $G\alpha_q$-coupled receptors but in a way that is not mimicked by either FSK or dbcAMP (Lezcano & Bergson, 2002). Furthermore, a PKA antagonist did block potentiation (Fig. 5.15), although it appeared to have too many potential other effects on signalling in general (Fig. 5.15 and Fig. 5.16) to be considered a particularly valid tool. As proposed above, the effects on potentiation may be secondary to an inhibitory effect on $G\alpha_q$-mediated signalling downstream of the muscarinic M3 receptor. This implicates an alternative mechanism in defining this crosstalk that does not involve either enhancement of phosphoinositide production or enhancement of the efficiency of Ca^{2+} release from $InsP_3$-sensitive stores. However, it should be noted that the kinetics of cAMP elevation by β2 adrenoceptor agonists may be different to that by forskolin or dbcAMP. This may underlie the failure of forskolin and dbcAMP to mimic the potentiation seen with isoproterenol due to an inability to raise cAMP levels in the same spatio-temporal pattern as this agonist. It would be useful to investigate the effects of an agent such as 2-APB on the crosstalk between muscarinic M3 receptors and β2 adrenoceptors to determine whether $InsP_3$ is involved anywhere in this interaction. $InsP_3$

levels may still be crucial to this interaction, but may be regulated by a decrease in the metabolism of $InsP_3$ rather than by an increase in its production by PLCβ. In this respect, the demonstration that phosphoinositide production is not enhanced in this situation is perhaps of limited value, since this gives no specific indication of inositol trisphosphate levels, and in particular the level of $Ins(1,4,5)P_3$. Indeed, in smooth muscle cells, $Ins(1,4,5)P_3$ elevation has been shown to be highly transient, and is followed by sustained increases in the accumulation of other inositol trisphosphate species, suggesting a rapid metabolism of $Ins(1,4,5)P_3$ into other inositol trisphosphates such as $Ins(1,3,4)P_3$ (Chilvers *et al*, 1991). Furthermore, it has been shown that increased $InsP_3$ metabolism (by transient transfection of $InsP_3$ 3-kinase) markedly reduces subsequent Ca^{2+} responses compared to non-transfected cells (Nash *et al*, 2002). This suggests that *inhibition* of such enzymes may have a substantial enough effect to significantly enhance both $InsP_3$ levels and, subsequently, $InsP_3$-dependent Ca^{2+} release. However, little is known about the ability of GPCRs to act in this way to modulate $InsP_3$ metabolism. There is a suggestion that cAMP may activate $InsP_3$ 3-kinase activity (Abdel-Latif, 1996; Ding *et al*, 1997), a function that would oppose any role of the $G\alpha_s$-coupled (cAMP-elevating) β2 adrenoceptor in mediating Ca^{2+}-enhancing crosstalk by decreasing $InsP_3$ metabolism.

If $InsP_3$ is not involved, candidate mechanisms for $InsP_3$-independent GPCR crosstalk may be the model of receptor redistribution dependent on dimerisation, or may involve one of the Ca^{2+} stores accessed by alternative Ca^{2+}-release pathways that were shown not to have any role in the interaction between P2Y2 receptors and CXCR2. Alternatively, it is interesting to note that in the primary neuronal cultures referred to above (Lezcano & Bergson, 2002), the D1/D5 dopamine receptor-induced Ca^{2+} response, while insensitive to artificial elevation of cAMP, was sensitive to PKC inhibition, suggesting that this enzyme may have a role in this interaction and perhaps to other examples of crosstalk involving Gs-coupled GPCRs.

This chapter addresses particular potential models of GPCR crosstalk for their involvement in the potentiation of Ca^{2+} signalling seen following joint stimulation of P2Y2 receptors and CXCR2. The findings of this investigation were that the mechanism was rather more complex than a simple pooling of some signalling mediator (i.e. $G\beta\gamma$), and had no dependence on phosphatase activity, thus ruling out the resensitisation of P2Y2 as an explanation for the secondary response observed upon addition of IL-8. In addition, the dependence of the phenomenon on the continued activation of $G\alpha_q$ subunits and presence of $G\beta\gamma$ subunits strongly supports the model proposed involving co-operativity between these proteins in terms of activating PLCβ such that phosphoinositide production and, subsequently, Ca^{2+} responses are enhanced. It was also concluded that the failure of P2Y1 to initiate an equally impressive potentiation was because this receptor did not fulfil the requirement for supply of activated $G\alpha_q$, being desensitised at the point of addition of IL-8. Finally, it was discovered that the crosstalk between P2Y2 receptors and CXCR2 was notably different from that between muscarinic M3 receptors and β2 adrenoceptors, since there existed certain fundamental differences in the characteristics defining each of these. The interaction between muscarinic M3 receptors and β2 adrenoceptors is therefore likely to involve a different mechanism of communication to execute the enhancement of Ca^{2+} signalling seen following concurrent stimulation of these receptors.

Chapter 6 – Search for crosstalk in physiologically-relevant cell types

Section 6.1.1: Introduction

This study has been focused on defining the mechanisms underlying two crosstalk phenomena, one occurring in a CXCR2-transfected HEK cell line, the other occurring in the HEK-WT cell. These studies provide valuable information about the ways in which GPCRs interact with one another in the course of signal transduction, and have allowed the exploration of the mechanisms underlying these crosstalks. Communication between GPCRs is reasonably commonplace in recombinant systems, but less information is available regarding parallel interactions in more physiological settings, in primary cell types endogenously expressing these receptors. Some examples have been documented, in cells derived from the central nervous system and systemic tissues. These include the enhancement of muscarinic receptor-induced excitatory post-synaptic potentials (EPSPs) by μ-opioid receptor agonists in hippocampal neurons (Kearns *et al*, 2001) and the augmentation of GABA release in rat periaqueductal gray neurons co-stimulated with 5-HT_{1A} serotonergic and μ-opioid receptor agonists (Kishimoto *et al*, 2001). $GABA_B$ receptors have been shown to enhance mGluR-mediated Ca^{2+} signalling and, consequently, to facilitate excitatory transmission in cerebellar synapses (Hirono *et al*, 2001). In the periphery, the range of effects of receptor interactions is somewhat broader. Activation of ($G\alpha_s$-coupled) parathyroid hormone receptors causes potentiated $[Ca^{2+}]_i$ responses to activation of $G\alpha_q$-coupled P2Y1 nucleotide agonists in rat osteoblasts (Buckley *et al*, 2001). In addition, interleukin-1β (IL-1β) causes potentiation of phosphoinositide hydrolysis and Ca^{2+} signalling downstream of the bradykinin B2 receptor in canine tracheal smooth muscle cells (Yang *et*

al, 2001). Further, the chemokine, monocyte chemotactic protein-1 (MCP-1), up-regulates vascular smooth muscle cell proliferation induced by serotonergic agonists (Watanabe *et al*, 2001b). It is clear, therefore, that GPCR crosstalk is of considerable relevance to the functioning of a variety of cell types and signal pathways. The aim of work described in this chapter was to extend the studies from the recombinant system into a series of primary cell types expressing both CXCR2 and P2Y receptors in order to assess the potential physiological impact of this crosstalk on their functionality.

CXCR2 expression has been demonstrated in numerous cell types of the central nervous system and in many systemic cell types, mainly in the leukocytes that provide an immune barrier against infection and tissue damage. However, many of these cell types can be excluded from any investigation here given their lack of expression of P2Y2 receptors. CXCR2 and P2Y2 receptor expression patterns have been covered in detail in the main introduction to this thesis, but consideration must be paid to those cells that express both types of receptor. Most leukocytes express one or more members of the P2Y nucleotide receptor family, including P2Y2, which is expressed in neutrophils and monocytes (Boarder *et al*, 1995; Jin *et al*, 1998). These cells also express appreciable levels of CXCR2, so represent a suitable physiological model in which to study CXCR2-P2Y2 receptor crosstalk. Co-expression of these receptors is also apparent in human microvascular endothelial cells (HMVECs) (Salcedo *et al*, 2000), hence these cells also provide a suitable experimental model. Cells co-expressing muscarinic M3 receptors and β2 adrenoceptors were not considered in this section for reasons of time restriction, although these perhaps represent more varied options for physiological cell models given their frequent co-expression in numerous cell types, perhaps most obviously in various types of smooth muscle (reviewed by O'Rourke & Vanhoutte, 1992; Abdel-Latif, 1996; Hegde & Eglen, 1999).

The significance of crosstalk at the level of Ca^{2+} signalling is unclear. At the simplest level, the increase in the magnitude of $[Ca^{2+}]_i$ may allow a greater response (e.g. stronger contraction, increased secretion, etc). Alternatively, the requirement for simultaneous stimulation of two receptors may afford a level of protection against inappropriate signalling. An extension of this would be that cellular responses could be modified depending on the presence or absence of a second agonist. Of relevance to this study is the possibility that a chemokine receptor could direct a leukocyte to perform a different function in the absence of ATP (e.g. in non-traumatic, inflammatory lesions) to that in the presence of ATP (e.g. in traumatic or necrotic lesions) as a result of crosstalk between $G\alpha_i$-coupled chemokine receptors and $G\alpha_q$-coupled P2Y nucleotide receptors. The ability to 'tailor' a response depending on a set of agonist criteria might serve to increase the flexibility of function in certain cells, and thus refine and widen the array of actions performed by a limited range of cell types. There are several benefits to having receptor signalling pathways that can variably converge and diverge, although there are few specific examples where potentiated Ca^{2+} signalling has been carried through to the up-regulation of a final cellular response. However, the infrequency of redundant interactions (i.e. interactions without physiological consequence) occurring in living cells would suggest that there is a definite role for receptor crosstalk given the accumulating evidence of crosstalk in endogenous systems.

Having elucidated a mechanism of interaction between GPCRs in a recombinant expression system, this chapter moves away from this cell line model to investigate the potential existence of this phenomenon in the more physiological setting of primary cell preparations co-expressing P2Y2 receptors and CXCR2. Initial investigations were aimed at replicating the crosstalk seen in the HEK-CXCR2 cells and demonstrating enhanced Ca^{2+} signalling in primary cells co-stimulated with nucleotide and chemokine. However, the demonstration of enhanced Ca^{2+} signalling resulting from co-stimulation of endogenously expressed receptors

is perhaps only the first step towards defining a role for this phenomenon in a physiological setting. The crucial question regards what downstream effects this modulation of Ca^{2+} signalling has to the final functional outputs of a cell. It has been discussed in earlier chapters that small fluctuations or modifications of a Ca^{2+} signal, in terms of both amplitude, frequency and spatio-temporal characteristics, can have disproportionate effects on the nature of the resultant response to that signal. For example, an interaction between nucleotide receptors and chemokine receptors may cause a shift in the behaviour of a leukocyte in terms of chemotaxis, activation and degranulation, behaviour that may require some degree of adaptation depending on circumstance. The combined presence of ATP and chemokine may then act as a type of coincidence detector. The consequences of Ca^{2+} signal modulation may differ between cell types also, depending on any existing Ca^{2+} signalling capacity in a given cell. This supports the concept of a Ca^{2+} signalling 'toolkit' proposed previously (Bootman *et al*, 2001) i.e. that a Ca^{2+} signal attains its striking diversity in terms of biological outputs by means of a cell assembling a unique profile of factors that determine that cell's response to elevation of $[Ca^{2+}]_i$. It was intended to extend any successful demonstration of crosstalk in primary cell types to an investigation of the functional consequences of this interaction. However, it is demonstrated in this chapter that, in three cell types co-expressing UTP-sensitive P2Y receptors and CXCR2, no enhancement of Ca^{2+} signalling could be observed upon co-stimulation. The possible reasons for this are explored in the discussion to this chapter and the final discussion, but this finding precluded the investigation of any downstream functional outputs.

Section 6.1.2: Experimental design

Certain changes were necessary in the pharmacological ligands and the protocols used to study Ca^{2+} changes in primary cells in order to optimise the selectivity of the assays and to minimise unwanted endogenous stimuli.

The preferred CXCR2 chemokine agonist was GROα. This was due to the non-specificity of IL-8 for CXCR2 over CXCR1. The lack of expression of CXCR1 in the HEK-CXCR2 cell line negated this effect in these cells, but all of the cell types used here (neutrophils, monocytes, HMVECs) express CXCR1 receptors (Murphy *et al*, 2000; Salcedo *et al*, 2000), and thus a more CXCR2-selective agonist was required.

The interference of endogenous ATP released from cells in response to stress has been addressed previously in Chapter 3. Release of ATP is likely to be an even greater concern in the study of leukocytes, in particular neutrophils, as isolation of these cells from whole blood requires the hypotonic lysis of red blood cells, which releases large amounts of ATP. The prolonged presence of ATP may cause desensitisation of P2Y2 receptors and therefore interfere with their subsequent ability to participate in crosstalk, or alternatively could 'pre-potentiate' the cells such that the observed response to GROα 'alone' is actually the combination of GROα and released ATP. This is a possible explanation for the profound variability in EC_{50} values for GROα stimulated Ca^{2+} release in neutrophils (Dr C. Grahames, AstraZeneca, personal communication), which may then be influenced by the level of ATP in the system prior to assay. Apyrase was thus employed to counter any effects of ATP in the physiological buffer during the period leading up to assay and during the assay itself, with the intention of removing ATP. To further investigate the effects of nucleotides (in a more controlled manner), the apyrase-resistant nucleotide analogue, ATPγS (whose P2Y subtype selectivity is almost identical to ATP (Nicholas *et al*, 1996; Troadec *et al*, 1999)), was used in the ATP-free system to investigate the ability of this nucleotide to modify CXCR2 signalling without external influence from released ATP. HMVECs did not require these measures as these were grown as monolayers and examined using a Ca^{2+} imaging system under constant perfusion to remove any ATP that was released.

One potential difficulty of using apyrase is that it degrades nucleotide polyphosphates to their base structures, which in the case of ATP is adenosine. Adenosine itself has been shown to have various cellular effects as an extracellular agonist including participation in crosstalk phenomena (Gerwins & Fredholm, 1992a and b), acting through three sub-families of adenosine receptors, namely A1, A2 and A3. These receptors modulate adenylyl cyclase activity, positively (*via* $G\alpha_s$) in the case of A2, negatively (*via* $G\alpha_i$) in the case of A1 and A3 (reviewed by Fredholm *et al*, 2001). Additional experiments were therefore performed to test whether this agonist influenced the observed response to GROα in the presence or absence of ATP and apyrase. Thus, the adenosine receptor antagonist, 8-(*p*-sulfophenyl)theophylline (8-PST) was included in some assays to block the actions of any adenosine generated by the action of apyrase.

Section 6.2: Results

Section 6.2.1: Effects of nucleotide pre-stimulation on GROα-induced $[Ca^{2+}]_i$ responses in neutrophils

The co-expression of Ca^{2+}-signalling nucleotide receptors and CXCR2 in neutrophils was confirmed by stimulating these cells with ATP (or ATPγS) and GROα, respectively. Neutrophils exhibited robust Ca^{2+} responses to both nucleotides and GROα (Fig. 6.1), indicating expression of functional receptors for each of these ligands.

In order to assess the potential effects of endogenous ATP released at the red blood cell lysis step of the neutrophil isolation procedure, experiments were performed in either the presence or absence of the nucleoside polyphosphate-degrading enzyme, apyrase[§]. The effects of apyrase on nucleotide responses are shown in Fig. 6.2. All responses are compared to the $[Ca^{2+}]_i$ elevation in response to 100μM ATP in the absence of any other agents or pre-stimulations. In the absence of apyrase, 100μM ATPγS gave an elevation of $[Ca^{2+}]_i$ (88±3%) that was comparable to that stimulated by 100μM ATP (100%). However, addition of apyrase (2U/ml) ablated the response to ATP (1±0.2% of control; $P<0.001$)), without affecting that to ATPγS. Buffer did not cause a Ca^{2+} response in either the presence or absence of the ATP degrading enzyme.

Following pre-treatment of neutrophils with either nucleotide agonists (or buffer as a control), the cells were stimulated with GROα (concentration range: 0.1-100nM) and the subsequent Ca^{2+} responses quantified (Fig. 6.3). After an addition of buffer (Fig. 6.3a),

[§] Apyrase was included in all solutions from the fluo-3/AM loading step onwards. It was also present in the agonist solutions added to the cell preparation during the experiment, meaning that apyrase had a period of ~2-5min prior to assay in which to degrade nucleotide polyphosphates in these solutions. It should be noted, therefore, that addition to the assay does not correspond to the first exposure of these agonists to apyrase, and that the dramatic reduction in responses to these agonists is not due to an almost instantaneous removal of nucleotide polyphosphates.

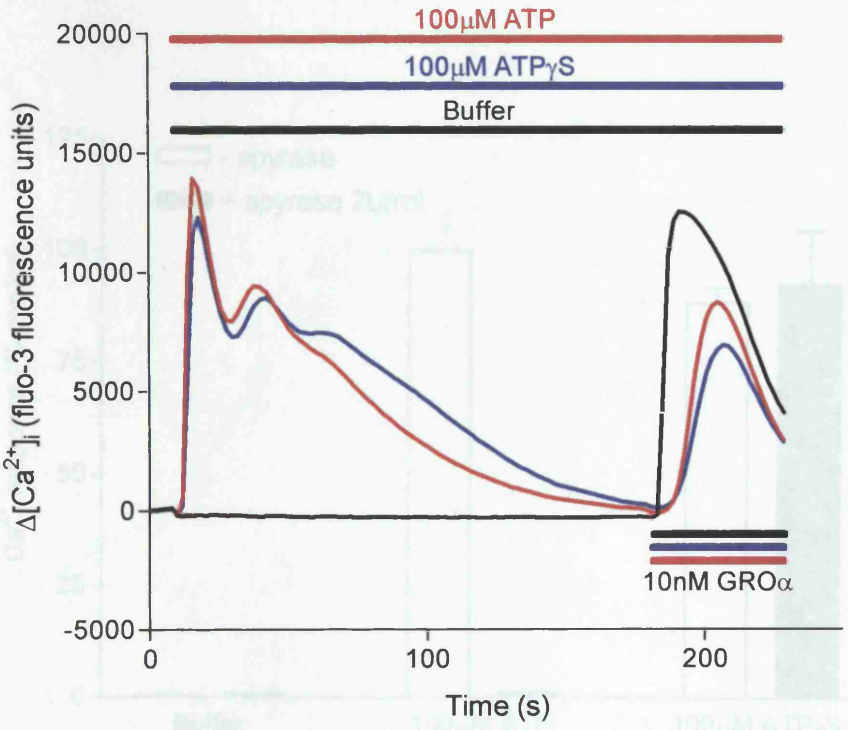


Figure 6.1: Effects of nucleotide agonists and IL-8 acting on human neutrophils. Human neutrophils were isolated from fresh whole blood by density gradient centrifugation (see Materials and Methods) and loaded with fluo-3 dye by incubating for 1hr with 5μM fluo-3/AM at room temperature. Stimulated increases in $[Ca^{2+}]_i$ were measured by FLIPR. Cells were stimulated with either 100μM ATP, 100μM ATPγS, or buffer alone, followed approximately 180s later by an addition of 10nM IL-8 in the continued presence of the pre-stimulating agonist (or buffer). Data are the mean of more than 15 similar experiments.

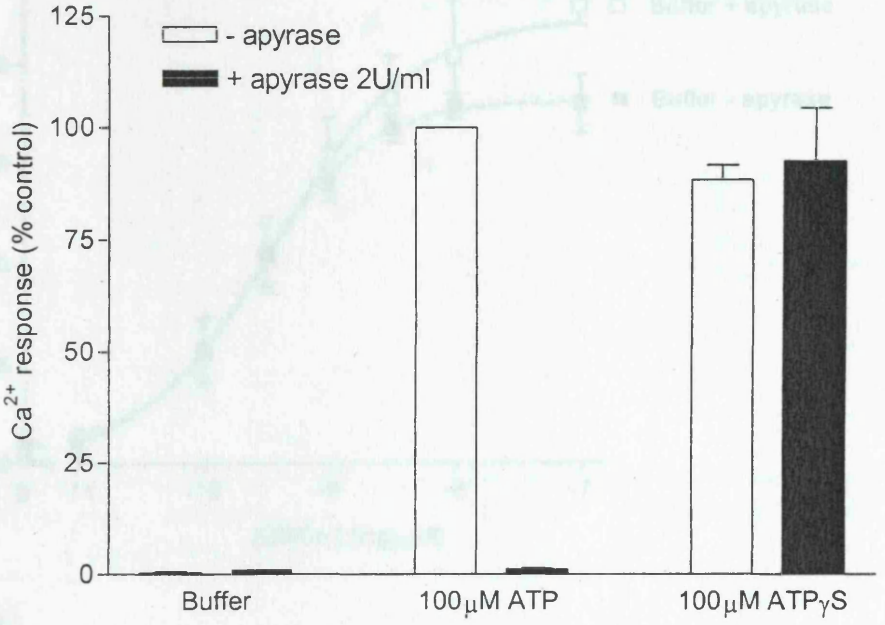


Figure 6.2: Effects of apyrase on nucleotide agonists acting on human neutrophils. Human neutrophils were isolated from fresh whole blood by density gradient centrifugation (see Materials and Methods) and loaded with fluo-3 dye by incubating for 1hr with 5μM fluo-3/AM at room temperature. To some samples was added 2U/ml apyrase for the duration of the loading step. Apyrase was included in all future additions to these cells. Stimulated increases in $[Ca^{2+}]_i$ were measured by FLIPR. Cells were stimulated with either 100μM ATP, 100μM ATPγS, or no agonist (Buffer), in the presence or absence of apyrase (2U/ml). Responses are expressed relative to the response to 100μM ATP in the absence of apyrase. Data are mean±s.e.m., n=5.

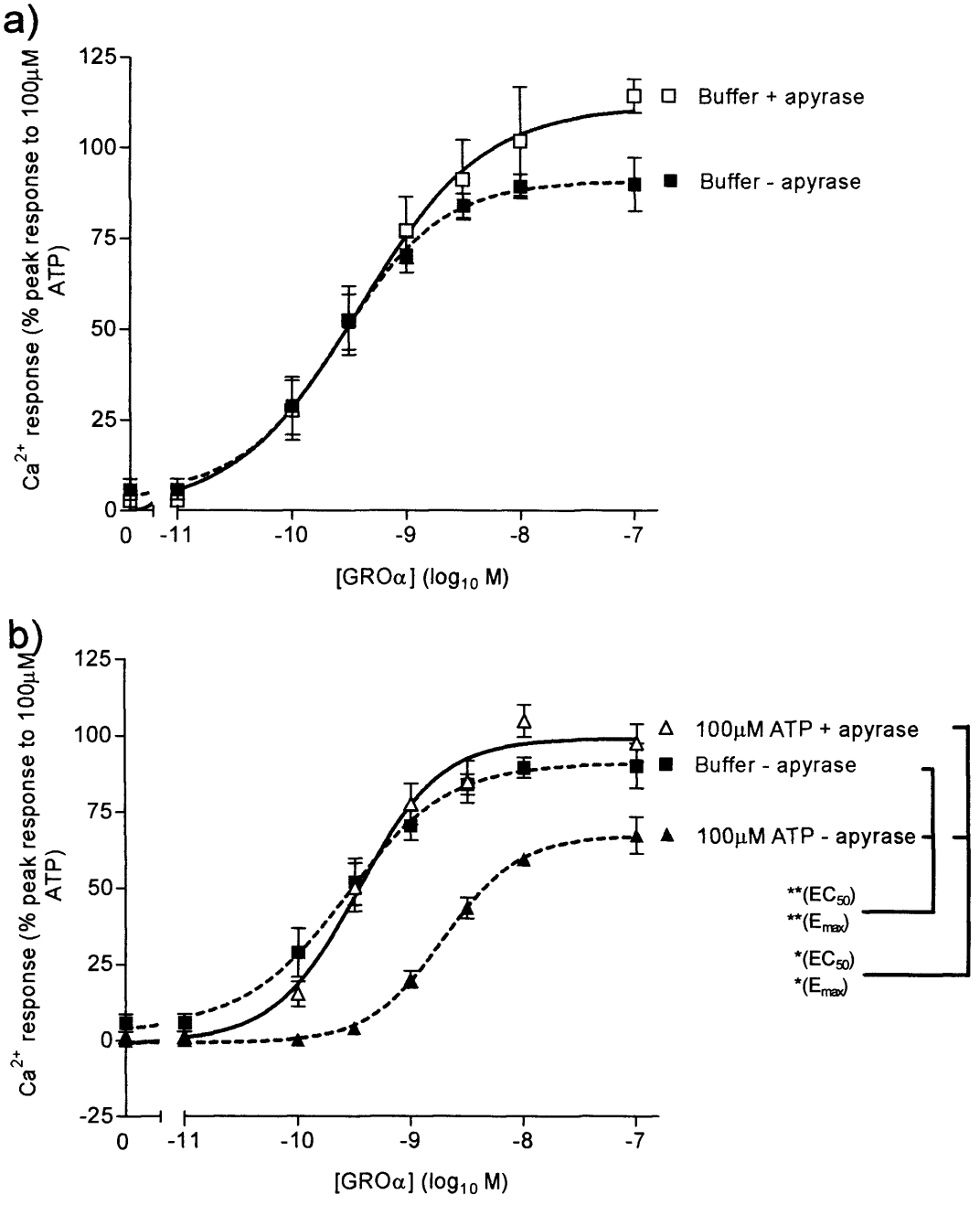
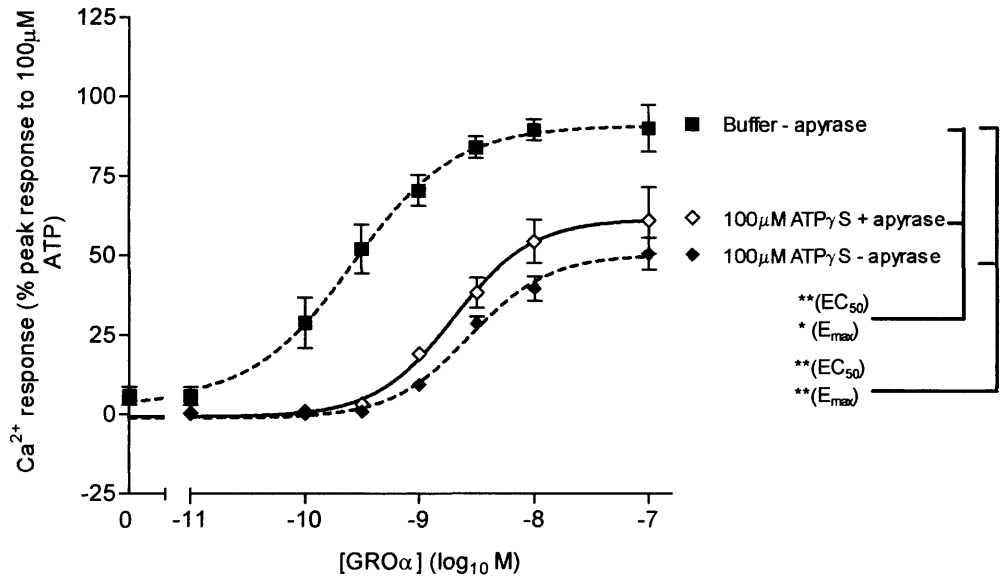


Figure 6.3 a) and b). See over for Fig. 6.3 c), summary table and legend.

c)



GROα pEC_{50} following pre-stimulation with:	- apyrase	+ apyrase (2U/ml)
Buffer	9.6±0.1	9.5±0.3
100μM ATP	8.7±0.1	9.5±0.1
100μM ATPγS	8.6±0.1	8.7±0.1

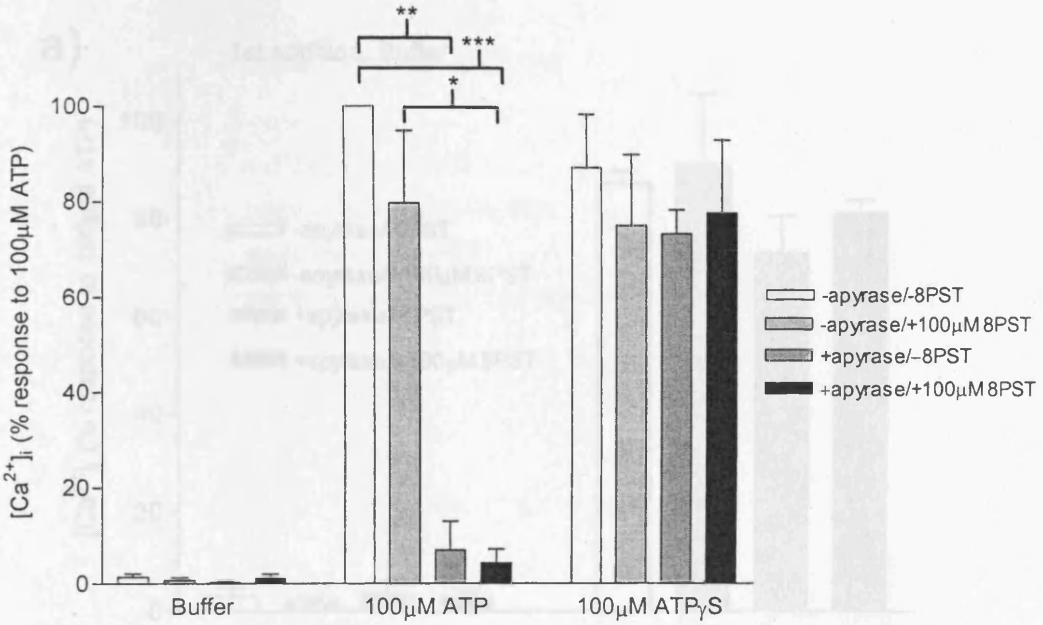
GROα E_{max} (% control) following pre-stimulation with:	-apyrase	+ apyrase (2U/ml)
Buffer	91±4	114±14
100μM ATP	67±3	99±5
100μM ATPγS	50±3	61±5

Figure 6.3: Effects of nucleotide agonists on GROα signalling in human neutrophils. Human neutrophils were isolated and assayed as in Fig. 6.1. Cells were pre-stimulated with either buffer (a), 100μM ATP (b), or 100μM ATPγS (c), followed 150s later by GROα. Values shown are responses to GROα expressed relative to the maximal response to 100μM ATP. A curve showing responses to GROα following buffer pre-addition in the absence of apyrase is included on all graphs as a comparison. Data are mean±s.e.m., n=5. *: $P<0.05$; **: $P<0.01$, by unpaired Student's t-test following two-way ANOVA. The tables summarise the pEC_{50} and E_{max} values for each of these treatments.

GROα stimulated $[Ca^{2+}]_i$ elevation with an pEC_{50} of 9.6±0.1 and an E_{max} of 91±4% of the maximal response to 100μM ATP. The presence of apyrase did not affect the pEC_{50} of this curve, but did cause a slight increase in the maximal response to GROα. Pre-stimulation of neutrophils with 100μM ATP caused a significant difference in the curve to GROα (Fig. 6.3b). Surprisingly, however, this was manifested as a significant *reduction* in both the pEC_{50} and the E_{max} of the GROα curve. The presence of apyrase restored the usual characteristics of the GROα curve. ATPγS also caused a significant reduction in the pEC_{50} and E_{max} of the GROα Ca^{2+} response, but these effects were resistant to apyrase (Fig. 6.3c).

To assess the potential effects of adenosine released by apyrase degradation of ATP, assays were performed in the presence or absence of the adenosine receptor antagonist, 8-(*p*-sulfophenyl)theophylline (8-PST) (Fig. 6.4). As shown previously, the presence of 2U/ml apyrase in the fluo-3/AM loading solution (and in all subsequent additions) significantly reduced the magnitude of the response to 100μM ATP, but had no significant effects on responses to 100μM ATPγS. The inclusion of 100μM 8-PST alongside apyrase had no significant effect on the observed responses to either ATP or ATPγS.

Similarly, 8-PST had no effect on the magnitude of responses to 10nM GROα following any of these pre-treatments (Fig. 6.5). As shown previously, 2U/ml apyrase had no significant effect on the maximal response to 10nM GROα in the absence of pre-stimulation (Fig. 6.5a). This situation was unaltered by the presence of 100μM 8-PST. Likewise, while the responses to GROα were inhibited by ATP pre-stimulation, and restored by the inclusion of apyrase during this pre-stimulation, there was no change under either of these conditions when 100μM 8-PST was included (Fig. 6.5b). Finally, 8-PST was without effect on GROα responses following pre-stimulation with 100μM ATPγS. ATPγS caused a significant



E_{max} (% control) of response to: ⇓	-apyrase		+ apyrase (2U/ml)	
	- 8PST	+ 8PST (100μM)	- 8PST	+ 8PST (100μM)
Buffer	1±1	1±1	0±0	0±0
100μM ATP	100	99±12	7±6	4±3
100μM ATPγS	94±6	94±12	83±3	98±6

Figure 6.4: Effects of adenosine receptor antagonism on nucleotide responses in apyrase-treated human neutrophils. Human neutrophils were isolated and assayed as in Fig. 6.1, except that 100μM 8-PST was included in some samples from the beginning of the loading period until the end of the assay. Stimulated increases in $[Ca^{2+}]_i$ were measured by FLIPR. Cells were stimulated with either 100μM ATP, 100μM ATPγS, or no agonist (Buffer), in the presence or absence of apyrase (2U/ml) and/or 8-PST (100μM). Responses are expressed relative to the response to 100μM ATP in the absence of apyrase. Data are mean±s.e.m., n=5. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$, by unpaired Student's t-test (where indicated) following two-way analysis of variance Table summarises E_{max} values for each treatment.

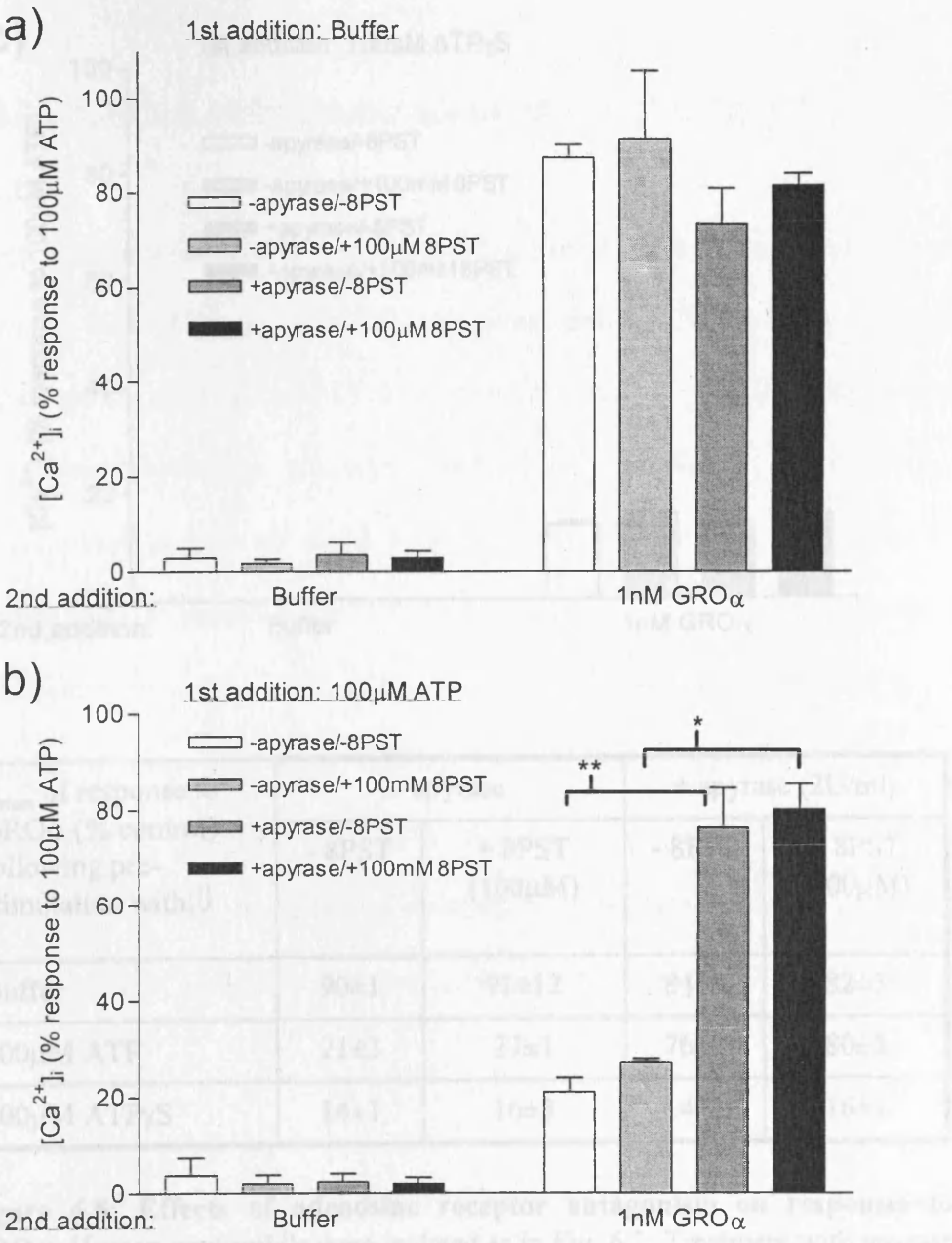
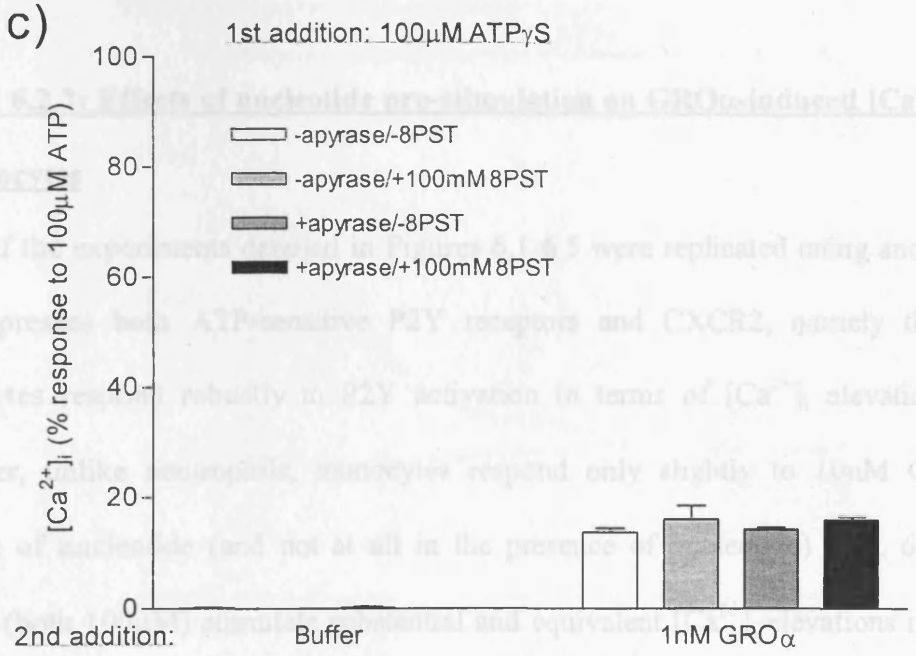


Figure 6.5 a) and b): See over for Fig. 6.6 c), summary table and legend.



E_{max} of response to GROα (% control) following pre-stimulation with:⇓	- apyrase		+ apyrase (2U/ml)	
	- 8PST	+ 8PST (100μM)	- 8PST	+ 8PST (100μM)
Buffer	90±1	91±12	84+8	82±3
100μM ATP	21±3	27±1	76±8	80±5
100μM ATPγS	14±1	16±3	14±1	16±1

Figure 6.5: Effects of adenosine receptor antagonism on responses to GROα. Human neutrophils were isolated as in Fig. 6.1. Treatment with apyrase (2U/ml) and/or 8-PST (100μM) was from the beginning of the fluo-3 loading period and continued until the end of the assay. Cells were pre-stimulated with either buffer (a), 100μM ATP (b), or 100μM ATPγS (c) and again, 150s later, with either buffer or 1nM GROα. Responses shown are to this second addition. Data are mean±s.e.m., n=3. *: $P<0.05$; **: $P<0.01$ by unpaired Student's t-test (where indicated) following one way ANOVA. Table summarises E_{max} values for GROα following each treatment.

reduction in the response to 10nM GROα in the absence or presence of apyrase, and the inclusion of 8-PST did not alter this in either the absence or presence of apyrase (Fig. 6.5c).

Section 6.2.2: Effects of nucleotide pre-stimulation on GROα-induced $[Ca^{2+}]_i$ responses in monocytes

Some of the experiments detailed in Figures 6.1-6.5 were replicated using another cell type that expresses both ATP-sensitive P2Y receptors and CXCR2, namely the monocyte. Monocytes respond robustly to P2Y activation in terms of $[Ca^{2+}]_i$ elevation (Fig. 6.6). However, unlike neutrophils, monocytes respond only slightly to 10nM GROα in the absence of nucleotide (and not at all in the presence of nucleotide) (Fig. 6.6). ATP and ATPγS (both 100μM) stimulate substantial and equivalent $[Ca^{2+}]_i$ elevations in the absence of any additional agents (Fig. 6.6 and Fig. 6.7). As in neutrophils, the response to ATP was ablated by the presence of apyrase, while apyrase was without significant effect on ATPγS responses (Fig. 6.7). The inclusion of 8-PST in these assays did not affect the responses to ATP or ATPγS in either the presence or absence of apyrase, although it did appear to increase the degree of variability in responses to ATPγS, as evidenced by the large standard errors.

The lack of response to GROα precluded the determination of pEC_{50} values for GROα in the presence or absence of nucleotides and apyrase (Fig. 6.8). Thus, all responses in Fig. 6.7 are quantified only by the magnitude of the peak $[Ca^{2+}]_i$ elevation (E_{max}) relative to the E_{max} of 100μM ATP in these cells. It also precluded any study of inhibitory effects of apyrase, 8-PST, or either of the nucleotide agonists. However, there was still the possibility that, as in neutrophils, apyrase may relieve some inhibitory action of endogenous ATP. Isolated monocytes were pre-stimulated with either 100μM ATP, 100μM ATPγS, or vehicle control, followed by an addition of GROα at a concentration of either 10nM (Fig. 6.9a) or 0.1nM

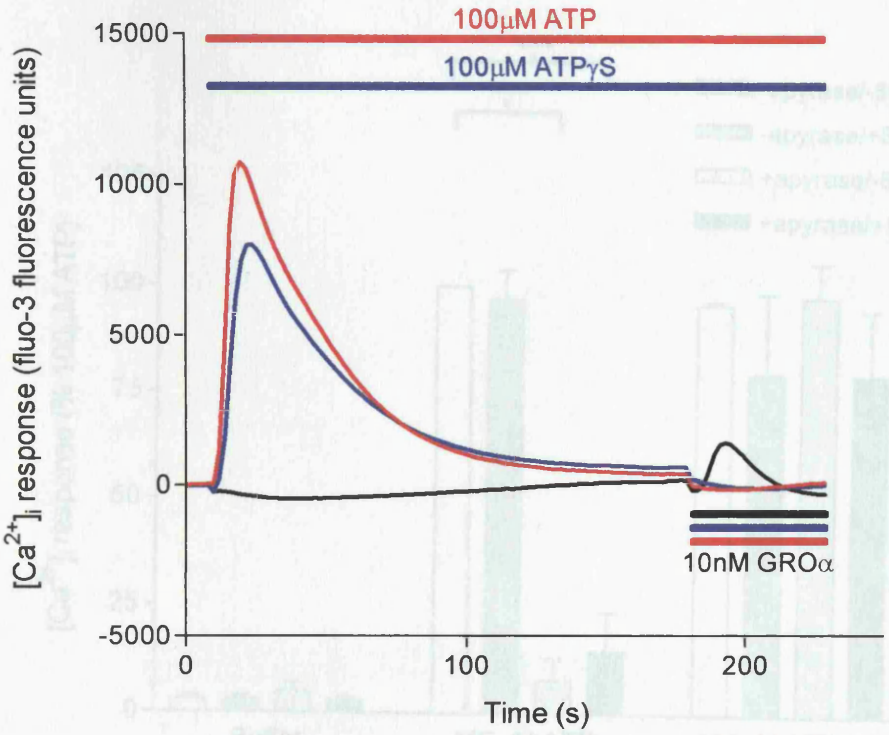
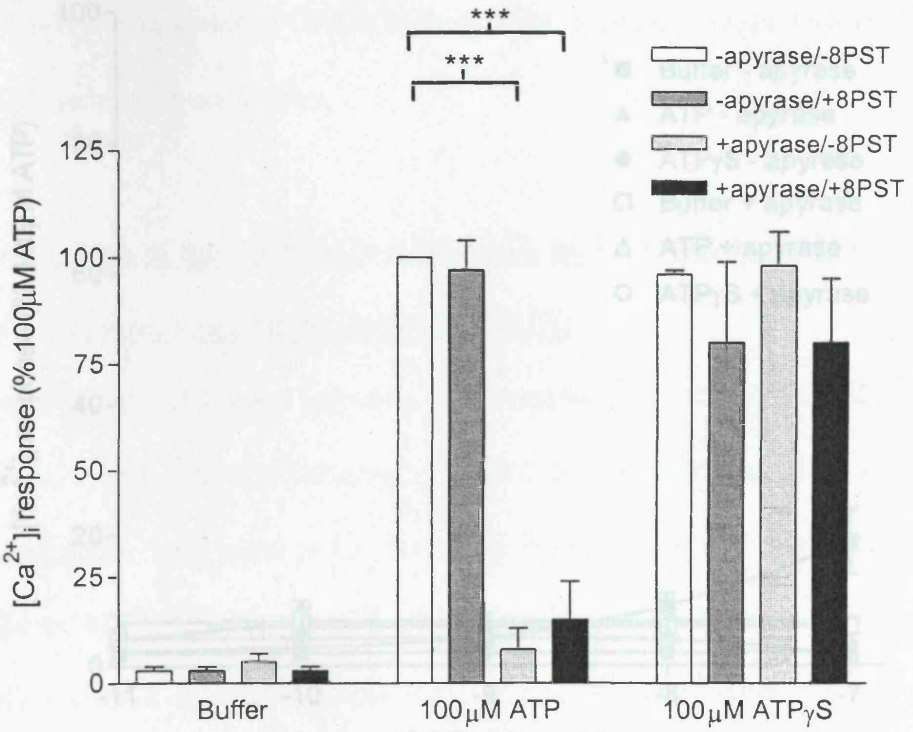


Figure 6.6: Effects of nucleotide agonists and IL-8 acting on human monocytes. Human monocytes were isolated from fresh whole blood (see Materials and Methods) and loaded with fluo-3 dye by incubating for 1hr with 5μM fluo-3/AM at room temperature. Stimulated increases in $[Ca^{2+}]_i$ were measured by FLIPR. Cells were stimulated with either 100μM ATP or 100μM ATPγS, followed approximately 180s later by an addition of 10nM IL-8 in the continued presence of the pre-stimulating agonist (or buffer). Responses are expressed relative to the response to 100μM ATP. Data are the mean of more than 15 identical experiments.



E_{max} (% control) of response to: ⇓	- apyrase		+ apyrase (2U/ml)	
	- 8PST	+ 8PST (100μM)	- 8PST	+ 8PST (100μM)
Buffer	3±1	3±1	5±2	2±1
100μM ATP	100	97±7	8±5	15±9
100μM ATPγS	96±1	80±19	98±8	80±15

Figure 6.7: Effects of nucleotide agonists in apyrase-treated human monocytes. Human monocytes were isolated by density gradient centrifugation, loaded for 1hr prior to assay with fluo-3/AM (5μM, room temperature) and plated onto poly-D-lysine-coated FLIPR plates. In some cases, apyrase (2U/ml and/or 8-PST (100μM)) was included in samples from the beginning of the loading period until the end of the assay. Stimulated increases in $[Ca^{2+}]_i$ were measured by FLIPR. Cells were stimulated with either 100μM ATP, 100μM ATPγS, or no agonist (Buffer), in the presence or absence of apyrase (2U/ml) and/or 8-PST (100μM). Responses are expressed relative to the response to 100μM ATP in the absence of apyrase. Data are mean±s.e.m., n=5. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$ by unpaired Student's t-test following two-way analysis of variance.

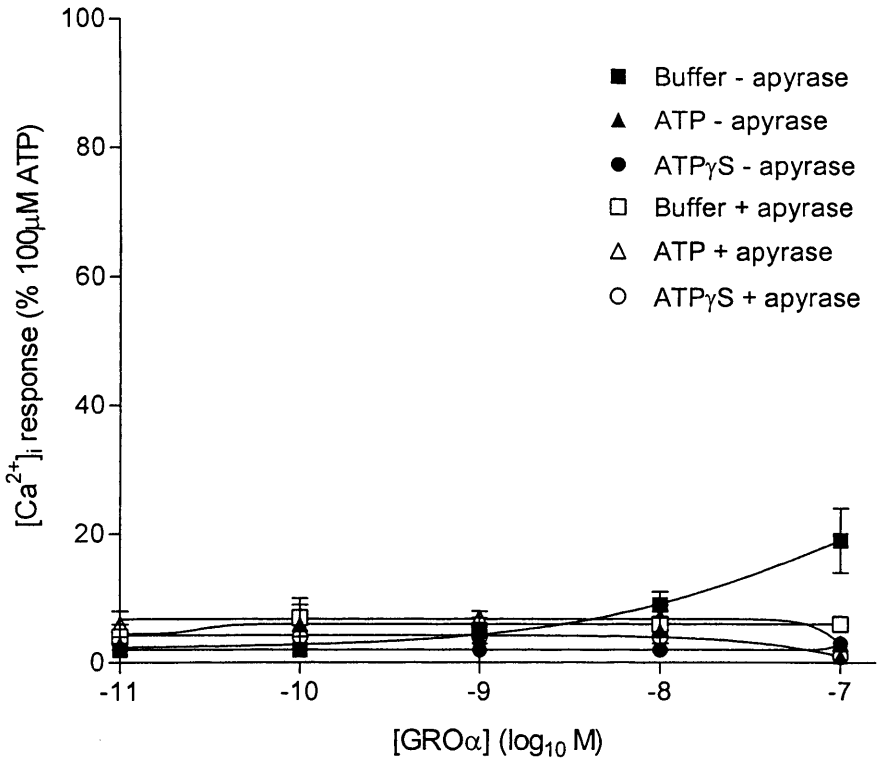


Figure 6.8: Effects of nucleotide agonists on GROα signalling in human monocytes. Human monocytes were isolated by density gradient centrifugation, loaded for 1hr prior to assay with fluo-3/AM (5μM, room temperature) and plated onto poly-D-lysine-coated FLIPR plates. In some cases, apyrase (2U/ml) and/or 8-PST (100μM) were included in samples from the beginning of the loading period until the end of the assay. Stimulated increases in $[Ca^{2+}]_i$ were measured by FLIPR. Cells were pre-stimulated with either buffer (■□), 100μM ATP (▲△), or 100μM ATPγS (●○), in the absence (filled symbols) or presence (open symbols) of 2U/ml apyrase. Pre-treatment was followed 150s later by a stimulation with GROα at the concentrations shown. Responses to GROα are expressed relative to the maximal response to 100μM ATP in the absence of apyrase. Data are mean±s.e.m., n=3.

(Fig. 6.9b). Experiments were performed in the absence or presence of apyrase (2U/ml) and/or 8-PST (100μM). The control responses to 10nM and 0.1nM GROα were 9±2% and 2±1%, respectively. Responses to GROα in these cells were not significantly changed under any of the aforementioned conditions.

Section 6.2.3: Effects of nucleotide pre-stimulation on GROα-induced $[Ca^{2+}]_i$ responses in human microvascular endothelial cells (HMVECs)

CXCR2 expression has also been recorded in the endothelial cells of the microvasculature (Murdoch *et al*, 1999). Primary cultures of HMVECs were obtained from Clonetics (*via* www.cambrex.co.uk), and cultured as described in Materials and Methods. Studies on primary cultures of these cells using flow cytometry and a phytoerythritin-tagged anti-CXCR2 antibody have showed that the level of CXCR2 expression changes relative to passage number, but also changes depending on the confluency of the cell monolayer, both absolutely and in relation to CXCR1 expression. Therefore, CXCR2 expression is comparatively high in sub-confluent monolayers, but decreases as confluency increases. Over this same period, CXCR1 becomes predominant (G.Andrews and I. Scott, AstraZeneca R&D Charnwood, personal communication). Experiments using HMVECs therefore took into account the state of confluence at the time of assay in order to assess whether there was any functional difference in CXCR2 signalling.

A typical Ca^{2+} response to UTP in HMVECs is shown in Fig. 6.10.1. Time courses of $[Ca^{2+}]_i$ elevation are also shown (Fig. 6.10.2). The state of confluence appears to have some effect on the size and shape of the Ca^{2+} response to UTP. Responses to 1μM UTP in both confluent and sub-confluent monolayers are oscillatory, as has been observed previously in other cells with low concentrations of UTP (Davis *et al*, 1999). Increasing the concentration of UTP to 100μM converts this response into a sustained elevation of $[Ca^{2+}]_i$, although in sub-confluent

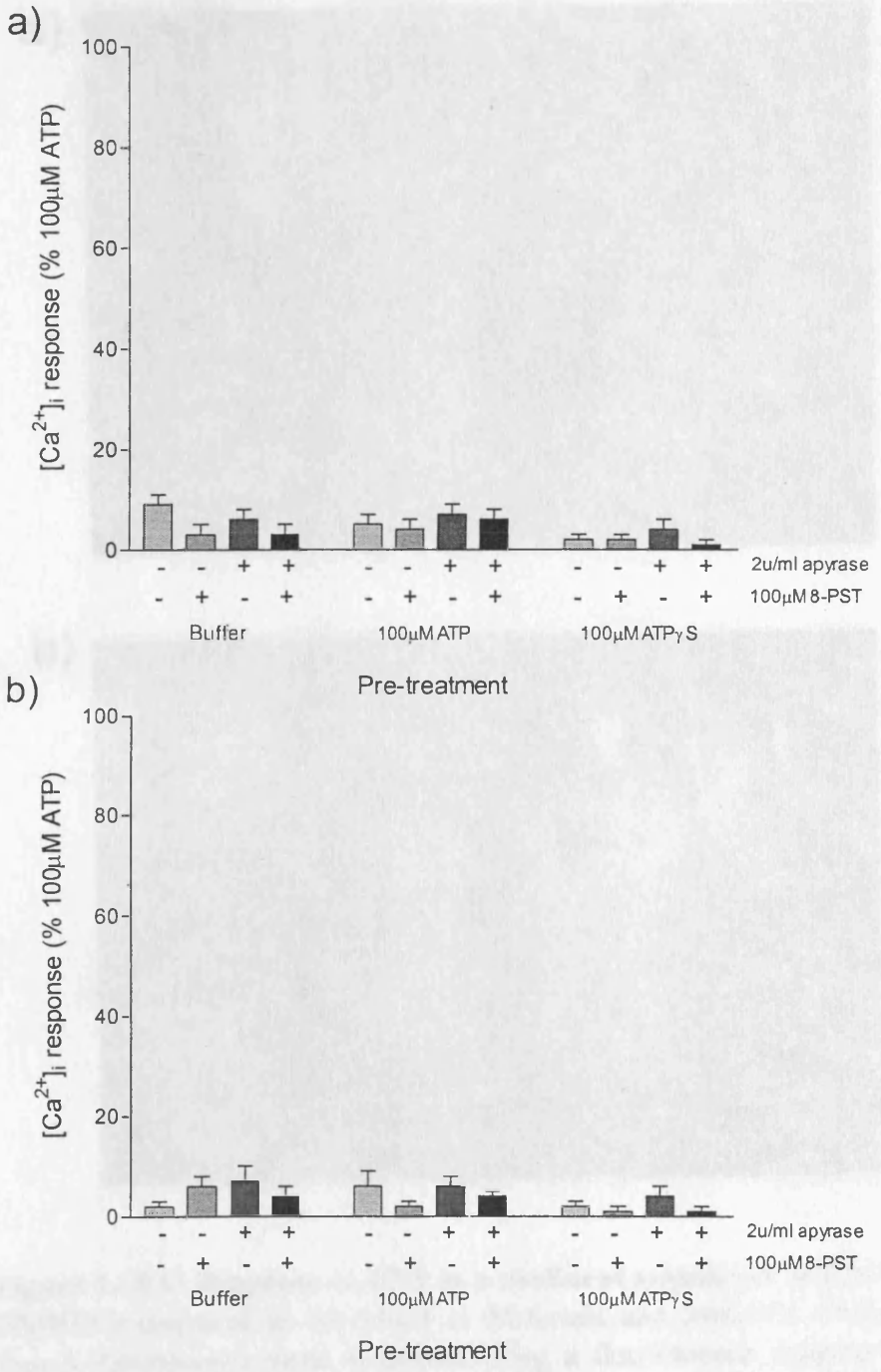


Figure 6.9: Effects of apyrase and 8-PST on GROα responses in human monocytes pre-stimulated with nucleotide. Human monocytes were isolated, prepared and assayed as described earlier (Fig. 6.7). Cells were pre-stimulated with either buffer, 100μM ATP, or 100μM ATPγS, in the absence or presence of 2U/ml apyrase and/or 100μM 8PST. Pre-treatment was followed 150s later by a stimulation with GROα (a: 10nM, b: 0.1nM). Responses to GROα are expressed relative to the maximal response to 100μM ATP in the absence of apyrase. Data are mean±s.e.m., n=3.

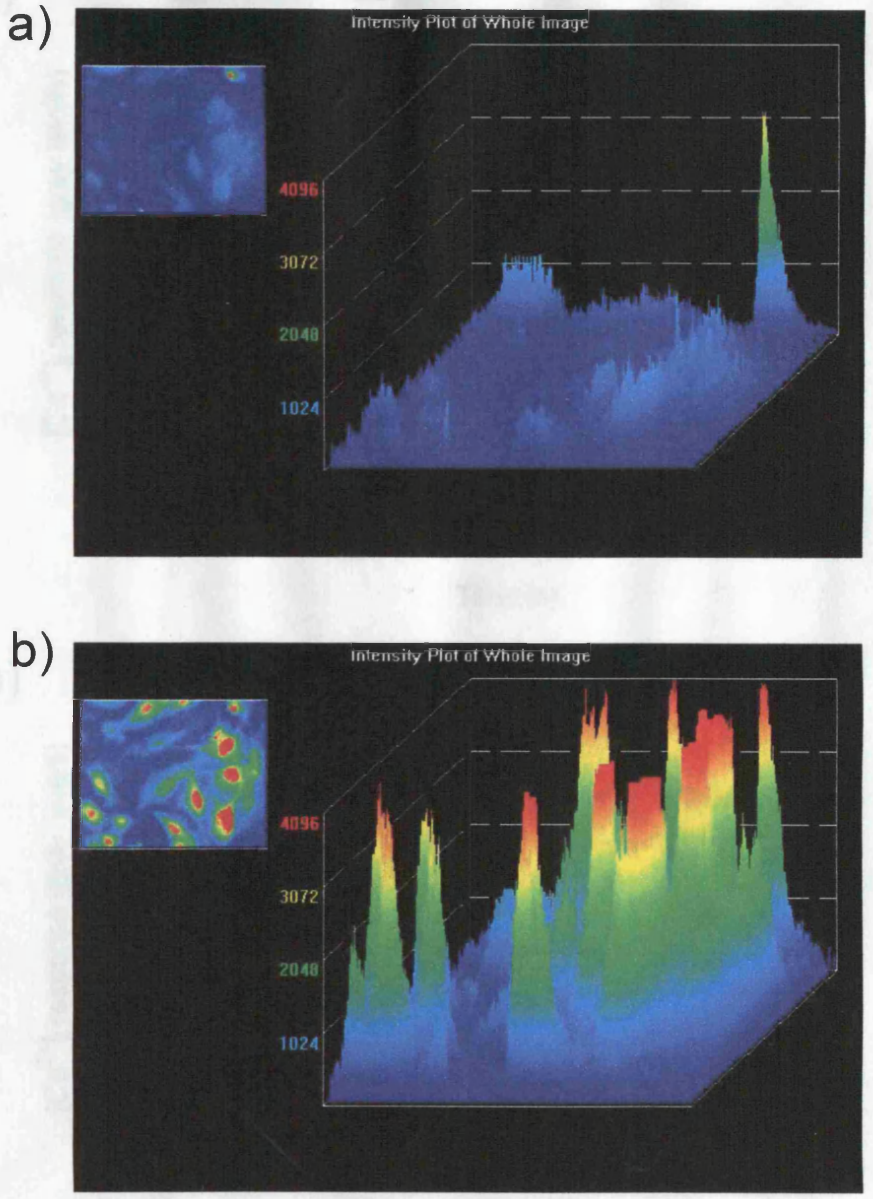


Figure 6.10.1: Response to UTP in a confluent monolayer of HMVECs. HMVECs prepared as described in Materials and Methods. Changes in fluo-3 fluorescence were measured using a fluorescence imaging set-up (see Materials and Methods). Under constant perfusion, cells were stimulated with 100μM UTP. Shown is a representative pseudocolour image of $[Ca^{2+}]_i$ at basal levels (a) and at peak magnitude following stimulation (b).

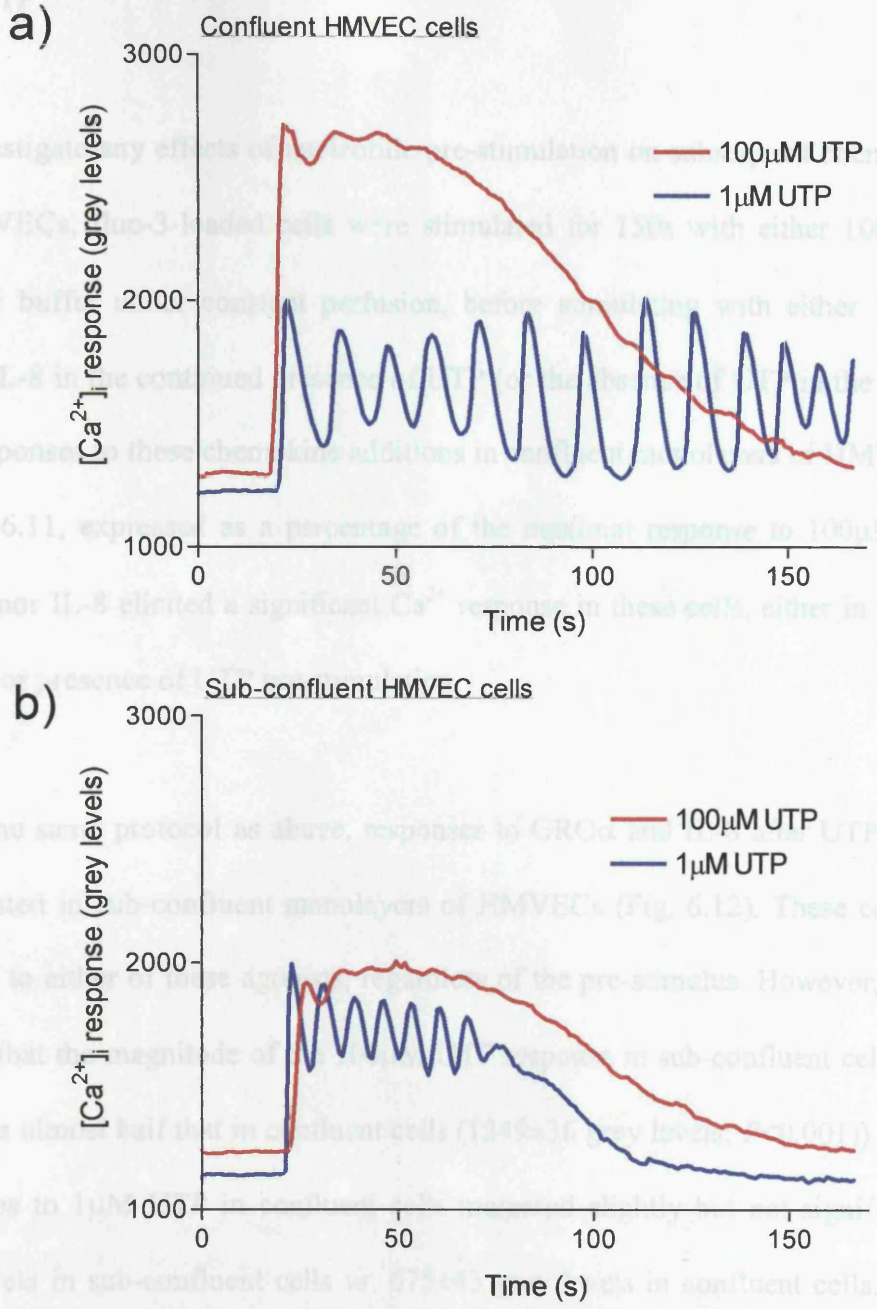


Figure 6.10.2: Response to UTP in confluent and sub-confluent monolayers of HMVECs. HMVECs prepared as described in Materials and Methods, grown either to 60% confluence, or to full confluence. Following fluo-3/AM loading, a fluorescence imaging set-up was used to measure changes in fluo-3 fluorescence as an index of $[Ca^{2+}]_i$ (see Materials and Methods). Under constant perfusion, cells were stimulated with UTP (100μM (red traces) or 1μM (blue traces)). Shown are typical traces showing responses to these agonist additions in both confluent (a) and sub-confluent (b) HMVEC monolayers.

cells the peak magnitude of this response appears to be no larger than that in response to 1μM UTP.

To investigate any effects of nucleotide pre-stimulation on subsequent chemokine responses in HMVECs, fluo-3-loaded cells were stimulated for 150s with either 100μM UTP, 1μM UTP or buffer under constant perfusion, before stimulating with either 10nM GROα or 10nM IL-8 in the continued presence of UTP (or the absence of UTP in the vehicle control). The responses to these chemokine additions in confluent monolayers of HMVECs are shown in Fig. 6.11, expressed as a percentage of the maximal response to 100μM UTP. Neither GROα nor IL-8 elicited a significant Ca^{2+} response in these cells, either in the absence (not shown) or presence of UTP pre-stimulation.

Using the same protocol as above, responses to GROα and IL-8 after UTP pre-stimulation were tested in sub-confluent monolayers of HMVECs (Fig. 6.12). These cells also did not respond to either of these agonists, regardless of the pre-stimulus. However, it is interesting to note that the magnitude of the 100μM UTP response in sub-confluent cells (677±25 grey levels) is almost half that in confluent cells (1249±36 grey levels; $P<0.001$)) (Fig. 6.13). The responses to 1μM UTP in confluent cells increased slightly but not significantly (560±40 grey levels in sub-confluent cells *vs.* 675±43 grey levels in confluent cells; $P=0.116$). The relevance of this is addressed in the Discussion to this chapter. This difference had no effect on the estimation of the GROα and IL-8 responses relative to this standard since these are essentially absent anyway.

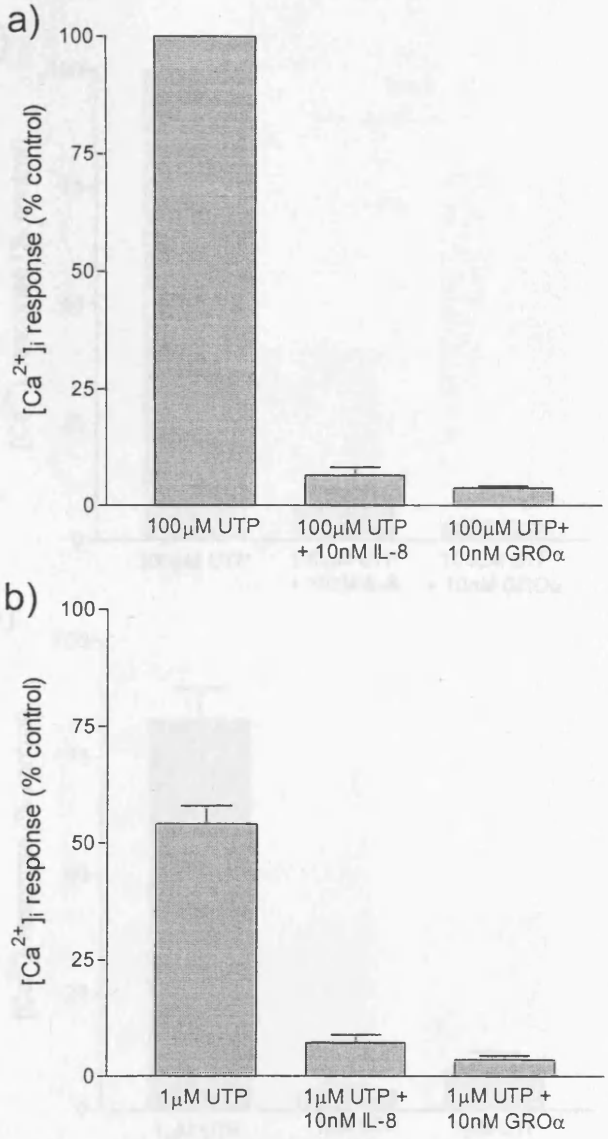


Figure 6.11: Responses to IL-8 and GROα in a confluent monolayer of HMVECs following UTP pre-stimulation. HMVECs were plated onto poly-D-lysine-coated 22mm glass coverslips and grown to full confluence, then loaded with fluo-3 fluorescent dye (5μM, room temperature) for 1hr prior to assay. Changes in fluo-3 fluorescence were measured using a fluorescence imaging set-up (see Materials and Methods section). Under constant perfusion, cells were stimulated with UTP (a: 100μM, b: 1μM), then stimulated 150s later with either IL-8 or GROα (both 10nM) in the continued presence of UTP. Responses to this secondary addition are expressed relative to the maximal response to 100μM UTP. Data are mean±s.e.m., n≥3.

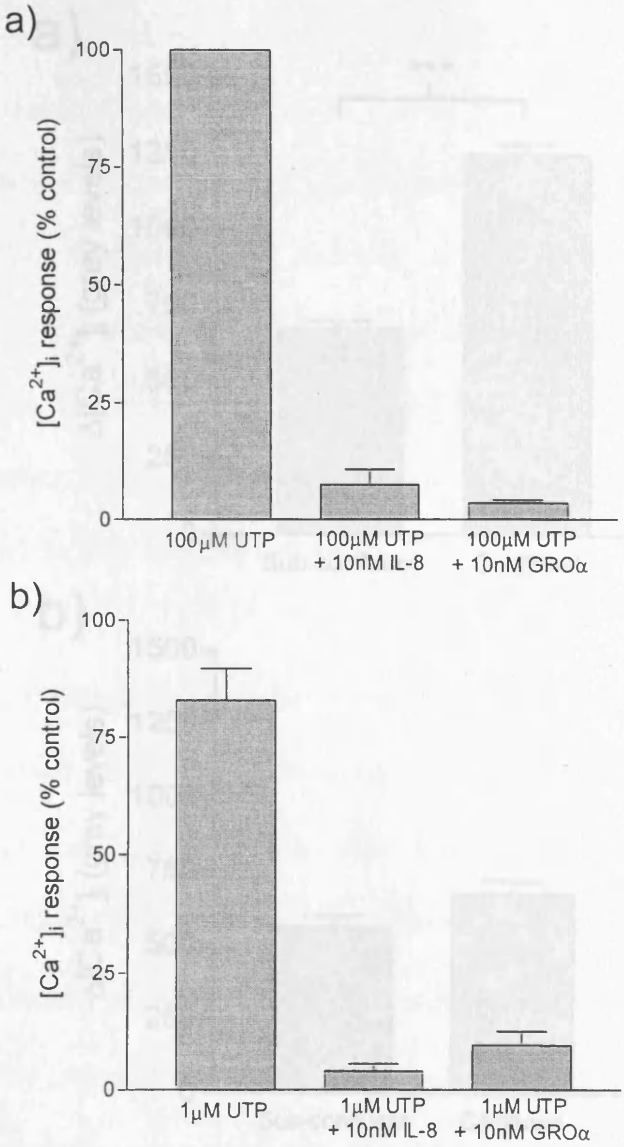


Figure 6.12: Responses to IL-8 and GROα in a sub-confluent monolayer of HMVECs following UTP pre-stimulation. HMVECs were plated onto poly-D-lysine-coated 22mm glass coverslips and grown to approximately 60% confluence, then loaded with fluo-3 fluorescent dye (5μM, room temperature) for 1hr prior to assay. Changes in fluo-3 fluorescence were measured using a fluorescence imaging set-up (see Materials and Methods section). Under constant perfusion, cells were stimulated with UTP (a: 100μM, b: 1μM), then stimulated 150s later with either IL-8 or GROα (both 10nM) in the continued presence of UTP. Responses to this secondary addition are expressed relative to the maximal response to 100μM UTP. Data are mean±s.e.m., n≥3.

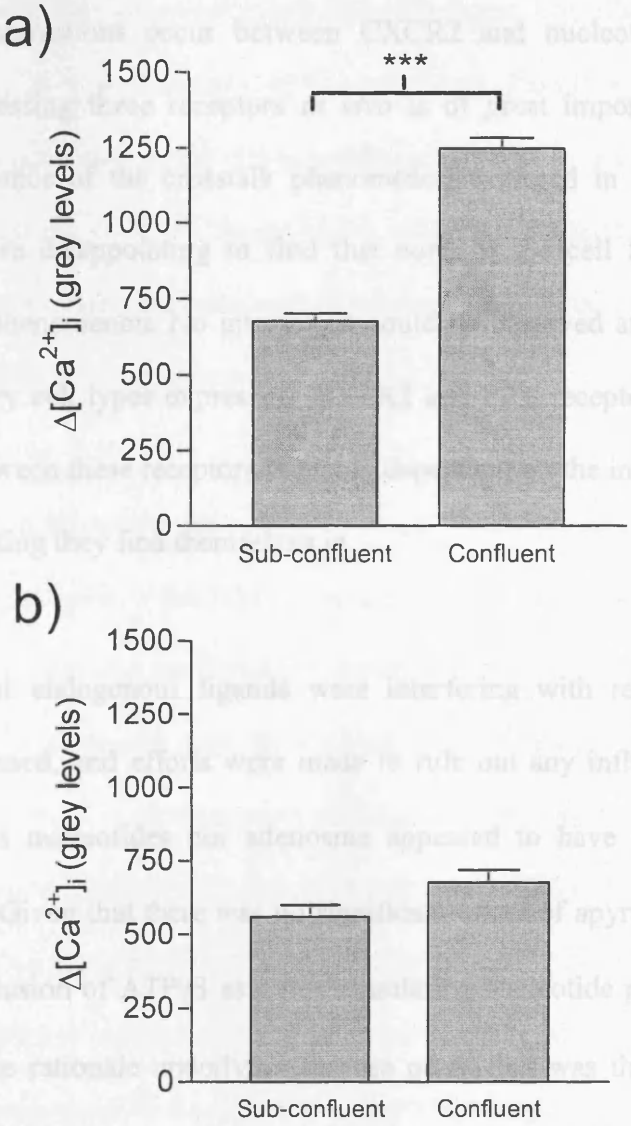


Figure 6.13: Comparison of responses to UTP in confluent and sub-confluent monolayers of HMVECs. HMVECs were plated onto poly-D-lysine-coated 22mm glass coverslips and grown to full confluence or to approximately 60% confluence, then loaded with fluo-3 fluorescent dye (5μM, room temperature) for 1hr prior to assay. Changes in fluo-3 fluorescence were measured using a fluorescence imaging set-up (see Materials and Methods section). Under constant perfusion, cells were stimulated with either 100μM UTP (a) or 1μM UTP (b). Responses to these additions are expressed as changes in fluo-3 fluorescence units (grey levels). Data are mean±s.e.m., n=8. ***: $P<0.001$, by unpaired Student's t-test.

Section 6.3: Discussion

Whether or not interactions occur between CXCR2 and nucleotide receptors in cells endogenously expressing these receptors *in vivo* is of great importance in assessing the physiological relevance of the crosstalk phenomenon explored in earlier sections of this study. It is therefore disappointing to find that none of the cell types investigated here demonstrated this phenomenon. No interaction could be observed at the level of $[Ca^{2+}]_i$ in any of three primary cell types expressing CXCR2 and P2Y receptors, suggesting that the communication between these receptors is highly dependent on the intracellular environment or physiological setting they find themselves in.

The possibility that endogenous ligands were interfering with responses to exogenous agonists was addressed, and efforts were made to rule out any influence of these factors. Neither endogenous nucleotides nor adenosine appeared to have any effect on GROα-induced responses. Given that there was no significant effect of apyrase on the responses to chemokine, the inclusion of ATPγS as a pre-stimulating nucleotide proved, in hindsight, to be unnecessary. The rationale underlying the use of ATPγS was that if endogenous ATP could potentiate chemokine responses, and that this could be reduced by the presence of apyrase, pre-addition of the non-hydrolysable nucleotide should restore chemokine potency to a level similar to that in the absence of any exogenous additions. However, the absence of any significant effects of endogenous ATP precluded against any attempt to confirm this.

Section 6.3.1: Profile and level of receptor expression

There are a number of possible explanations as to why there may be a lack of interaction in these cells specifically. The first is that the relative expression of the two receptors may be at an undesirable ratio, and that crosstalk may not occur under these conditions. It has been

found that the expression of CXCR2 decreases (both in absolute terms and in relation to CXCR1 expression) in confluent monolayers of HMVECs (Mr Glen Andrews, AstraZeneca R&D Charnwood, personal communication). For this reason, HMVECs were studied both at full confluence and at approximately 60% confluence. However, it was apparent that potentiation was not occurring under either condition, suggesting that some other factor was limiting the enhancement of Ca^{2+} signalling. It was concluded that the failure of these receptors (CXCR2 and nucleotide receptors) to interact was not due to diminution of CXCR2 expression as a result of over-confluence.

The identity of the ATP-sensitive nucleotide receptors in the leukocytes, and the UTP-sensitive nucleotide receptor in the HMVECs, is unclear. There are no data available from these studies on the expression profile of nucleotide receptors in any of these cell types, but previous studies have suggested that, in addition to P2Y2 receptors, neutrophils express P2Y4 and P2Y6 receptors, and monocytes and endothelial cells express P2Y1, P2Y4 and P2Y6 receptors (Jin *et al*, 1998). UTP and ATP both have affinity for P2Y4 receptors, while P2Y6 receptors display some sensitivity to UTP (Nicholas *et al*, 1996a and b). Having shown in Chapter 3 that P2Y1 receptors are markedly less able to mediate potentiation than P2Y2 receptors, it is of importance to know the receptor responsible for the observed nucleotide responses in these cells. Involvement of a non-P2Y2 receptor subtype may underlie the inability of these cells to host crosstalk of the kind demonstrated in HEK cells.

Section 6.3.2: Intracellular Ca^{2+} stores

The organisation of intracellular Ca^{2+} stores may be different in these primary cell types compared to the HEK-CXCR2 cell line. It could be argued that the store in the primary cells is either smaller than in HEKs or is more easily released, and thus there is insufficient Ca^{2+} remaining at the time of chemokine addition to permit any further release. However, the

finding that there is no potentiation of chemokine responses even by a sub-maximal concentration of UTP (Fig. 6.11b and Fig. 6.12b) suggests that the failure to potentiate is due to a fundamental deficiency in the receptors and/or their lines of communication rather than in the Ca^{2+} store, which would be only partially depleted under these conditions. The phenomenon of quantal Ca^{2+} release demonstrated in HEK cells may not be applicable to these primary cells. As such, the initial addition of nucleotide may have caused a partial but global desensitisation of Ca^{2+} responses that could not be overcome by interaction between P2Y receptors and CXCR2. This would be in contrast to the model of full but local desensitisation predicted by quantal Ca^{2+} release that should be overcome by increasing the volume, rate or sensitivity of some factor in the Ca^{2+} release pathway (see Chapter 4).

In relation to the Ca^{2+} store, it is interesting to note the difference in the size of the maximal Ca^{2+} responses in sub-confluent cells compared to confluent cells (Fig. 6.13). While Ca^{2+} responses to sub-maximal concentrations of UTP were similar in sub-confluent cells to those in confluent monolayers, the response to 100μM UTP seemed to be attenuated in sub-confluent cells. This may suggest that the magnitude of the Ca^{2+} response is limited in sub-confluent cells by the size of the Ca^{2+} pool. This would be expected to impact upon any crosstalk between these receptors and CXCR2. A severely diminished Ca^{2+} store may limit the amount of Ca^{2+} available to any potentiated CXCR2 signal, and an interaction may become unobservable due to lack of Ca^{2+}. Whether this is still limiting even in confluent cells, where the restriction appears to be less repressive (i.e. the maximal response to 100μM UTP is larger), and whether this would prevent against any synergy between nucleotide receptors and CXCR2 is unclear. Nevertheless, it does highlight that cell-specific differences in terms of receptor expression or Ca^{2+} storage may be crucial in the ability of a cell to mediate crosstalk such as that demonstrated in HEK cells in Chapters 3-5.

Section 6.3.3: PLCβ expression profile

Another potential reason why these cells fail to exhibit potentiation is that the primary cells may express PLCβ isoforms that are incompatible with this interaction. Having shown that the potentiation of CXCR2 signalling by nucleotide receptors may be underpinned by a convergence and co-operativity between $G\alpha_q$ and $G\beta\gamma$ at PLCβ, it is important to consider if this convergence may be affected by the intrinsic sensitivity of each PLC isoform to $G\beta\gamma$. PLCβ2 demonstrates high sensitivity to $G\beta\gamma$ in the absence of any $G\alpha_q$ assistance (Rhee, 2001). It would therefore be unlikely to observe a large increase in this sensitivity in the presence of $G\alpha_q$, especially as PLCβ2 responsiveness to $G\alpha_q$ in terms of activation by this G-protein subunit is relatively low anyway. Chemokines are well known to produce robust $G\beta\gamma$-mediated Ca^{2+} responses in neutrophils, suggesting the predominance of the $G\beta\gamma$-sensitive PLCβ isoforms (PLCβ2/3), whereas HEK cells (despite being shown to express nearly all isoforms of PLC (Dare *et al*, 1998)) may express PLCβ1 predominantly given their unresponsiveness to CXCR2 in non-pre-stimulated cells. This isoform, having a close relationship with $G\alpha_q$ in terms of activation, may be the most suitable candidate in which to witness a $G\alpha_q$-directed co-operativity interaction, which would explain the existence of potentiation in the HEK-CXCR2 cell background. Whether co-expression of CXCR2, P2Y receptors and PLCβ1 occurs in any primary cell type is unknown. It is possible that the phenomenon studied in HEK cells may be more predictive of Gi-Gq interactions in general than of P2Y-CXCR2 interactions specifically. Nevertheless, the elucidation of its mechanism of action is a valuable addition to the knowledge of GPCR signalling in general, even if this particular reaction is not apparent in more physiological circumstances.

Section 6.3.4: Agonist trafficking

It is also interesting to note that CXCR2 mediates agonist-specific responses in neutrophils (Damaj *et al*, 1996b). It was shown that EGTA or Ca^{2+}-entry blockers attenuated the response to GROα but not that to IL-8, suggesting that although GROα does appear to have an intracellular Ca^{2+} component, it additionally couples to Ca^{2+} entry, whereas IL-8 couples exclusively to intracellular release. It is potentially significant to this study that the choice of GROα in these studies of primary cell types (to minimise any interference from CXCR1) may have caused a fundamental change in the signalling properties of CXCR2 such that it was less able to couple to Ca^{2+} release and therefore unable to participate in potentiation of signalling at this level. On the other hand, GROα appeared to couple to Ca^{2+} release and potentiation with very similar potency to IL-8 in the initial studies performed on the HEK-CXCR2 cells (data not shown), suggesting that, in apparent contradiction to the previously published work (Damaj *et al*, 1996b), this agonist trafficking may not occur in these cells.

In addition to those discussed above, there are a number of other possible explanations for the differences between the observations made in the HEK-CXCR2 cell line and those made in primary cells, ranging from the existence of unsuitable isoforms or splice variants of crucial signalling factors to differences in membrane compartmentalisation of signalling partners. Many of these are excellently reviewed by Dumont *et al* (2002), and will not be discussed further here, except to state that while the HEK-CXCR2 cell line has proven invaluable for studying an interaction between nucleotide receptors and CXCR2, full physiological relevance will not be realised unless it is replicated in physiological settings. This by no means detracts from the work contained herein, which gives vital clues towards understanding how GPCRs may interact, but may suggest that these clues may be of more relevance in generic terms than specifically to the functioning of CXCR2 in its normal environment.

While it is perhaps, therefore, unsurprising that none of the primary cell types studied here displayed a crosstalk phenomenon such as that detailed in HEK-CXCR2 cells in Chapters 3-5, it is nonetheless encouraging that there are numerous possible reasons as to why this may be the case. Analysis of receptor expression levels in primary cell types, and identification of the P2Y receptor expression profile would be useful in defining the differences between these and immortalised cell lines that may underlie their contrasting abilities to mediate potentiation. In addition, analysis of the PLC expression profile by Western blotting would also give important information regarding whether signal convergence at PLCβ is precluded by the predominance of an unsuitable PLCβ isoform. The demonstration of numerous crosstalks in primary cells between a wide array of endogenously expressed receptors (see Chapter 1) suggests that crosstalk is a very real phenomenon, and that the failure to demonstrate a similar potentiation in leukocytes or endothelial cells owes more to cell-specific differences than to any deficiency in the concept of GPCR crosstalk.

Chapter 7 - Concluding discussion

Section 7.1: Summary of data

This thesis aimed to explore the interaction between two differentially coupled GPCRs ($G\alpha_q$-coupled P2Y2 nucleotide receptors and $G\alpha_i$-coupled CXCR2 chemokine receptors) that led to the enhancement of intracellular Ca^{2+} signalling. It found that stimulation of recombinant CXCR2 with IL-8 does not elevate $[Ca^{2+}]_i$ in HEK-CXCR2 cells unless these cells are pre-stimulated with nucleotides acting at metabotropic P2Y nucleotide receptors endogenously expressed in these cells. Facilitation of CXCR2 Ca^{2+} signalling by nucleotides occurred in a nucleotide concentration-dependent manner, but was not reciprocal, thus IL-8 was not able to potentiate the signalling by P2Y receptors. The interaction was dependent on intracellular Ca^{2+} stores, given that the CXCR2-mediated Ca^{2+} response was sensitive to thapsigargin pre-treatment but not to the withdrawal of extracellular Ca^{2+}. Interestingly, protocols that allowed removal of the pre-stimulating agonist showed that P2Y receptor activation did not set in motion a chain of events that persisted for any time after the removal of agonists at these receptors. Experiments were performed in an attempt to determine the mechanism underlying the crosstalk between CXCR2 and P2Y receptors, and data from these showed that the enhancement of CXCR2 Ca^{2+} signalling required activation of the PLC/$InsP_3$/Ca^{2+} release pathway, as demonstrated by the activity of an inhibitor of $InsP_3$ receptors, and also following interruption of the supply of the PLCβ substrate, PtdIns(4,5)P_2. Further evidence for the involvement of PLC was the demonstration that co-stimulation of HEK-CXCR2 cells with nucleotide and chemokine agonists also caused a marked potentiation in the production of inositol phosphate species, suggesting that Ca^{2+} signal potentiation occurs as a direct result of enhanced inositol phosphate generation. Data indicating the involvement of Gβγ and the

potential requirement for continued activation of $G\alpha_q$ support a hypothesis that these G-protein subunits co-operate in their modulation of PLCβ activity, causing enhanced inositol phosphate production and, consequently, enhanced Ca^{2+} signalling. The exhibition of quantal Ca^{2+} release in this HEK cell line confirmed that, even in cells where nucleotide-sensitive Ca^{2+} stores had been emptied, further increase in $InsP_3$ was sufficient to elicit additional Ca^{2+} release.

A similar crosstalk between $G\alpha_q$-coupled muscarinic M3 receptors and $G\alpha_s$-coupled β2 adrenoceptors was also characterised in HEK-WT cells, where both receptors are endogenously expressed. These experiments also demonstrated that β2 adrenoceptor-mediated Ca^{2+} signalling was concentration-dependently enhanced by pre-stimulation with carbachol. However, despite some similarity between this interaction and that described above, there was crucially no evidence for enhancement of PLC activity following co-stimulation of muscarinic M3 receptors and β2 adrenoceptors as determined by total inositol phosphate accumulation. This suggests that the two phenomena were mediated by different mechanisms. An obvious candidate effector enzyme for the interaction between muscarinic M3 receptors and $G\alpha_s$-coupled β2 adrenoceptors is PKA. However, an inhibitor of PKA had no effect on the response to stimulation of these receptors, and artificial elevation of cAMP with forskolin or dbcAMP was also ineffective, suggesting that PKA was not relevant to this particular crosstalk.

There are potentially important consequences of GPCR crosstalk in physiological systems in terms of cell signalling and function. However, the impact of nucleotide stimulation of cells natively expressing CXCR2 was found to be negligible in terms of enhancing Ca^{2+} elevation in response to chemokine agonists. Indeed, chemokine signalling appeared to be depressed by prior stimulation with nucleotide, possibly due to Ca^{2+} store depletion. Thus, the extent of

GPCR crosstalk involvement in physiological function is unclear, as no downstream events could be examined. However, this work clearly demonstrates that GPCR crosstalk can be of profound consequence in cellular systems, and that there is more than one mechanism by which Ca^{2+}-potentiating crosstalk can occur.

Section 7.2: Discussion of experimental designs

The work presented here demonstrates and characterises an interaction between a recombinant receptor (CXCR2) and another GPCR with limited homology to CXCR2 expressed endogenously in the host cell line (P2Y2). Major advances have been made here regarding the understanding of how this, and perhaps other, combinations of receptors may be able to communicate *in vivo*. However, interactions such as the crosstalk between nucleotide receptors and chemokine receptors presented here are often dismissed as artifacts of receptor over-expression, or simply of expression of a recombinant receptor in an essentially alien environment. Much evidence exists to suggest that it is not artifactual, as similar phenomena have been observed in cells endogenously expressing combinations of GPCRs that communicate to enhance Ca^{2+} signalling (Jimenez *et al*, 1999; Buckley *et al*, 2001; Hirono *et al*, 2001; Kearns *et al*, 2001; Watanabe *et al*, 2001a and b). It was initially proposed here that the crosstalk between muscarinic M3 receptors and β2 adrenoceptors, both of which are endogenously expressed in HEK cells, was good evidence that HEK cells could support such communications, and that data generated using these recombinant cells was valid. In hindsight, this conclusion could be contested with the evidence that the muscarinic M3 receptor-β2 adrenoceptor interaction may be *via* a different pathway, and therefore may not comparable to the relationship between P2Y2 receptors and CXCR2.

In this respect, it is perhaps important to scrutinise the data that supports the conclusion that the two mechanisms are in fact different. The pivotal piece of data here was the

demonstration that while co-administration of UTP and IL-8 caused potentiation of phosphoinositide production, the addition of carbachol together with isoproterenol did not. At face value, this suggests that the P2Y2 receptor-CXCR2 mechanism involves convergence at or upstream of PLCβ, while the interaction between muscarinic M3 receptors and β2 adrenoceptors may be downstream of this effector, causing potentiation without affecting $InsP_3$ levels. Yet, it also raises the question of whether the assay measuring inositol phosphate accumulation is sensitive enough to detect changes under all circumstances, and also whether potentiation of phosphoinositide generation over a timescale of many minutes is an accurate reflection of the interaction that causes enhanced Ca^{2+} signalling within a matter of seconds. There are a number of arguments that support the conclusion that the interactions between P2Y receptors and CXCR2 and between muscarinic M3 receptors and β2 adrenoceptors are a consequence of different mechanisms. Firstly, whilst the two interactions cause similar magnitudes of potentiation at the level of Ca^{2+}, it seems strange that the two mechanisms could involve such markedly different profiles of potentiation at the level of phosphoinositide signalling. However, complicating factors such as response desensitisation may become relevant during an extended time-course, and thus any doubts over whether changes in phosphoinositide accumulation at 30min can really be compared to changes in $[Ca^{2+}]_i$ at 10s become particularly pertinent here. The critical time for potentiation may be in the first few seconds following addition of the secondary agonist, a point at which the $[^3H]$-$InsP_x$ accumulation assay is not sensitive enough to accurately detect changes. Rapid desensitisation after this point may preclude further $[^3H]$-$InsP_x$ accumulation such that at 30min, there is no apparent difference between cells stimulated with one or both agonists. However, it is interesting to note that even the P2Y1 nucleotide receptor, which is shown here to stimulate limited potentiation of IL-8-mediated Ca^{2+} signalling, is still capable of causing a small but significant degree of potentiation at the level of $[^3H]$-$InsP_x$ accumulation. Although possible, it seems unlikely that any interaction between muscarinic M3 receptors

and β2 adrenoceptors could be so spatially localised and so rapidly desensitising that it could cause the level of Ca^{2+} signal potentiation it does without registering even a small increase in [^{3}H]-$InsP_x$ accumulation.

The desensitisation issue raised above is an important consideration. Differences between receptors in the rate of response desensitisation may have marked effects on the overall accumulation of [^{3}H]-$InsP_x$ over a timescale of many minutes. However, this assay is able to highlight the point at which PLCβ activity ceases, this being the point at which no further [^{3}H]-$InsP_x$ accumulation occurs. This property actually proved advantageous in revealing that the potentiation of phosphoinositide signalling by co-stimulation with UTP and IL-8 could be split into two distinct components: 1) an accelerated rate of inositol phosphate production and 2) an extended period of PLC activity. It is noted, however, that the sensitivity of the [^{3}H]-$InsP_x$ assay may not be sufficient to draw firm conclusions regarding the initial rate of PLC activity. An assay that measures changes in phosphoinositide levels over a much shorter timescale or, ideally, in real time would be preferable in this situation (see discussion of the eGFP-$PH_{PLC}\delta$ $InsP_3$ biosensor below). Nevertheless, while the extended duration of PLC activity would not be relevant in the time-course over which changes in $[Ca^{2+}]_i$ were studied, it does provide reasonable evidence for an enhanced activation of PLCβ. Taken together, these data strongly suggest that the muscarinic M3-β2 adrenoceptor crosstalk is indeed mediated by a different mechanism to the P2Y2 receptor-CXCR2 interaction. Furthermore, although the experiments performed do not allow a reasoned hypothesis regarding the nature of the muscarinic M3 receptor-β2 adrenoceptor interaction, any involvement of enhancement of PLCβ activity is unlikely.

The aforementioned sensitivity of the [^{3}H]-$InsP_x$ assay would not be an issue given an assay for direct measurement of $InsP_3$ in a similar time frame to that used to study changes in

$[Ca^{2+}]_i$ with rather higher sensitivity than the $InsP_3$ mass assay (Fig. 6.11). In this respect, it is interesting to note the development of a fluorescent 'biosensor' of intracellular $InsP_3$ levels (Stauffer *et al*, 1998; Várnai & Balla, 1998; Hirose *et al*, 1999). Consisting of the pleckstrin homology domain of PLCδ1 tagged with enhanced green fluorescent protein (eGFP), this construct (eGFP-$PH_{PLC\delta}$) has high affinity for PtdIns(4,5)P_2, and anchors into the cell membrane under resting conditions. However, it is believed to have higher affinity for $InsP_3$, and agonist stimulation of PLCβ causes translocation of this construct into the cytosol in response to elevations in cytosolic [$InsP_3$] (as opposed to a reduction in PtdIns(4,5)P_2 content in the membrane) (Hirose *et al*, 1999). This translocation of GFP fluorescence from the membrane into the cytosol can be quantified as a measure of cytosolic $InsP_3$ levels, and can be imaged in real time in parallel with fura-2 measurement of $[Ca^{2+}]_i$ (Stauffer *et al*, 1998; Várnai and Balla, 1998; Hirose *et al*, 1999; Nash *et al*, 2001). This technique may have been of use here in determining changes in [$InsP_3$] without the need to adopt a protocol with such limitations as the quantification of [^{3}H]-$InsP_x$. However, it should also be noted that the use of these constructs is not without drawbacks. It has been demonstrated that the binding of PLC-PH domains into the phospholipid membrane can impede access to these lipids by PLC itself, and consequently inhibit hydrolysis of phosphoinositides and generation of $InsP_3$ (Várnai and Balla, 1998). Such assays were attempted during the course of the study described in this thesis (data not shown), but no translocation could be observed even at high concentrations of UTP. This suggests that the eGFP-$PH_{PLC\delta}$ system may not be amenable to use in the HEK-CXCR2 cell line due to an inherent inability to detect the $InsP_3$ elevations in these cells. This suggests that while the PLC-PH domain construct may be useful in terms of studying the spatio-temporal characteristics of $InsP_3$ responses, its use may still be limited by its apparent lack of sensitivity to low concentrations of $InsP_3$.

There is a body of evidence presented here supporting the co-operative interaction between $G\alpha_q$ and $G\beta\gamma$ at PLCβ as the mechanism underlying Ca^{2+} signal potentiation seen following co-stimulation of P2Y receptors and CXCR2 (discussed in Chapter 5). Having concluded that the crosstalk between muscarinic M3 receptors and β2 adrenoceptors exhibits fundamental differences to that between P2Y receptors and CXCR2, the obvious question remains of exactly what this mechanism is. The demonstration that a crosstalk involving β2 adrenoceptors was sensitive to the actions of CTX (Chapter 3) implicated the activation of some part of the G_s-$\alpha\beta\gamma$ heterotrimer as a major factor in the interaction. $G\alpha_s$-mediated actions tend to be cAMP-dependent, occurring either by activation of PKA, through Epac (exchange protein directly activated by cAMP) nucleotide exchange factors (de Rooij *et al*, 1998 and 2000; Bos *et al*, 2001), or causing direct effects through cAMP. Having shown that neither forskolin nor dbcAMP are able to substitute for the β2 adrenoceptor, this suggests that none of the actions of PKA are responsible for any enhancement of the muscarinic M3 receptor-mediated release of Ca^{2+}. It also suggests that cAMP-activated Epacs also cannot be involved. This would presumably rule out the most obvious actions of $G\alpha_s$ in this interaction, and thus predicts that $G\beta\gamma$ derived from Gs proteins is the most likely executor of the muscarinic M3-β2 adrenoceptor crosstalk. A similar crosstalk between β2 adrenoceptors (or adenosine A2B receptors) and P2Y receptors in astrocytes was proposed to be dependent on $G\beta\gamma$ subunits and, as here, independent of cAMP (Jiménez *et al*, 1999). However, a role for $G\beta\gamma$ in Ca^{2+} signal potentiation in the absence of any effects on PLC activity is difficult to envisage given current knowledge. Most models of $G\beta\gamma$-dependent crosstalk would be expected to enhance PLC activity. These include the co-operativity between $G\alpha_q$ and $G\beta\gamma$ discussed in this thesis, and the potential translocation of PLCβ to the plasma membrane following $G\beta\gamma$ stimulation of the activity of certain PI 3-kinase isoforms (Stephens *et al*, 1997) and the enrichment of PLC-anchoring 3'-phosphorylated phosphoinositides in the

plasma membrane (Barker *et al*, 1998 and 1999; Bobe *et al*, 2001). It is likely that further investigation of this pathway would begin with the study of the effects of $G\alpha_{t1}$ on the muscarinic M3 receptor-β2 adrenoceptor crosstalk. This would enable confirmation of whether Gβγ has a role to play in this interaction. If Gβγ is indeed involved, further exploration would be required into the ways in which Gs-derived Gβγ subunits may be involved in this mechanism in a way that is dissimilar to the proposed co-operativity between $G\alpha_q$ and Gβγ in the P2Y2 receptor-CXCR2 crosstalk. It is interesting to note, however, that the CTX experiment was performed on crosstalk between β2 adrenoceptors and the P2Y2 nucleotide receptor, not the muscarinic M3 receptor. It is therefore possible that the muscarinic M3 receptor-β2 adrenoceptor interaction may not be CTX-sensitive, and may act through a G-protein-independent mechanism. This raises the possibility that the identity of the $G\alpha_q$-coupled receptor may dictate the pathway by which crosstalk occurs. P2Y2 receptors may preferentially adopt a Gβγ-dependent co-operativity mechanism (hence the sensitivity to G-protein-inactivating toxins such as PTX and CTX) as supported by the evidence of Jiménez *et al* (1999), while muscarinic M3 receptors may utilise a distinct pathway that is independent of G-protein activation. Exploration of the muscarinic M3 receptor-β2 adrenoceptor interaction would also necessarily assess the effects of CTX on this crosstalk to discover whether this crosstalk does involve Gs-protein activation.

The idea that the precise mode of crosstalk is dictated by the $G\alpha_q$-coupled GPCR may explain the inability of some such GPCRs to participate in these interactions. Theoretically, $G\alpha_q$ should be able to co-operate with Gβγ, irrespective of the receptor type from which it ($G\alpha_q$) is derived. However, this is clearly not the case. It is shown here that P2Y1 receptors, while eliciting peak Ca^{2+} responses equivalent to those stimulated by P2Y2 receptors, are substantially less able to cause potentiation of IL-8 signalling. It was proposed earlier

(Chapter 5) that while P2Y1 receptor desensitisation may play a part in its inability to interact with CXCR2, it was not the only factor, given that moves to minimise desensitisation of the P2Y1 receptor gave somewhat equivocal results. Another factor could be that P2Y1 receptors fundamentally cannot communicate with CXCR2 in the HEK cell environment. In addition, other $G\alpha_q$-coupled receptors, both endogenously- and recombinantly expressed, make markedly different contributions to potentiating β2 adrenoceptor-mediated Ca^{2+} signals. Two forms of the rat gonadotrophin releasing hormone (GnRH) receptor transfected into HEK-WT cells stimulated robust increases in $[Ca^{2+}]_i$, yet neither could facilitate the Ca^{2+} signalling of the β2 adrenoceptor (data not shown), although stimulation of the native muscarinic M3 receptor in the same cells could (this study). In contrast, recombinant Gq-coupled human neuromedin U receptors, NmU-R1 and NmU-R2, were capable of facilitating β2-adrenoceptor-mediated Ca^{2+} signalling (Paul Brighton, Dept. of Cell Physiology and Pharmaclogy, University of Leicester, personal communication). Furthermore, a similar crosstalk between β-adrenoceptors (or adenosine A2B receptors) and P2Y receptors in astrocytes was proposed to be dependent on Gs-derived Gβγ subunits (Jiménez *et al*, 1999). This suggests, perhaps, that β-adrenoceptor interaction with P2Y receptors may utilise a $G\alpha_q$-Gβγ co-operativity mechanism such as that described here downstream of P2Y2 receptors. In contrast, muscarinic M3 receptor communication with other GPCRs often appears to be without effect on phosphoinositide responses (Short & Taylor, 2000; Yeo *et al*, 2001; this study), although this is not always the case (Connor *et al*, 1996; Chan *et al*, 2000). Therefore, it appears that both crosstalk pathways could be dictated by the identity of the $G\alpha_q$–coupled receptor. Whether this represents variable abilities to, for instance, form heterodimeric complexes, or to segregate into discrete membrane locations (e.g. caveolae, lipid rafts), is unclear.

From another perspective, such differences may arise due to fundamental differences between the cell backgrounds used to study specific combinations of receptors. A cell-specific Ca^{2+} signalling 'toolkit' has been mentioned previously to describe the ability of different cells to decode Ca^{2+} signals to transduce a highly diverse range of functions (Bootman *et al*, 2001). It is possible that different cell types also contain an analogous crosstalk 'toolkit' that allows interaction between specific receptor combinations in some cell types but not others. In support of this is evidence that δ-opioid receptors and muscarinic M3 receptors interact to enhance Ca^{2+} signals in both CHO cells and in neuroblastoma-derived SH-SY5Y cells, but only in the CHO cells is $InsP_x$ production concomitantly enhanced (Yeo *et al*, 2001). This suggests that CHO cells and SH-SY5Y cells may have different 'toolkits' and therefore mediate crosstalk rather differently.

The importance of cell-specific environments and of a $G\alpha_q$-mediated selection of crosstalk pathway to the results obtained from studies on GPCR activity is neatly illustrated in work on rat osteoblast-like cells (Buckley *et al*, 2001). This study found that a crosstalk between PTH receptors and P2Y1 nucleotide receptors was, similar to the β2 adrenoceptor-P2Y receptor interaction above (Jiménez *et al*, 1999, and this study), dependent on Gs, but was also shown to be independent of the actions of cAMP and PKA activation. Furthermore, it demonstrated that while agents such as forskolin and dbcAMP were not effective in mimicking potentiation, the phosphodiesterase inhibitor IBMX did potentiate nucleotide-stimulated Ca^{2+} responses. However, these authors also drew attention to the fact that other studies had found IBMX to be ineffective, in particular in the interaction between the PTH receptor and muscarinic M3 receptor in HEK cells (Short & Taylor, 2000). The cell-specific effects of IBMX were cited as a potential reason for the discrepancies in these results, namely that IBMX, by elevating cGMP, blocks $InsP_3$ receptor-mediated Ca^{2+} responses in some cells but not others (Komalavilas & Lincoln, 1994). Thus, the disparate effects of an

experimental pharmacological tool in different cell backgrounds may heavily influence both the results of some studies and their subsequent interpretation. However, it should be noted that these studies (Short & Taylor, 2000; Buckley *et al*, 2001) used different $G\alpha_q$-coupled receptors in their respective model systems. The mechanisms of interaction may be dictated by virtue of an effect of these different receptors in selecting the pathway by which they communicate with the PTH receptor (see above).

It is perhaps surprising that the potentiation phenomenon demonstrated in the HEK cell model was not apparent in isolated leukocytes or primary cultures of endothelial cells. As discussed in Chapter 6, there are experimental and physiological reasons as to why this may be the case, including high levels of ATP present during leukocyte isolation or the expression of incompatible isoforms of PLCβ. Nevertheless, there are numerous examples of GPCR crosstalk in primary cell types (see Section 1). The possible selection of the mode of interaction by the $G\alpha_q$-coupled receptor may preclude against CXCR2 communicating with P2Y2 receptors in leukocytes and endothelial cells simply because the machinery for P2Y2-type interactions is not present in those cells. CXCR2 may still interact with other $G\alpha_q$-coupled GPCRs in these cells, but only those for which suitable communication conditions exist. Likewise, P2Y2 receptors may only take part in potentiation situations in certain cells that will support them (e.g. HEK cells) but be silent in those that do not (e.g. leukocytes). It will be some time before the array of interacting GPCR combinations and the model cell systems used to study them is sufficient to search for trends to validate this hypothesis.

Section 7.3: Implications of this work for future experimental design

Mechanistically, crosstalk is not well defined. Numerous examples of GPCR communication have been reported to influence Ca^{2+} signalling (see Section 1, Table 1), but the machinery that performs this communication is decidedly poorly understood. Protocol design has

contributed to the generation of results that are somewhat ambiguous or of limited interpretability. For example, the use of non-washout procedures precludes against any conclusions regarding the dependence of a phenomenon on the continued presence of the pre-stimulating agonist. In addition, the wide array of potential loci for signal convergence means that few sets of data are thorough enough to be attributed to a single mechanism. However, the model of $G\alpha_q$-$G\beta\gamma$ co-operativity proposed here has been implicated previously from *in vitro* and *in vivo* studies (Smrcka & Sternweis, 1993; Schmidt *et al*, 1996c; Zhu and Birnbaumer, 1996; Yoon *et al*, 1999).

The lack of literature regarding mechanisms of Ca^{2+}-potentiating GPCR crosstalk is due in large part to the choices of experimental design in previous experiments that precluded firm conclusions being drawn regarding the machinery underlying such interactions. For instance, most early experiments on these types of interactions used either a co-addition strategy or a sequential addition protocol without washout of the initial agonist. Neither procedure allows any conclusions to be made regarding the potential dependence of the communication on the continued activation of the $G\alpha_q$-coupled receptor, such as that shown here. Furthermore, there are examples of possible mis-interpretation of data from recombinant systems due to unaccounted stimuli in the assay system. For example, Ca^{2+} stimulating activity has been attributed to Gi-coupled GPCRs in preparations of cells where ATP release is high, but any crosstalk between these receptors and endogenous ATP-selective P2Y receptors is only occasionally recognised (Akerman *et al*, 1998; this study). Nonetheless, there are many examples of experiments that are highly valuable for their demonstration of involvement of certain factors (such as $G\beta\gamma$ (Selbie *et al*, 1997; Tomura *et al*, 1997; Dickenson *et al*, 1998b; Olianas *et al*, 2000) or PKA (Hoiting *et al*, 1996. Liu & Simon, 1996)) but that are unable to propose exactly how these may be mediating the observed effects on Ca^{2+} signalling. This, together with the potential for ambiguity in some experimental designs, is probably

somewhat responsible for the relative deficiency of literature confirming precise models for GPCR crosstalk. The implications of differences between cell types and their effects on interactions between transduction components in normal and abnormal environments is excellently discussed by Dumont *et al* (2002).

From a physiological perspective, also, crosstalk is still something of a mystery. Its patho-physiological roles are unclear and research into this area is in its infancy. In addition, the discontinuity between findings in cell lines and in primary cells hinders progress towards understanding them. The choice of model cell system is likely to be important in unravelling the complex relationships between GPCRs, with the chosen system having to closely reflect the native cell it is modelling. For instance, a study of CXCR2 interactions with other GPCRs (not limited to P2Y2) may be better served by a neutrophil-like cell line such as the HL-60, which expresses CXCR2 endogenously when differentiated (Lippert *et al*, 1998) and is likely to provide a more accurate approximation of the normal neutrophil environment of CXCR2. Commonly used cell lines such as HEK and CHO cells may be inadequate for these purposes in that they may lack some crucial component(s) of these interactions. A variety of possible explanations for tissue specificity exists, including alternative isoform expression or splicing of a crucial signalling factor, expression levels of receptor or effector proteins, the variable existence of suitable scaffold/signalling complex partners or modulatory proteins, and different patterns of compartmentalisation between cells.

However, this aside, these cell models are of great benefit in evolving hypotheses regarding mechanisms of crosstalk between GPCRs which can then be forwarded for testing in more physiological (but perhaps less 'malleable') cell types. Furthermore, there is a key role for these types of cells and interactions in assisting high throughput screening (HTS) of compounds for novel therapeutic ligands. Measurement of intracellular Ca^{2+} is perhaps the

most straightforward functional test of receptor activity, and with equipment such as the FLIPR, researchers can have enormous HTS power. This phenomenon of crosstalk provides a mechanism through which $G\alpha_i$- or $G\alpha_s$- coupled GPCRs may be coupled to Ca^{2+} signalling thereby accelerating the search for agonist and antagonist ligands at these receptors.

Section 7.4: Future directions

There are clear routes for continuation of this study to further define the crosstalks demonstrated herein. The P2Y2 receptor-CXCR2 interaction has been proposed to be the consequence of co-operativity between $G\alpha_q$ and $G\beta\gamma$ subunits at the level of PLCβ activation, but this has not been conclusively proven. Molecular strategies such as PLCβ knockout, or the use of catalytically deficient PLCβ mutants (Meij & Ross, 1996), may prove more effective than the inhibition of PLC enzymes using U73122, and may give a better indication of the involvement of these enzymes in this mechanism. Furthermore, co-immunoprecipitation experiments may enable the demonstration that $G\beta\gamma$ association with PLCβ is enhanced by the presence of $G\alpha_q$. Refinement, also, of a strategy using a constitutively-active $G\alpha_q$ sub-unit ($G\alpha_q$-Q209L (Heximer *et al*, 1997)) may confirm the requirement for activated $G\alpha_q$ (and perhaps substitute for P2Y2 receptor activation). However, this would need to be carefully controlled to assess the effects of this construct on, for example, basal $InsP_3$ levels and PLCβ desensitisation.

The interaction between muscarinic M3 receptors and β2 adrenoceptors needs full characterisation. However, special attention should perhaps be paid to the use of 2-APB or eGFP-$PH_{PLC}\delta$ to determine the involvement of $InsP_3$. It is expected that $InsP_3$ would have some role in this relationship, implicating perhaps $InsP_3$ receptor sensitisation as a candidate mechanism. The difficulty in interpreting results using thimerosal perhaps precludes the use

of this agent in investigating this possibility, and the use of $InsP_3$/BM (see Chapter 5 Discussion) may be of more benefit. However, $InsP_3$ receptor sensitisation (or sensitisation of $InsP_3$-releasable stores to $InsP_3$) is usually a PKA-dependent process (Enouf *et al*, 1987; Burgess *et al*, 1991; Hajnóczky *et al*, 1993; Wojcikiewicz and Luo, 1998; Bruce *et al*, 2002), and the results disputing a role for PKA are therefore at odds with this mechanism. It is unclear what alternative mechanisms may be involved if $InsP_3$ is shown not to be required, except perhaps a diversion of signalling to an alternative Ca^{2+} store, or the movement of Ca^{2+} by an $InsP_3$-independent mechanism into a depleted $InsP_3$-sensitive store ('store-shifting'). However, while these types of mechanism have been hypothesised previously (Short & Taylor, 2001), there is no firm evidence for their existence. Indeed, the concept of store shifting has since been abandoned by the group that proposed it (Goraya *et al*, 2001).

Section 7.5: Summary and conclusions

This thesis was aimed at uncovering and characterising possible models for modulation of CXCR2 signalling, and assessing their relevance to this receptor in physiological settings. It is concluded here that these receptors communicate with P2Y nucleotide receptors in a recombinant cell system in a manner that seems to require co-operativity between G-protein subunits at the level of PLC activation and subsequently leads to enhancement of $InsP_3$-dependent Ca^{2+} signalling. However, the study of interactions between alternative combinations of GPCRs highlights that crosstalk in these cells is by somewhat divergent mechanisms. It becomes especially apparent, when compared to the interaction between muscarinic M3 receptors and β2 adrenoceptors, that cells seem to possess a number of distinct modes of modulating Ca^{2+} signalling, and this raises several questions regarding the nature, function and tissue specificity of these. This tissue specificity is likely to have had a heavy influence on the inability to demonstrate a similar crosstalk between P2Y receptors and CXCR2 in primary leukocytes and endothelial cells, and it is anticipated that GPCR

communication will be found to have key roles in the patho-physiological functioning of these and other cells. The study of these receptors in a recombinant system has revealed a deeper understanding of how their signalling pathways may converge to influence Ca^{2+} signalling. Future work will surely take place in physiological systems to elucidate the role of GPCR crosstalk to the normal and pathological functioning of cells and tissues. The work presented here provides valuable evidence for the ways in which GPCRs can communicate *via* 'crosstalk'.

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