Crosstalk between P2Y2 nucleotide receptors and CXCR2 resulting in enhanced Ca\(^{2+}\) signalling involves enhancement of phospholipase C activity, and is enabled by incremental Ca\(^{2+}\) release in HEK cells*

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Abbreviations
BSS, balanced salts solution; [Ca$^{2+}$]$_i$, intracellular Ca$^{2+}$ concentration; CXCR2, CXC chemokine receptor 2 or IL-8 receptor B; fluo-3/AM, fluo-3 acetoxymethyl ester; fura-2/AM, fura-2 acetoxymethyl ester; GPCR, G-protein-coupled receptor; GFP, green fluorescent protein; GTP, guanosine triphosphate; HEK, human embryonic kidney cell; HEK-CXCR2, HEK cell with stable expression of recombinant human CXCR2; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; IL-8, interleukin-8; Ins(1,4,5)P$_3$, inositol 1,4,5-trisphosphate; InsP$_x$, inositol phosphates; PIP, phosphatidylinositol 4-phosphate; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PI 3-kinase, phosphatidylinositol 3-kinase; PI 4-kinase, phosphatidylinositol 4-kinase; PKC, protein kinase C; PLC, phospholipase C; RGS protein, regulator of G-protein signalling protein; UTP, uridine 5’-triphosphate.

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ABSTRACT

We have shown previously that activation of endogenously expressed, $G_{\alpha_{q/11}}$-coupled P2Y$_2$ nucleotide receptors with UTP reveals an intracellular Ca$^{2+}$ response to activation of recombinant, $G_{\alpha_4}$-coupled CXC chemokine receptor 2 (CXCR2) in HEK cells. Here we characterise further this crosstalk and demonstrate that phospholipase C (PLC) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$)-dependent Ca$^{2+}$ release underlies this potentiation. The putative Ins(1,4,5)P$_3$ receptor antagonist, 2-aminoethoxydiphenyl borane (2-APB) reduced the response to CXCR2 activation by interleukin-8 (IL-8), as did sustained inhibition of phosphatidylinositol 4-kinase with wortmannin, suggesting the involvement of phosphoinositides in the potentiation. Against a Li$^+$-block of inositol monophosphatase activity, co-stimulation of P2Y$_2$ nucleotide receptors and CXCR2 caused phosphoinositide accumulation that was significantly greater than that following activation of P2Y$_2$ nucleotide receptors or CXCR2 alone, and was more-than-additive. Thus, PLC activity, as well as Ca$^{2+}$ release, was enhanced. In these cells, agonist-mediated Ca$^{2+}$ release was incremental in nature, suggesting that a potentiation of Ins(1,4,5)P$_3$ generation in the presence of co-activation of P2Y$_2$ nucleotide receptors and CXCR2 would be sufficient for additional Ca$^{2+}$ release. Potentiated Ca$^{2+}$ signalling by CXCR2 was markedly attenuated by expression of either regulator of G-protein signalling 2 (RGS2) or the $G_{\beta\gamma}$-scavenger, $G_{\alpha_{11}}$ (transducin $\alpha$ subunit), indicating the involvement of $G_{\alpha_4}$ and $G_{\beta\gamma}$ subunits, respectively.
Through a variety of intracellular signal transduction pathways, diverse ligands of G-protein-coupled receptors (GPCRs) are able to regulate many different aspects of cell function. Typically, each GPCR is considered to preferentially activate a specific signal transduction pathway but it is clear that promiscuity and crosstalk can occur. Thus, a GPCR may couple to more than one pathway, whilst activation of a GPCR is often able to influence the signalling by another co-expressed receptor. Such crosstalk can have either positive or negative effects on receptor function and may serve to allow coincidence detection, thereby integrating signals from multiple receptor types.

A phenomenon of particular interest in terms of positive GPCR crosstalk is the enhancement of intracellular Ca\(^{2+}\) release arising as a consequence of the concomitant or sequential stimulation of two types of GPCR that are preferentially coupled to different G-proteins. For example, \(\alpha_i\) or \(\alpha_s\)-coupled receptors can markedly enhance the Ca\(^{2+}\) signalling of simultaneously activated \(\alpha_{q/11}\)-coupled receptors in clonal cell lines (Dickenson and Hill, 1994; Yeo et al., 2001). Such crosstalk has also been demonstrated in cells derived from both the central nervous system (Jimenez et al., 1999; Hirono et al., 2001) and peripheral tissues (Shah et al., 1999; Cilluffo et al., 2000; Buckley et al., 2001) where potentiated cellular Ca\(^{2+}\) signalling may have important physiological consequences (Hirono et al., 2001; Buckley et al., 2001).

A number of studies have demonstrated an involvement of \(\alpha_{q/11}\)-coupled P2Y nucleotide receptors in potentiated Ca\(^{2+}\) signalling (Jimenez et al., 1999; Quitterer and Lohse, 1999). We have also recently demonstrated in HEK cells that activation of endogenously expressed P2Y\(_2\) nucleotide receptors reveals a Ca\(^{2+}\) signal to stimulation of recombinantly expressed, \(\alpha_i\)-coupled human CXC chemokine receptor 2 (CXCR2) (Werry et al., 2002). The ability of CXCR2 to mediate a Ca\(^{2+}\) response is absolutely dependent upon the presence of P2Y\(_2\) nucleotide receptor agonists. Although the Ca\(^{2+}\)
response to activation of P2Y2 nucleotide receptors is insensitive to pertussis toxin (PTX), the potentiated CXCR2-mediated response is PTX-sensitive, showing the involvement of Gαq. Furthermore, the potentiated CXCR2 response is independent of extracellular Ca2+, demonstrating that, following the release of Ca2+ by P2Y2 nucleotide receptors, activated CXCR2 are able to play a role in the mobilization of an additional or discrete intracellular store of Ca2+ that is inaccessible following the stimulation of either CXCR2 or P2Y2 nucleotide receptors alone. The identity of this store and the mechanism through which Ca2+ is mobilised from it remain to be identified and provides the focus of the current study. A large variety of mechanisms exist that have the potential to mediate such crosstalk (Werry et al., 2003) but specific pathways have rarely been identified and have proved difficult to interrogate. In this study, we demonstrate that phospholipase C (PLC) activity and inositol 1,4,5-trisphosphate (Ins(1,4,5)P3)-mediated Ca2+ release are crucial to the enhanced release of Ca2+ resulting from crosstalk and that it is dependent upon both Gαq and Gβγ subunits. Furthermore, we demonstrate that activation of Gαq/11-coupled receptors in these cells results in incremental Ca2+ release. As a consequence of this we suggest that potentiated inositol phosphate production is sufficient to account for the crosstalk at the level of Ca2+ release and discuss mechanisms through which this may occur.
METHODS

Materials

Cell culture reagents were obtained from Gibco BRL Life Technologies (Paisley, Scotland). Cell culture plastics were obtained from Nalgene (Europe) Ltd. (Hereford, UK). Genejuice\textsuperscript{®} transfection reagent was from Novagen (through CN Biosciences, Nottingham, UK), and pTracer expression vector was supplied by Invitrogen (Inchannan, Scotland, UK). Fura-2 acetoxyethyl ester (fura-2/AM), uridine 5'-triphosphate (UTP), thapsigargin, ryanodine and caffeine were obtained from Sigma Aldrich (Poole, UK). Fluo-3 acetoxyethyl ester (fluo-3/AM) was from TEF Labs (Austin, TX, USA). Interleukin-8 (IL-8) was supplied by R & D Systems (Abingdon, UK). Staurosporine, 2-aminoethoxydiphenyl borane (2-APB), cyclosporin A and okadaic acid were from Calbiochem (through CN Biosciences, Nottingham, UK). All other reagents were of analytical grade and were obtained from Sigma Aldrich (Poole, UK) or Fisher Scientific (Loughborough, UK).

Cloned cDNA encoding either the bovine transducin G\(\alpha\) subunit (G\(\alpha_{t1}\)) or human RGS2 were kind gifts from Prof. G. Milligan (University of Glasgow, UK) and Dr. C. Doupnik (University of South Florida, Tampa, FL, USA), respectively.

Assay buffer used in all experiments was a balanced salts solution (BSS) composing 130mM NaCl, 5.4mM KCl, 16mM NaHCO\(_3\), 1.3mM NaH\(_2\)PO\(_4\), 0.8mM MgCl\(_2\), 1.8mM CaCl\(_2\), 10mM HEPES, 5.5mM D-glucose, pH 7.4.

Cell culture - HEK-CXCR2 cell line

The HEK cell line expressing recombinant human CXCR2 at approximately 50,000 sites per cell was generated and selected as previously described (Werry et al., 2002). This cell line (HEK-CXCR2) was maintained in Dulbecco's Modified Eagle Medium (containing 25mM D-glucose, 4mM L-alanyl-L-glutamine and 1mM sodium pyruvate)
supplemented with 10% foetal calf serum, 1% non-essential amino acids, 50µg/ml gentamicin and 400µg/ml G-418 at 37°C in a 5% CO2 humidified atmosphere.

Generation of HEK-CXCR2 cells expressing either Gαt1 or RGS2

The coding sequence for Gαt1 was ligated into pTracer using EcoR1 and Xho1 restriction sites. The cDNA encoding RGS2 was ligated into pTracer using BstX1 restriction sites. HEK-CXCR2 cells were transfected with one of these pTracer constructs, or were control-transfected (using pTracer containing neither Gαt1 nor RGS2 cDNA) as a control. Transfection was performed using Genejuice® transfection reagent according to the manufacturer’s instructions. Non-clonally selected cell lines containing either Gαt1, RGS2 or pTracer were created using the blasticidin-resistance property of pTracer, incubating for 3 weeks with growth medium containing 5µg/ml blasticidin. Untransfected HEK-CXCR2 cells were treated identically as a positive control for the ability of blasticidin to kill non-transfected cells. The pTracer control cell line was used as the control for all experiments using either RGS2- or Gαt1-transfected cells.

Measurement of the intracellular [Ca2+] ([Ca2+]i)

HEK-CXCR2 cells were seeded onto 22mm diameter poly-D-lysine-coated glass coverslips and cultured for 48hrs. Cells were then loaded with fluo-3/AM or fura-2/AM (5µM, 1hr, room temperature) and the coverslips mounted in a perfusion 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 a monochromator, cells were excited at 488nm (fluo-3) or at 340nm and 380nm (1s intervals; fura-2) by light from a xenon lamp (PerkinElmer Life Sciences, Cambridge, UK). Fluorescence emissions at 510nm (fluo-3) or above 510nm (fura-2) were
detected by a CCD camera at a rate of 0.75 frames per second (flu-3) or 0.5 frame per second (fura-2) and converted into on-screen images by UltraVIEW imaging software (PerkinElmer Life Sciences, Cambridge, UK). Fluo-3 was used preferentially, but fura-2 was used in experiments utilising cells expressing green fluorescent protein (GFP) as there was significant ‘bleed-through’ of fluorescence in the emission spectra of GFP and fluo-3. This bleed-through was substantially reduced when fura-2 was used. Measurements were made by averaging fluorophore fluorescence levels across a field of ~10-20 cells. All responses were internally controlled, being normalised against the response to a high concentration of nucleotide (100µM UTP or similar, as indicated in individual figures) in the presence of extracellular Ca\textsuperscript{2+}.

**Total \([^3]H\)-inositol phosphate generation**

Cells were grown for 48hrs in the presence of 3µCi/ml \([^3]H\)-myo-inositol. Following washing and pre-incubation (20 min, 37°C) with BSS containing 10mM Li\textsuperscript{+} to inhibit inositol monophosphatase activity, cells were stimulated for the required time before the reaction was stopped with an equivalent volume of ice-cold, 1M trichloroacetic acid. The reaction mix (1ml final volume) was added to 250µl 10mM EDTA together with 1ml of a freshly-prepared 1:1 (v/v) mixture of tri-n-octyl-amine and 1,1,2-trichloro-trifluoroethane and mixed thoroughly by vortexing. A 700µl aliquot of the upper aqueous layer was removed and added to 50µl 250mM NaHCO\textsubscript{3}. Soluble inositol phosphates in this aqueous fraction were subsequently isolated using strongly basic Dowex chloride anion exchange columns (8% cross linkage, 100-200 dry mesh; Sigma no. 1X8-200) by adding the sample to the column, and washing firstly with water and then with 25mM ammonium formate. \([^3]H\)-InsP\textsubscript{X} were eluted from the columns using 1M HCl, and quantified using liquid scintillation counting.
RESULTS

Potentiation of CXCR2-mediated Ca\(^{2+}\) signalling by P2Y\(_2\) nucleotide receptor activation.

Repeated short exposure of HEK-CXCR2 cells to 100µM UTP (which activates P2Y\(_2\) nucleotide receptors in these cells (Werry et al., 2002)) in the absence of extracellular Ca\(^{2+}\) resulted in the gradual loss of UTP-mediated [Ca\(^{2+}\)]\(_i\) elevation (Fig. 1a). A similar protocol performed in the presence of extracellular Ca\(^{2+}\) did not result in diminished responses to UTP (Fig. 1b) indicating an absence of P2Y\(_2\) nucleotide receptor desensitization to repetitive short exposures to UTP over this time-frame. Thus, in the absence of extracellular Ca\(^{2+}\), UTP is able to fully drain the intracellular Ca\(^{2+}\) store to which it has access. Following drainage of the UTP-sensitive store, 10nM IL-8 is unable to elevate [Ca\(^{2+}\)]\(_i\) unless it is added with UTP (Fig. 1a). The magnitude of this Ca\(^{2+}\) response relative to that of the addition of UTP to naïve cells is comparable to that seen in our previous studies using a fluorescent light imaging plate reader (FLIPR) in which IL-8 was added following, but in the continued presence of UTP (Werry et al., 2002). These data demonstrate that a store of Ca\(^{2+}\) is accessed following co-addition of UTP and IL-8 that is inaccessible to UTP or IL-8 alone.

Potentiated Ca\(^{2+}\) responses following co-addition of UTP and IL-8 require a thapsigargin-sensitive Ca\(^{2+}\) store but not ryanodine receptors

Cells were treated with the sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) inhibitor, thapsigargin (2µM), before stimulating with a single addition of either 100µM UTP, or a co-addition of 100µM UTP and 10nM IL-8. Thapsigargin abolished the Ca\(^{2+}\) responses to both of these stimulations (Fig. 2a).

To test whether Ca\(^{2+}\) stores gated by activation of ryanodine receptors are involved in the Ca\(^{2+}\) response to co-activation of CXCR2 and P2Y\(_2\) nucleotide receptors, store-depleted
cells were incubated for 5 min (from point A, Fig. 1) in the presence or absence of 30µM ryanodine before a co-addition of 100µM UTP and 10nM IL-8. Ryanodine had no significant effect on the response to this co-addition (Fig. 2b). This concentration of ryanodine was shown to be effective at blocking ryanodine receptors since it inhibited the Ca²⁺ elevation seen following stimulation with 10mM caffeine (Fig. 2c).

**Inositol phosphate generation is enhanced by co-stimulation of P2Y₂ nucleotide receptors and CXCR2**

Agonist-mediated accumulation of [³H]-inositol phosphates ([³H]-InsP₃) against a Li⁺ block of inositol monophosphatase was determined as an index of PLC activity. HEK-CXCR2 cells loaded with [³H]-myo-inositol were stimulated in the presence of Li⁺ with either 100nM IL-8, 1mM UTP, or a co-addition of both agonists for varying durations (range: 0-30 min). IL-8 alone evoked little or no accumulation of [³H]-InsP₃ (Fig. 3). In contrast, UTP caused an accumulation of [³H]-InsP₃ 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. Furthermore, in the presence of both agonists, accumulation continued for approximately 15 min compared to an accumulation that only continued for approximately 5 min in the presence of UTP alone (Fig. 3). We also measured mass levels of Ins(1,4,5)P₃ using a radioreceptor assay exactly as previously described (Willars et al., 1998). Basal levels were 33±5 (n=4) pmol/mg protein but were not consistently elevated by either 1mM UTP alone or co-addition of 1mM UTP and 10nM IL-8 suggesting small, localised production and/or rapid metabolism of Ins(1,4,5)P₃.

The role of PLC was also explored using the putative PLC inhibitor U73122. Following store drainage, a 5min incubation with 10µM U73122 (from Point A, Fig. 1) reduced the Ca²⁺ response to co-addition of 100µM UTP and 10nM IL-8 from 64±3%
(n=5) (of the response to 100µM UTP in naïve cells) to 9±4% (n=5). In contrast 10µM U73343 (the aminosteroid negative control to U73122) did not affect the response to co-addition (64±8%, n=5). Qualitatively similar results were obtained using a co-addition of 3µM UTP and 10nM IL-8 (control 38±6% of the response to 100µM UTP in naïve cells, U73122 treated 0%, U73343 treated 38±2%; n=5 for each). Despite this, a variety of data were obtained that were not consistent with the specific inhibition of PLC by 10µM U73122 in these cells. As an example, U73122 had no effect on the accumulation of [3H]-InsP₃ following stimulation of muscarinic M₃ receptors, yet inhibited muscarinic receptor-mediated Ca²⁺ responses, as did U73343. These data confirm previous observations that U73122 is unreliable as a specific inhibitor of PLC (Taylor and Broad, 1998; Walker et al., 1998) and emphasize caution in the interpretation of experiments in which it is used.

Potentiated Ca²⁺ responses following co-addition of UTP and IL-8 are inhibited by 2-aminoethoxydiphenylborane (2-APB)

To examine the involvement of Ins(1,4,5)P₃ receptors in the potentiated response to CXCR2 activation, the effects of the putative Ins(1,4,5)P₃ receptor inhibitor, 2-APB, were investigated. Responses to 100µM UTP in the presence and absence of 100µM 2-APB (5 min pre-incubation) in non-store depleted cells were measured as a positive control to the action of 2-APB. Under these circumstances, 2-APB markedly reduced the Ca²⁺ responses to 100µM UTP (Fig. 4b). Following Ca²⁺ store depletion using 100µM UTP (see Fig. 1), cells were exposed to either 100µM 2-APB or buffer alone for 5 min (from point A, Fig. 1) and subsequently, in the continued presence or absence of 2-APB, stimulated sequentially with 100µM UTP, 10nM IL-8 and a co-addition of these agonists, removing each agonist before addition of the next. Consistent with the data above, co-addition resulted in a robust Ca²⁺ response but this was significantly reduced in the presence of 2-APB (Fig. 4a).
Neither agonist alone elevated [Ca$^{2+}$]$_i$ in the presence or absence of 2-APB, ruling out the possibility that any Ca$^{2+}$ store refilling had occurred during incubation with 2-APB (data not shown).

**Chronic, but not acute, inhibition of phosphatidylinositol 4-kinase inhibits potentiation of Ca$^{2+}$ responses following co-addition of UTP and IL-8**

Activation of phosphatidylinositol 3-kinase (PI 3-kinase) in HEK cells is blocked by wortmannin at a concentration of 100-300nM (Meier et al., 1997; van der Kaay et al., 1997; Sweeney et al., 2001). Pre-incubation of our HEK-CXCR2 cells for 20 min with 300nM wortmannin inhibited the PI 3-kinase-mediated activation of extracellular signal-regulated kinase (ERK) 1/2 by foetal bovine serum by 73% as assessed using an in vitro kinase assay exactly as described previously (Wylie et al., 1999). However, 300nM wortmannin had no effect on the Ca$^{2+}$ responses to 10nM IL-8 when IL-8 was added with either 3µM UTP (42±3% vs. 38±5% in controls, where responses are expressed as a percentage of the response to addition of 100µM UTP alone in naïve cells) or 100µM UTP (56±8% vs. 65±10% in controls), indicating the lack of any involvement of PI 3-kinase.

At higher concentrations than those used to block PI 3-kinase, wortmannin inhibits phosphatidylinositol 4-kinase (PI 4-kinase) (Nakanishi et al., 1995; Willars et al., 1998), a crucial enzyme in maintaining the supply of the PLC$\beta$ substrate, phosphatidylinositol 4,5-bisphosphate (PIP$_2$). As inhibition of PI 3-kinase had no impact on the potentiated Ca$^{2+}$ response to IL-8 in the presence of UTP (see above), we used wortmannin at 10µM to inhibit PI 4-kinase activity and limit the supply of PIP$_2$ (Nakanishi et al., 1995; Willars et al., 1998). A 20 min pre-incubation with 10µM wortmannin had no effect on the response to IL-8 when this agonist was added following 150s pre-stimulation with 100µM UTP (Fig. 5a). When the pre-stimulation with UTP was extended to 7 min in the absence of
wortmannin, the Ca\textsuperscript{2+} response seen following the subsequent addition of IL-8 was similar to that seen following 150s pre-stimulation with UTP (Fig. 5a), but if cells were pre-incubated with wortmannin for 20 min and then stimulated with UTP for 7 min in the continued presence of wortmannin, the subsequent Ca\textsuperscript{2+} response following IL-8 addition was significantly reduced (Fig. 5a). This suggests that the response observed following co-addition of UTP and IL-8 is dependent upon phosphoinositides, but that significant depletion of PIP\textsubscript{2} requires exposure to wortmannin and sustained UTP signalling and is unlikely to be relevant over the usually short time-course of our experiments.

To confirm that PIP\textsubscript{2} levels were not limiting to acute UTP signalling in these cells, repeated short stimulations with UTP were performed in the presence or absence of 10\mu M wortmannin. Responses to these stimulations with UTP (in the presence of extracellular Ca\textsuperscript{2+}) did not progressively reduce in the presence of wortmannin, even when cells were additionally stimulated for 200s by carbachol to activate an endogenously expressed G\alpha\textsubscript{q}-coupled muscarinic M\textsubscript{3} receptor (thus further depleting the cellular PIP\textsubscript{2} pool but without sustained activation and potential desensitization of the P2Y\textsubscript{2} nucleotide receptors) (Fig. 5b).

\textit{Incremental Ca\textsuperscript{2+} release occurs in HEK-CXCR2 cells}

Cells were repetitively stimulated (in the absence of extracellular Ca\textsuperscript{2+}, to prevent store refilling) with progressively increasing concentrations of UTP (Fig. 6). The aim of this protocol was to progressively increase the generation of Ins(1,4,5)P\textsubscript{3} to investigate whether incremental increases in Ins(1,4,5)P\textsubscript{3} resulted in a corresponding fractional release of Ca\textsuperscript{2+} from intracellular stores. Stimulation with a train of 20s pulses of 1\mu M UTP (with 20s perfusion of nominally Ca\textsuperscript{2+}-free buffer separating each) caused progressively diminished responses until no further significant Ca\textsuperscript{2+} release was observed (Fig. 6). These cells were
then stimulated with 20s pulses of progressively higher concentrations of UTP (10µM then 100µM), until again no further response was seen to either concentration. Finally, the cells were co-stimulated with 100µM UTP and 10nM IL-8. At the point at which 1µM UTP could cause no further increase in [Ca^{2+}]_i, a robust elevation was seen following subsequent stimulation with 10µM UTP. At the point at which no Ca^{2+} response was seen to 10µM UTP, there was little response to stimulation with 100µM UTP (Fig. 6). However, following depletion of the UTP-sensitive Ca^{2+} store using this protocol, co-addition of 100µM UTP and 10nM IL-8 evoked a further robust elevation of [Ca^{2+}]_i (Fig. 6).

*Expression of recombinant RGS2 or Gα_{11} inhibit the P2Y_2 nucleotide receptor-dependent Ca^{2+} response to CXCR2 activation*

In order to assess the participation of Gα_q subunits in the potentiated response to CXCR2, HEK-CXCR2 cells were transfected with RGS2, a GTPase activating protein that attenuates Gα_q signalling by selectively accelerating the intrinsic GTPase activity of Gα_q (Heximer et al., 1997). HEK-CXCR2/RGS2 cells (or cells transfected with the empty pTracer vector) 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 100µM UTP was reduced by approximately 65% compared to control cells (Fig. 7).

To test the involvement of Gβγ subunits in the potentiated response to CXCR2, HEK-CXCR2 cells were transfected with the Gβγ-scavenger, Gα_{11}. Expression of Gα_{11} caused a significant reduction in the magnitude of the response to 10nM IL-8 in the presence of 100µM UTP (Fig. 7).
Potentiated Ca\(^{2+}\) responses following co-addition of UTP and IL-8 are not a simple consequence of the pooling of G\(\beta\gamma\)-subunits

In our HEK cells, ongoing activation of P2Y\(_2\) nucleotide receptors also reveals a robust Ca\(^{2+}\) response to activation of endogenously expressed G\(\alpha_s\)-coupled \(\beta_2\)-adrenoceptors (Werry et al., 2002). Despite this, co-activation of CXCR2 with 10nM IL-8 and \(\beta_2\)-adrenoceptors with 10\(\mu\)M isoproterenol did not influence \([\text{Ca}^{2+}]_i\) (Fig. 8).

Potentiated Ca\(^{2+}\) responses following co-addition of UTP and IL-8 are unaffected by inhibition of protein kinase C-, tyrosine kinase- or phosphatase-activities

Cells were treated with either the protein kinase C (PKC) and tyrosine kinase inhibitor staurosporine (3\(\mu\)M), or with vehicle (0.1% DMSO) for 20 min prior to assay. This treatment with staurosporine blocks PKC activity in these cells (Ferrari et al., 1999). The UTP-sensitive intracellular Ca\(^{2+}\) store was then drained by repeated stimulation with 100\(\mu\)M UTP in the absence of extracellular Ca\(^{2+}\) (see Fig. 1) in the continued presence of staurosporine or vehicle control. Consistent with all the data above, following store drainage the addition of either 100\(\mu\)M UTP or 10nM IL-8 alone did not elevate \([\text{Ca}^{2+}]_i\), and in four experiments this was unaffected by staurosporine. Co-addition of UTP and IL-8 evoked a Ca\(^{2+}\) response that was equivalent in the presence and absence of treatment with staurosporine (Fig. 9).

Using the protocol described above (Fig. 1), the UTP-sensitive store was drained and cells were exposed to either 10\(\mu\)M okadaic acid (an inhibitor of protein phosphatases 1 and 2A) or 10\(\mu\)M cyclosporin A (an inhibitor of protein phosphatase 2B) for 5 min (from point A, Fig. 1). These concentrations (or lower) have been shown previously to effectively inhibit the activity of these phosphatases (Groblewski et al., 1994; Otero et al., 2000).
Neither compound had any effect on the response to a subsequent co-addition of 100μM UTP and 10nM IL-8 (Fig. 9).
DISCUSSION

There are many reports of crosstalk between Gαi/o- and Gαq/11-coupled receptors that results in enhanced Ca^{2+} signalling. A diversity of mechanisms have been suggested to account for such crosstalk (Werry et al., 2003), but in general they are poorly defined and often not easily tested. Furthermore, it is likely that multiple mechanisms exist and precisely which are involved may depend on factors such as the receptors studied and the cellular background used. In any particular instance of crosstalk, determining the source of Ca^{2+} and whether the enhanced Ca^{2+} signalling is associated with increased PLC activity may give some indication of the mechanism involved.

We have shown previously that crosstalk between P2Y₂ nucleotide receptors and CXCR2 requires co-stimulation of the receptors, is PTX-sensitive, and is independent of extracellular Ca^{2+} (Werry et al., 2002). Furthermore our previous work demonstrates that the ability of P2Y₂ nucleotide receptors to potentiate Ca^{2+} signalling by CXCR2 is dependent upon the concentration of UTP with an EC₅₀ of approximately 10µM. In the present study we have used either maximal (100µM) or sub-maximal (3µM) concentrations of UTP with maximal concentrations of IL-8 (10nM) to further investigate this crosstalk. Our data show that the potentiated CXCR2-mediated Ca^{2+} response is dependent upon a thapsigargin-sensitive intracellular Ca^{2+} store but independent of ryanodine receptors, suggesting that this crosstalk may be Ins(1,4,5)P₃-dependent. Inhibition of the potentiated Ca^{2+} response by the putative Ins(1,4,5)P₃ receptor antagonist, 2-APB, supports this conclusion. Although 2-APB can have non-selective effects on Ca^{2+} handling, particularly the block of Ca^{2+} channels other than Ins(1,4,5)P₃ receptors (Bootman et al., 2002), our experiments were conducted in the absence of extracellular Ca^{2+} to negate any impact on Ca^{2+} entry. Furthermore, the enhanced accumulation of [³H]-InsP₃ during co-stimulation with UTP and IL-8 (compared to UTP alone) indicates that potentiated PLC activity is
associated with co-activation of P2Y2 nucleotide receptors and CXCR2. It is of interest that following longer stimulation with UTP in the presence of 10µM wortmannin (to reduce PIP2 levels in the plasma membrane), the Ca2+ responses to receptor co-activation were reduced. This confirms that PIP2 is required, presumably for the generation of Ins(1,4,5)P3.

If levels of PIP2 are rate-limiting for PLC activity, then an increase in its supply could account for enhanced PLC activity and Ca2+ signalling. In this respect, an increase in PIP2 levels mediated via PTX-sensitive G-proteins, enhances muscarinic receptor-mediated Ca2+ signalling in HEK cells (Schmidt et al., 1996). Thus, an increased supply of PIP2 in response to activation of CXCR2 could result in enhanced P2Y2 nucleotide receptor-mediated Ins(1,4,5)P3 generation. PI 4-kinase may limit the supply of PIP2 (Willars et al., 1998) and we therefore determined the effect on the crosstalk of PI 4-kinase inhibition. We found that stimulation of PI 4-kinase by CXCR2 is unlikely to be involved in potentiation given the lack of effect of PI 4-kinase inhibition on Ca2+ responses to co-addition following a relatively short pre-stimulation with UTP. However, other phosphoinositide kinases may play a role in the maintenance of PIP2 levels and thus a role for, for example, phosphatidylinositol 4-phosphate 5-kinase cannot be excluded.

In the absence of Ca2+ store refilling, consecutive increases in the concentration of UTP caused further release of intracellular Ca2+. This is consistent with quantal Ca2+ release (Bootman, 1994) or incremental detection (Meyer and Stryer, 1990). Thus, the intracellular Ca2+ store appears to be functionally divided into fractions that are released incrementally according to the concentration of Ins(1,4,5)P3. The presence of incremental Ca2+ release in these cells suggests that other mechanisms such as agonist-dependent shifting of Ca2+ between stores (Short and Taylor, 2000) and sensitization of the Ins(1,4,5)P3 receptor (Tovey et al., 2003) are not required to mediate crosstalk resulting in enhanced Ca2+ mobilization when PLC activity and Ins(1,4,5)P3 generation are potentiated. It is also possible that Ca2+ is released from stores that are accessed according to the locality of
Ins(1,4,5)P$_3$ generation. However, at the resolution of our imaging equipment we were unable to distinguish any spatial differences in the Ca$^{2+}$ signalling mediated by either UTP alone or a co-addition of UTP and IL-8.

Crosstalk between CXCR2 and P2Y$_2$ nucleotide receptors is abolished by PTX treatment (Werry et al., 2002). Given that CXCR2 is coupled to G$_{\alpha_i}$ this may explain the PTX-sensitivity of the crosstalk although there remains the possibility that coupling of P2Y2 nucleotide receptors to G$_{\alpha_i}$ may be important. However, inhibition of crosstalk by the G$_{\alpha_i}$-specific RGS protein, RGS2, indicates a role for G$_{\alpha_i}$. One possible mechanism is that co-incident receptor stimulation enhances activation of G$_{\alpha_i}$ through mechanisms such as heterodimerization and/or G-protein switching (Lawler et al., 2001; Mellado et al., 2001). We attempted to measure the activation of G$_{\alpha_i}$ directly in cell membranes using the binding of [35S]-GTP$\gamma$S and specific immunoprecipitation. However, this was not possible as UTP competed effectively with GTP for binding to G$_{\alpha_i}$.

The vast majority of PLC-coupled GPCRs undergo either full or partial desensitization within seconds of agonist addition and this may occur at the receptor and/or post-receptor level (Ferguson, 2001). A reduction or reversal of desensitization could account for potentiated signalling. Indeed, the accumulation of [$^3$H]-InsP$_x$ during co-stimulation with UTP and IL-8 was more prolonged than during stimulation with UTP alone (Fig. 3) suggesting that crosstalk may protect PLC activity from desensitization. Independent of the molecular site, the common denominator in desensitization is often a change in the phosphorylation state of proteins and we therefore disrupted pathways by which phosphorylation states can be altered. Firstly, staurosporine was used under conditions shown to inhibit PKC in these cells (Ferrari et al., 1999). PKC is key in the feedback inhibition of signalling by many PLC-coupled GPCRs (Chuang et al., 1996) and the lack of effect of staurosporine demonstrates that inhibition of PKC activity does not mediate
crosstalk between CXCR2 and P2Y2 nucleotide receptors. Staurosporine also inhibits tyrosine kinases (Ohmichi et al., 1992), indicating that CXCR2 does not act through, or via a reversal of, a tyrosine kinase-dependent phosphorylation event.

Rapid desensitization is predominantly through receptor phosphorylation via one or more of a family of GPCR kinases (GRKs) and de-phosphorylation is required for receptor resensitization (Ferguson, 2001). For many GPCRs, de-phosphorylation is mediated by phosphatases such as the protein phosphatase (PP)-2A family (Pitcher et al., 1995). Phosphorylation of the C-terminus of the P2Y2 nucleotide receptor by a kinase other than PKC mediates agonist-induced receptor desensitization, and inhibition of PP1/PP2A with okadaic acid inhibits resensitization (Otero et al., 2000). Here we show that inhibition of either PP1/PP2A with okadaic acid, or PP2B with cyclosporin A does not influence the potentiated Ca2+ responses, suggesting that P2Y2 nucleotide receptor de-phosphorylation and any associated re-sensitization is not the mechanism of crosstalk.

Inhibition of potentiated Ca2+ signalling by expression of Goq indicates a role for Gβγ subunits, consistent with their role in other examples of crosstalk (Selbie et al., 1997; Chan et al., 2000). We believe that the facilitated CXCR2 Ca2+ response is not, however, a simple consequence of pooling Gβγ subunits from two co-activated receptor populations. Thus, while Goq-coupled β2-adrenoceptors are able to elevate [Ca2+]i, if P2Y2 nucleotide receptors are also activated in these cells (Werry et al., 2002), co-activation of CXCR2 and β2-adrenoceptors did not influence [Ca2+]i.

The requirement for both Goq and Gβγ may be at distinct sites. Alternatively, they could converge to enhance PLC activity directly. The Gβγ-sensitive isoforms of PLC (β1-3) have distinct binding sites for Goq and Gβγ, and stimulation of PLC by PTX-insensitive Go proteins and Gβγ can be additive (Smrcka and Sternweis, 1993) or even synergistic (Zhu and Birnbaumer, 1996), providing a mechanism for receptor crosstalk. Indeed, Goq
may prime PLCβ to subsequent activation by Gβγ subunits derived from δ-opioid receptors in NG108-15 cells (Yoon et al., 1999). The precise mechanism of sensitization is not clear but could involve a conformational change in PLCβ following Gαq binding that relieves a steric hindrance to Gβγ binding. Such a mechanism would account for the need for ongoing activation of P2Y2 nucleotide receptors required for this potentiation, and also for the dependence on Gαq and Gβγ.

This study demonstrates that crosstalk between P2Y2 nucleotide receptors and CXCR2 results in the release of Ca²⁺ from a thapsigargin-sensitive, Ins(1,4,5)P₃-dependent intracellular store. Furthermore, crosstalk results in the potentiation of PLC activity and our data suggest that the enhanced generation of Ins(1,4,5)P₃ may be sufficient to account for potentiated Ca²⁺ release. Our data are entirely consistent with enhanced PLC activity through synergistic actions of Gαq and Gβγ-subunits derived from Gαi. The most straightforward interpretation of this is that Gαq and Gβγ-subunits are derived from activated P2Y2 nucleotide receptors and CXCR2 respectively. There are alternatives and indeed a large and expanding array of mechanisms that could account for crosstalk have been described (for review see Werry et al., 2003). Further investigation of these is required to define whether or not they are able to contribute to crosstalk under this or any other example of crosstalk.
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Figure 1. Potentiation of IL-8-mediated elevation of $[\text{Ca}^{2+}]_i$ in HEK-CXCR2 cells following nucleotide-mediated Ca$^{2+}$ store depletion. **a)** Protocol used to demonstrate the potentiation of Ca$^{2+}$ signalling. Using a Ca$^{2+}$ imager with a perfusion system as described in ‘Methods’, fluo-3-loaded cells were initially challenged with 100$\mu$M UTP as a reference response. Subsequently, cells were repeatedly stimulated with 100$\mu$M UTP for the times indicated (black bars) in the absence of extracellular Ca$^{2+}$ until no Ca$^{2+}$ response was observed. Cells were then stimulated with 10nM IL-8 (to demonstrate the lack of response) followed by a co-addition of 100$\mu$M UTP and 10nM IL-8. Shown is a representative trace tracking changes in fluo-3 fluorescence in a small population of cells (<20) as an index of $[\text{Ca}^{2+}]_i$. Arrow A indicates the point at which some of the inhibitors were added (see text and other figures for details). **b)** Fluo-3-loaded cells were prepared as in Fig. 1a and perfused with 100$\mu$M UTP as indicated (black bars). Shown is a representative trace tracking changes in fluo-3 fluorescence in a small population of cells (<20) as an index of $[\text{Ca}^{2+}]_i$. In contrast to Fig. 1a, this experiment was carried out in the presence of extracellular Ca$^{2+}$ to demonstrate that short, repetitive exposure to 100$\mu$M UTP does not result in a desensitization of the response.
Figure 2. The Ca\(^{2+}\) store accessed by IL-8 in the presence of UTP requires a) a thapsigargin-sensitive, but b) is not ryanodine-sensitive.  

a) HEK-CXCR2 cells were incubated with or without 2\(\mu\)M thapsigargin for 5 min prior to assay. Cells were then stimulated with a single addition of either 100\(\mu\)M UTP or a co-addition of 100\(\mu\)M UTP and 10nM IL-8. Using a Ca\(^{2+}\) imaging system, changes in fluo-3 fluorescence were measured as an index of [Ca\(^{2+}\)]. Responses are expressed as a percentage of the maximal response to 100\(\mu\)M UTP in the absence of thapsigargin pre-treatment. Data are mean±s.e.m., n=4 and * and ** denote \(P<0.05\) and \(P<0.01\) determined by unpaired Student’s t-test. 

b) Using the store depletion protocol and Ca\(^{2+}\) imaging (Fig. 1), cells were incubated with or without 30\(\mu\)M ryanodine following store depletion in the absence of extracellular Ca\(^{2+}\) (point A, Fig. 1) before stimulating with a co-addition of 100\(\mu\)M UTP and 10nM IL-8. Responses to this co-addition were measured as changes in fluo-3 fluorescence and are expressed as a percentage of the maximal response to 100\(\mu\)M UTP before store drainage. Data are mean±s.e.m., n=4 

c) As a positive control for the effect of ryanodine, fluo-3-loaded cells were stimulated with 10mM caffeine in the absence or presence of 30\(\mu\)M ryanodine (with a 5 min preincubation). Responses to caffeine stimulation are expressed as fluo-3 fluorescence. Data are mean±s.e.m., n=4 and * denotes \(P<0.05\) determined by unpaired Student’s t-test.
Figure 3. Co-stimulation of P2Y$_2$ nucleotide receptors and CXCR2 also potentiates phosphoinositide hydrolysis. HEK-CXCR2 cells loaded with $[^3]$H-myoinositol were stimulated with the indicated agonist additions for varying periods in the presence of 10mM Li$^+$. Accumulated $[^3]$H-InsP$_x$ were isolated by phase separation and quantified by liquid scintillation counting. Shown are the accumulations of $[^3]$H-InsP$_x$ for each stimulation at each time point, expressed as fold-of-basal. Data are mean±s.e.m., n=5. The responses were compared using two-way analysis of variance ($P<0.001$) followed by a comparison of the maximal accumulations by Student’s t-test (*** denotes $P<0.001$).
Figure 4. Antagonism of Ins(1,4,5)P₃ receptors with 2-APB inhibits potentiation. a) Following depletion of the UTP-sensitive Ca²⁺ store (see Fig. 1), cells were incubated with or without 2-APB (100µM; 5 min) before resuming stimulations as in Fig. 1, in the continued presence (or absence) of 2-APB. Shown are responses to co-addition of 100µM UTP and 10nM IL-8, using fluo-3 fluorescence as an index of [Ca²⁺]ᵢ. Data are expressed as a percentage of the initial response to 100µM UTP, mean±s.e.m., n=4 and * denotes P<0.05 determined by unpaired Student’s t-test. b) As a positive control for the effect of 2-APB, cells were stimulated with 100µM UTP by perfusion for 30s, followed by washout for 90s with buffer containing Ca²⁺ to allow refilling of intracellular Ca²⁺ stores. Cells were then re-stimulated twice more with 100µM UTP, firstly in the absence of 2-APB (to demonstrate reproducibility of responses) and subsequently following a 5 min incubation with 100µM 2-APB (during which time extracellular Ca²⁺ was present to facilitate store refilling). Data are expressed and analysed as in main figure; n=4 (** denotes P<0.001).
Figure 5. **Effects of the inhibition of PI 4-kinase on crosstalk between P2Y$_2$ nucleotide receptors and CXCR2.**

**a)** Cells were pre-incubated for 20 min with 10μM wortmannin or vehicle (0.1% DMSO). Using a protocol that maintained the presence of UTP (Werry et al., 2002), cells were stimulated (in the continued presence of 10μM wortmannin, or vehicle) by perfusion of 100μM UTP for either 150s or 7 min. Following this, cells were then stimulated with 10nM IL-8 in the continued presence of UTP. Responses shown are those to the addition of IL-8, expressed as a percentage of the maximal response to 100μM UTP, using changes in fluo-3 fluorescence as an index of [Ca$^{2+}$]. Data are mean±s.e.m., n=4 and * denotes $P<0.05$ by Student’s unpaired t-test vs. the response in the absence of wortmannin.

**b)** Cells were pre-incubated for 20 min with 10μM wortmannin, then stimulated (in the continued presence of wortmannin) with 100μM UTP (black bars) and 100μM carbachol (CCh; dark grey bars), separated by periods of agonist washout with buffer. Shown is a representative trace of three experiments. Data are expressed as changes in fluo-3 ‘grey levels’ as an index of [Ca$^{2+}$].
**Figure 6. HEK cells exhibit incremental Ca\(^{2+}\) release.** Using a Ca\(^{2+}\) imaging system that allowed continuous perfusion of the cell chamber, cells 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 the same cells were stimulated until there was again no further Ca\(^{2+}\) response, and then likewise with 100µM UTP. Finally cells were challenged with a co-addition of 100µM UTP and 10nM IL-8. Values shown are the sequential responses to each pulse of agonist, measured as changes in fluo-3 fluorescence. Data are mean±s.e.m., n=3.
**Figure 7. Expression of RGS2 or Gα₁₁ inhibits potentiation of Ca²⁺ signalling.** HEK-CXCR2 cells were transfected with cDNA encoding Gα₁₁ or RGS2 in pTracer vector, or mock-transfected with ‘empty’ pTracer vector. Cells were then selected using blasticidin but clones were not isolated. Using a Ca²⁺ imager and perfusion system, cells were stimulated with 100µM UTP followed (150s later) by 10nM IL-8 (in the continued presence of UTP). Shown are responses to IL-8, expressed as a percentage of the initial maximal response to UTP. Data are mean±s.e.m., n=5 and * denotes *P*<0.05 by Duncan’s multiple range test following one-way analysis of variance that gave *P*<0.05.
Figure 8: Co-activation of $G_{\alpha_i}$ and $G_{\alpha_s}$-coupled receptors does not result in $Ca^{2+}$ signalling. $Ca^{2+}$ stores were drained as in Fig. 1a. 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 the maximal response to 100µM UTP (“Control”). Data are mean±s.e.m., n=4.
Figure 9. Ca\textsuperscript{2+} responses to co-activation of P2Y\textsubscript{2} nucleotide receptors and CXCR2 are unaffected by inhibition of either protein kinase C, tyrosine kinase or phosphatase activities. Cells were pre-treated for 20 min with 3\mu M staurosporine (Staur). In its continued presence, the UTP-sensitive Ca\textsuperscript{2+} store was then depleted using the Ca\textsuperscript{2+} imager perfusion protocol (Fig. 1) in the absence of extracellular Ca\textsuperscript{2+}. Cells were then stimulated with a co-addition of 100\mu M UTP and 10nM IL-8. Alternatively, cells were Ca\textsuperscript{2+} store-depleted and then incubated with either 10\mu M okadaic acid (OA) or 10\mu M cyclosporin A (CsA) for 5 min (from point A shown in Fig. 1). Cells were subsequently stimulated with a co-addition of 100\mu M UTP and 10nM IL-8. In all cases the response to co-addition of UTP and IL-8 is shown expressed as a percentage of the maximal Ca\textsuperscript{2+} response to an addition of 100\mu M UTP prior to store drainage. Control cells were treated with vehicle only (0.01% DMSO) for the same duration as each inhibitor. Shown is the control response to staurosporine (0.01% DMSO for 20 min prior to and throughout assay). Controls to OA and CsA are not shown but were identical to the staurosporine control. Data are mean±s.e.m., n=4.
Figure 1

(a) Graph showing Fluo-3 fluorescence over time with different treatments.

(b) Graph showing Fluo-3 fluorescence over time with UTP treatments.

Time (s)

Fluo-3 fluorescence (grey levels)
Figure 2
Figure 3
Figure 4

(a) Response to co-addition of UTP and IL-8

(b) Response to addition of UTP alone
a) Response to a co-addition of 100μM UTP and 10nM IL-8 at:

- 150s
- 150s
- 7 min
- 7 min

[Ca^{2+}] response (% control)

b) 10μM Wortmannin

Figure 5
Figure 7
Figure 8