Cross Talk between P2Y₂ Nucleotide Receptors and CXC Chemokine Receptor 2 Resulting in Enhanced Ca²⁺ Signaling Involves Enhancement of Phospholipase C Activity and Is Enabled by Incremental Ca²⁺ Release in Human Embryonic Kidney Cells

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ABSTRACT
We have shown previously that activation of endogenously expressed, \( G_{q/11} \)-coupled P2Y₂ nucleotide receptors with UTP reveals an intracellular Ca²⁺ response to activation of recombinant, \( G_{o/1} \)-coupled CXC chemokine receptor 2 (CXCR2) in human embryonic kidney cells. Here, we characterize further this cross talk and demonstrate that phospholipase C (PLC) and inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] dependent Ca²⁺ release underlies this potentiation. The putative Ins(1,4,5)P₃ receptor antagonist 2-aminoethoxydiphenyl borane reduced the response to CXCR2 activation by interleukin-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, costimulation of P2Y₂ nucleotide receptors and CXCR2 caused phosphoinositide accumulation that was significantly greater than that after activation of P2Y₂ nucleotide receptors or CXCR2 alone, and was more than additive. Thus, PLC activity, as well as Ca²⁺ release, was enhanced. In these cells, agonist-mediated Ca²⁺ release was incremental in nature, suggesting that a potentiation of Ins(1,4,5)P₃ generation in the presence of coactivation of P2Y₂ nucleotide receptors and CXCR2 would be sufficient for additional Ca²⁺ release. Potentiated Ca²⁺ signaling by CXCR2 was markedly attenuated by expression of either regulator of G protein signaling 2 or the \( G_{q/1} \) transducin α subunit, indicating the involvement of \( G_{o/1} \) and \( G_{q/1} \) subunits, respectively.

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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 cross talk can occur. Thus, a GPCR may couple to more than one pathway, whereas activation of a GPCR is often able to influence the signaling by another coexpressed receptor. Such cross talk 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 cross talk is the enhancement of intracellular Ca²⁺ 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, \( G_{q/1} \)- or \( G_{o/1} \)-coupled receptors can markedly enhance the Ca²⁺ signaling of simultaneously activated \( G_{q/11} \)-coupled receptors in clonal cell lines (Dickenson and Hill, 1994; Yeo et al., 2001). Such cross talk 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., 2000).
Materials and Methods

Materials. Cell culture reagents were obtained from Invitrogen (Paisley, Scotland). Cell culture plastics were obtained from Nalgene (Europe) Ltd. (Hereford, UK). Genejuice transfection reagent was from Novagen (through CN Biosciences, Nottingham, UK), and pTracer expression vector was supplied by Invitrogen (Inchannan, Scotland). Fura-2 acetoxymethyl ester (fura-2/AM), UTP, thapsigargin, ryanodine, and caffeine were obtained from Sigma Chemical (St. Louis, MO). Fura-2 acetoxymethyl ester (fura-2/AM), UTP, thapsigargin, ryanodine, and caffeine were obtained from Sigma Chemical (St. Louis, MO). Cell permeabilization reagent according to the manufacturer's instructions. Noncloned cell lines containing either Gα11, RGS2 or pTracer were created using the blasticidin-resistance property of pTracer, incubating for 3 weeks with growth medium containing 5 μg/ml blasticidin. Untransfected HEK-CXCR2 cells were treated identically as a positive control for the ability of blasticidin to kill nontransfected cells. The pTracer control cell line was used as the control for all experiments using either RGS2- or Gα11-transfected cells.

Measurement of the Intracellular Ca2+ Concentration (Ca2+). HEK-CXCR2 cells were seeded onto 22-mm-diameter poly-D-lysine-coated glass coverslips and cultured for 48 h. Cells were then loaded with fluo-3/AM or fura-2/AM (5 μM, 1 h, room temperature) and the coverslips mounted in a perfusion chamber on the stage of an IX70-S1F inverted microscope (Olympus, Tokyo, Japan). The chamber was perfused at a rate of 5 ml/min with BSS or drug solutions and the temperature maintained at 37°C using a Peltier unit. Using a monochromator, cells were excited at 488 nm (fura-3) or at 340 and 380 nm (1-s intervals; fura-2) by light from a xenon lamp (PerkinElmer Life Sciences, Cambridge, UK). Fluorescence emissions at 510 nm (fura-3) or above 510 nm (fura-2) were detected by a charge-coupled device camera at a rate of 0.75 frames/s (fura-3) or 0.5 frames/s (fura-2) and converted into on-screen images by UltraVIEW imaging software (PerkinElmer Life Sciences). Fluo-3 was used preferentially, but fura-2 was used in experiments using cells expressing green fluorescent protein (GFP) from pTracer because 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 to 20 cells. All responses were internally controlled, being normalized against the response to a high concentration of nucleotide (100 μM ATP or similar, as indicated in individual figures) in the presence of extracellular Ca2+. Total [3H]inositol Phosphate Generation. Cells were grown for 48 h in the presence of 3 μCi/ml [3H]myo-inositol. After washing and preincubation (20 min, 37°C) with BSS containing 10 mM Li+ to inhibit inositol monophosphatase activity, cells were stimulated for the required time before the reaction was stopped with an equivalent volume of ice-cold, 1 M trichloroacetic acid. The reaction mix (1-ml final volume) was added to 250 μl of 10 mM EDTA together with 1 ml of a freshly prepared 1:1 (v/v) mixture of tri-n-octylamine and 1,1,2-trichloro-trifluoroethane and mixed thoroughly by vortexing. A 700-μl aliquot of the upper aqueous layer was removed and added to 50 μl of 250 mM NaHCO3. 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 1 × 8-200) by adding the sample to the column, and washing with firstly and then with 25 mM ammonium formate. [3H]inositol phosphates ([3H]InsP3) were eluted from the col-

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umns using 1 M HCl and quantified using liquid scintillation counting.

Results

Potentiation of CXCR2-Mediated Ca\(^{2+}\) Signaling by P2Y\(_2\) Nucleotide Receptor Activation. Repeated short exposure of HEK-CXCR2 cells to 100 \(\mu M\) 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. After drainage of the UTP-sensitive store, 10 nM 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 naive cells is comparable with that seen in our previous studies using a fluorescent light im-

Potentiated Ca\(^{2+}\) Responses after Coaddition of UTP and IL-8 Require a Thapsigargin-Sensitive Ca\(^{2+}\) Store but Not Ryanodine Receptors. Cells were treated with the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin (2 \(\mu M\)), before stimulating with a single addition of either 100 \(\mu M\) UTP, or a coaddition of 100 \(\mu M\) UTP and 10 nM 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 coactivation 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 30 \(\mu M\) ryanodine before a coaddition of 100 \(\mu M\) UTP and 10 nM IL-8. Ryanodine had no significant effect on the response to this coaddition (Fig. 2b). This concentration of ryanodine was shown to be effective at blocking

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Fig. 1. Potentiation of IL-8-mediated elevation of [Ca\(^{2+}\)]\(_i\) in HEK-CXCR2 cells after nucleotide-mediated Ca\(^{2+}\) store depletion. a, protocol used to demonstrate the potentiation of Ca\(^{2+}\) signaling. Using a Ca\(^{2+}\) imager with a perfusion system as described under Materials and 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 10 nM IL-8 (to demonstrate the lack of response) followed by a coaddition of 100 \(\mu M\) UTP and 10 nM IL-8. Shown is a representative trace tracking changes in fluo-3 fluorescence in a small population of cells (\(<20\)) as an index of [Ca\(^{2+}\)]. Arrow A indicates the point at which inhibitors were added (see text and other figures for details). b, fluo-3-loaded cells were prepared as in a 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 [Ca\(^{2+}\)]. In contrast to a, 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.
ryanodine receptors because it inhibited the Ca\(^{2+}\) elevation seen after stimulation with 10 mM caffeine (Fig. 2c).

**Inositol Phosphate Generation Is Enhanced by Co-stimulation of P2Y₂ Nucleotide Receptors and CXCR2.**

Agonist-mediated accumulation of \(^{[3]H}\)InsP₄ against a Li⁺ block of inositol monophosphatase was determined as an index of PLC activity. HEK-CXCR2 cells loaded with \(^{[3]H}\)myo-inositol were stimulated with either 100 nM IL-8, 1 mM UTP, or a coaddition of both agonists for varying durations (range 0–30 min). IL-8 alone evoked little or no accumulation of \(^{[3]H}\)InsP₄ (Fig. 3). In contrast, UTP caused an accumulation of \(^{[3]H}\)InsP₄ to a maximum of 1.6 ± 0.09-fold of basal, whereas coaddition 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 with 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 described previously (Willars et al., 1998). Basal levels were 33 ± 5 (n = 4) pmol/mg protein but were not consistently elevated by either 1 mM UTP alone or coaddition of 1 mM UTP and 10 nM IL-8, suggesting small, localized production and/or rapid metabolism of Ins(1,4,5)P₃.

The role of PLC was also explored using the putative PLC inhibitor U73122. After store drainage, a 5-min incubation with 10 mM U73122 (from point A, Fig. 1) reduced the Ca\(^{2+}\) response to coaddition of 100 μM UTP and 10 nM IL-8 from 64 ± 3% (n = 5) (of the response to 100 μM UTP in naive cells) to 9 ± 4% (n = 5). In contrast 10 μM U73343 (the aminosteroid negative control to U73122) did not affect the response to coaddition (64 ± 8%; n = 5). Qualitatively similar results were obtained using a coaddition of 3 μM UTP and 10 nM IL-8 (control 38 ± 6% of the response to 100 μM UTP in naive 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 10 μM U73122 in these cells. As an example, U73122 had no effect on the accumulation of \(^{[3]H}\)InsP₄ after stimulation of muscarinic M₃ receptors, yet inhibited muscarinic receptor-mediated Ca\(^{2+}\) 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 Ca\(^{2+}\) Responses after Coaddition of UTP and IL-8 Are Inhibited by 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 100 μM UTP in the presence and absence of 100 μM 2-APB (5-min preincubation) in nonstore-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 100 μM UTP (Fig. 4b). After Ca2+ store depletion using 100 μM UTP (Fig. 1), cells were exposed to either 100 μM 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 100 μM UTP, 10 nM IL-8, and a coaddition of these agonists, removing each agonist before addition of the next. Consistent with the data mentioned above, coaddition resulted in a robust Ca2+ response, but this was significantly reduced in the presence of 2-APB (Fig. 4a). Neither agonist alone elevated [Ca2+]i, in the presence or absence of 2-APB, ruling out the possibility that any Ca2+ store refilling had occurred during incubation with 2-APB (data not shown).

Chronic, but Not Acute, Inhibition of Phosphatidylinositol 4-Kinase Inhibits Potentiation of Ca2+ Responses after Coaddition 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 to 300 nM (Meier et al., 1997; van der Kaay et al., 1997; Sweeney et al., 2001). Preincubation of our HEK-CXCR2 cells for 20 min with 300 nM wortmannin inhibited the PI 3-kinase-mediated activation of extracellular signal-regulated kinase 1/2 by fetal bovine serum by 73% as assessed using an in vitro kinase assay exactly as described previously (Wylie et al., 1999). However, 300 nM wortmannin had no effect on the Ca2+ responses to 10 nM IL-8 when IL-8 was added with either 3 μM UTP (42 ± 3 versus 38 ± 5% in controls, where responses are expressed as a percentage of the response to addition of 100 μM UTP alone in naive cells) or 100 μM UTP (56 ± 8 versus 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 PLCβ substrate, phosphatidylinositol 4,5-bisphosphate (PIP2). As inhibition of PI 3-kinase had no impact on the potentiated Ca2+ response to IL-8 in the presence of UTP (see above), we used wortmannin at 10 μM to inhibit PI 4-kinase activity and limit the supply of PIP2 (Nakanishi et al., 1995; Willars et al., 1998). A 20-min preincubation with 10 μM wortmannin had no effect on the response to IL-8 when this agonist was added after 150-s prestimulation with 100 μM UTP (Fig. 5a). When the prestimulation with UTP was extended to 7 min in the absence of wortmannin, the Ca2+ response seen after the subsequent addition of IL-8 was similar to that seen after 150-s prestimulation with UTP (Fig. 5a), but if cells were preincubated with wortmannin for 20 min and then stimulated with UTP for 7 min in the continued presence of wortmannin, the subsequent Ca2+ response after IL-8 addition was significantly reduced (Fig. 5a). This suggests that the response observed after coaddition of UTP and IL-8 is dependent upon phosphoinositides but that significant depletion of PIP2 requires exposure to wortmannin and sustained UTP signaling and is unlikely to be relevant over the usually short time course of our experiments.

To confirm that PIP2 levels were not limiting to acute UTP signaling in these cells, repeated short stimulations with UTP were performed in the presence or absence of 10 μM wortmannin. Responses to these stimulations with UTP (in the presence of extracellular Ca2+) did not progressively reduce in the presence of wortmannin, even when cells were additionally stimulated for 200 s by carbachol to activate an endogenously expressed Gαs-coupled muscarinic M2 receptor (thus further depleting the cellular PIP2 pool but without sustained activation and potential desensitization of the P2Y2 nucleotide receptors) (Fig. 5b).

Incremental Ca2+ Release Occurs in HEK-CXCR2 Cells. Cells were repetitively stimulated (in the absence of extracellular Ca2+, 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)P3 to investigate whether incremental increases in Ins(1,4,5)P3 resulted in a corresponding fractional release of Ca2+ from intracellular stores. Stimulation with a train of 20-s pulses of 1 μM UTP (with 20-s perfusion of nominally Ca2+-free buffer separating each) caused progressively di-

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**Fig. 4.** Antagonism of Ins(1,4,5)P3 receptors with 2-APB inhibits potentiation. a, after depletion of the UTP-sensitive Ca2+ store (Fig. 1), cells were incubated with or without 2-APB (100 μM; 5 min) before resuming stimulations as in Fig. 1, in the continued presence (or absence) of 2-APB. Shown are responses to coaddition of 100 μM UTP and 10 nM IL-8, using fluo-3 fluorescence as an index of [Ca2+]i. Data are expressed as a percentage of the initial response to 100 μM UTP, mean ± S.E.M., n = 4, with * 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 100 μM UTP by perfusion for 30 s, followed by washout for 90 s with buffer containing Ca2+ to allow refilling of intracellular Ca2+ stores. Cells were then restimulated twice more with 100 μM UTP, first in the absence of 2-APB (to demonstrate reproducibility of responses) and subsequently after a 5-min incubation with 100 μM 2-APB (during which time extracellular Ca2+ was present to facilitate store refilling). Data are expressed and analyzed as in main figure; n = 4 (***, P < 0.001).
absence of wortmannin. b, cells were preincubated for 20 min with 10 μM 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 10 μM wortmannin, or vehicle) by perfusion of 100 μM UTP for either 150 s or 7 min. After this, cells were then stimulated with 10 nM 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 100 μM UTP. In cells expressing RGS2, the response to 10 nM IL-8 in the presence of 100 μM UTP was reduced by approximately 65% compared with control cells (Fig. 7).

To test the involvement of Gβγ subunits in the potentiated response to CXCR2, HEK-CXCR2/RGS2 cells were transfected with the Gβγ-scavenger Goi1. Expression of Goi1 caused a significant reduction in the magnitude of the response to 10 nM IL-8 in the presence of 100 μM UTP (Fig. 7).

**Potentiated Ca2+ Responses after Coaddition of UTP and IL-8 Are Not A Simple Consequence of the Pooling of Gβγ-Subunits.** In our HEK cells, ongoing activation of P2Y2 nucleotide receptors also reveals a robust Ca2+ response to activation of endogenously expressed Gαq-coupled βγ-adrenoceptors (Werry et al., 2002). Despite this, coactivation of CXCR2 with 10 nM IL-8 and βγ-adrenoceptors with 10 μM isoproterenol did not influence [Ca2+]i (Fig. 8).

**Potentiated Ca2+ Responses after Coaddition 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 μM), or with vehicle (0.1% DMSO) for 20 min before assay. This treatment with staurosporine blocks PKC activity in these cells (Ferrari et al., 1999). The UTP-sensitive intracellular Ca2+ store was then drained by repeated stimulation with 100 μM UTP in the absence of extracellular Ca2+ (Fig. 1) in the continued presence of staurosporine or vehicle control. Consistent with all the data mentioned above, after store drainage the addition of either 100 μM UTP or 10 nM IL-8 alone did not elevate [Ca2+]i, and in four experiments this was unaffected by staurosporine. Coaddition of UTP and IL-8 evoked a Ca2+ 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 μM okadaic acid (an inhibitor of protein phosphatases 1 and 2A) or 10 μM 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 coaddition of 100 μM UTP and 10 nM IL-8 (Fig. 9).

**Discussion**

There are many reports of cross talk between Goq and Ga11-coupled receptors that results in enhanced Ca2+ signaling. A diversity of mechanisms have been suggested to account for such cross talk (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 cross talk, determining the source of Ca2+ and whether the enhanced Ca2+ signaling is associated

**Fig. 5.** Effects of the inhibition of PI 4-kinase on cross talk between P2Y2 nucleotide receptors and CXCR2. a, cells were preincubated for 20 min with 10 μM 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 10 μM wortmannin, or vehicle) by perfusion of 100 μM UTP for either 150 s or 7 min. After this, cells were then stimulated with 10 nM 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 100 μM UTP, using changes in fluo-3 fluorescence as an index of [Ca2+]i. Data are mean ± S.E.M., n = 4, with * P < 0.05 by Student’s unpaired t test versus the response in the absence of wortmannin. b, cells were preincubated for 20 min with 10 μM wortmannin, then stimulated (in the continued presence of wortmannin) with 100 μM UTP (black bars) and 100 μM carbachol (CCh; dark gray 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 “gray levels” as an index of [Ca2+]i.

minimized responses until no further significant Ca2+ release was observed (Fig. 6). These cells were then stimulated with 20-s pulses of progressively higher concentrations of UTP (10 μM then 100 μM), until again no further response was seen to either concentration. Finally, the cells were costimulated with 100 μM UTP and 10 nM IL-8. At the point at which 1 μM UTP could cause no further increase in [Ca2+]i, a robust elevation was seen after subsequent stimulation with 10 μM UTP. At the point at which no Ca2+ response was seen to 10 μM UTP, there was little response to stimulation with 100 μM UTP (Fig. 6). However, after depletion of the UTP-sensitive Ca2+ store using this protocol, coaddition of 100 μM UTP and 10 nM IL-8 evoked a further robust elevation of [Ca2+]i (Fig. 6).

**Expression of Recombinant RGS2 or Goi1 Inhibits the P2Y2 Nucleotide Receptor-Dependent Ca2+ Response to CXCR2 Activation.** To assess the participation of Gaq subunits in the potentiated response to CXCR2, HEK-CXCR2 cells were transfected with RGS2, a GTPase-activating protein that attenuates Gαq signaling by selectively accelerating the intrinsic GTPase activity of Gαq (Heximer et al., 1997). HEK-CXCR2/RGS2 cells (or cells transfected with the empty pTracer vector) were stimulated with 100 μM UTP followed, 150 s later, with 10 nM IL-8 in the continued presence of UTP. In cells expressing RGS2, the response to 10 nM IL-8 in the presence of 100 μM UTP was reduced by approximately 65% compared with control cells (Fig. 7).
of variance that gave P maxima (100/262 with 100 approximately 10/262/262 with 100/262 approximately 10/262/262 with 100/262 one way analysis of variance). 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, with *, p < 0.05 by Duncan's multiple range test after one-way analysis of variance that gave P < 0.05.

with increased PLC activity may give some indication of the mechanism involved.

We have shown previously that cross talk between P2Y2 nucleotide receptors and CXCR2 requires costimulation of the receptors, is PTX-sensitive, and is independent of extracellular Ca2++. (Werry et al., 2002). Furthermore, our previous work demonstrates that the ability of P2Y2 nucleotide receptors to potentiate Ca2+ signaling by CXCR2 is dependent upon the concentration of UTP with an EC50 value of approximately 10 μM. In the present study, we have used either maximal (100 μM) or submaximal (3 μM) concentrations of UTP with maximal concentrations of IL-8 (10 nM) to further investigate this cross talk. Our data show that the potentiated CXCR2-mediated Ca2+ response is dependent upon a thapsigargin-sensitive intracellular Ca2+ store but independent of ryanodine receptors, suggesting that this cross talk may be Ins(1,4,5)P3-dependent. Inhibition of the potentiated Ca2+ response by the putative Ins(1,4,5)P3 receptor antagonist 2-APB supports this conclusion. Although 2-APB can have nonselective effects on Ca2+ handling, particularly the block of Ca2+ channels other than Ins(1,4,5)P3 receptors (Bootman et al., 2002), our experiments were conducted in the absence of extracellular Ca2+ to negate any impact on Ca2+ entry. Furthermore, the enhanced accumulation of [3H]InsP3 during costimulation with UTP and IL-8 (compared with UTP alone) indicates that potentiated PLC activity is associated with coactivation of P2Y2 nucleotide receptors and CXCR2. It is of interest that after longer stimulation with UTP in the presence of 10 μM wortmannin (to reduce PI3-kinase activity) and 10 nM PGE2 (to reduce PI3-kinase activity), we observed a greater potentiation of PLC activity compared with UTP alone. This confirms that PI3-kinase is required, presumably for the generation of Ins(1,4,5)P3.

If levels of PI3-kinase are rate-limiting for PLC activity, then an increase in its supply could account for enhanced PLC activity and Ca2+ signaling. In this respect, an increase in PI3-kinase levels mediated via PTX-sensitive G proteins enhances muscarinic receptor-mediated Ca2+ signaling in HEK cells (Schmidt et al., 1996). Thus, an increased supply of PI3-kinase 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 PI3-kinase (Willars et al., 1998), and we therefore determined the effect of the cross...
is abolished by PTX treatment (Werry et al., 2002). Given that CXCR2 is coupled to Goαi, this may explain the PTX sensitivity of the cross talk, although there remains the possibility that coupling of P2Y2 nucleotide receptors to Goαi may be important. However, inhibition of cross talk by the Goαi-specific RGS protein RGS2 indicates a role for Goαi. One possible mechanism is that coincident receptor stimulation enhances activation of Goαi 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 Goαi directly in cell membranes using the binding of [35S]guanosine 5′-O-(3-thio)triphosphate and specific immunoprecipitation. However, this was not possible as UTP competed effectively with the radiolabeled compound for binding to Goαi.

The 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 postreceptor level (Ferguson, 2001). A reduction or reversal of desensitization could account for potentiated signaling. Indeed, the accumulation of [3H]InsP3 during costimulation with UTP and IL-8 was more prolonged than during stimulation with UTP alone (Fig. 3), suggesting that cross talk 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. First, staurosporine was used under conditions shown to inhibit PKC in these cells (Ferrari et al., 1999). PKC is key in the feedback inhibition of signaling 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 cross talk 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, and dephosphorylation is required for receptor resensitization (Ferguson, 2001). For many GPCRs, dephosphorylation 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 dephosphorylation and any associated resensitization is not the mechanism of cross talk.

Inhibition of potentiated Ca2+ signaling by expression of Goαi indicates a role for Gβγ subunits, consistent with their role in other examples of cross talk (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 coactivated receptor populations. Thus, although Goαi-coupled β2-adrenoceptors are able to elevate [Ca2+]i, if P2Y2 nucleotide receptors are also activated in these cells (Werry et al., 2002), coactivation of CXCR2 and β2-adrenoceptors did not influence [Ca2+]i.
The requirement for both \( \text{Go}_q \) and \( \text{G}\beta\gamma \) may be at distinct sites. Alternatively, they could converge to enhance PLC activity directly. The \( \text{G}\beta\gamma \)-sensitive isoforms of PLC (\( \beta1-3 \)) have distinct binding sites for \( \text{Go}_q \) and \( \text{G}\beta\gamma \), and stimulation of PLC by PTX-insensitive \( \text{Go} \) proteins and \( \text{G}\beta\gamma \) can be additive (Smrcka and Sternweis, 1993) or even synergistic (Zhu and Birnbaumer, 1996), providing a mechanism for receptor cross talk. Indeed, \( \text{Go}_q \) may prime PLC\( \beta \) to subsequent activation by \( \text{G}\beta\gamma \) subunits derived from \( \delta \) 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 PLC\( \beta \) after \( \text{Go}_q \) binding that relieves a steric hindrance to \( \text{G}\beta\gamma \) binding. Such a mechanism would account for the need for ongoing activation of P2Y2 nucleotide receptors for this potentiation and also for the dependence on \( \text{Go}_q \) and \( \text{G}\beta\gamma \).

This study demonstrates that cross talk between P2Y2 nucleotide receptors and CXCR2 results in the release of Ca\( ^{2+} \) from a thapsigargin-sensitive, Ins(1,4,5)P\( ^3 \)-dependent intracellular store. Furthermore, cross talk results in the potentiation of PLC activity, and our data suggest that the enhanced generation of Ins(1,4,5)P\( ^3 \) may be sufficient to account for potentiated Ca\( ^{2+} \) release. Our data are consistent with enhanced PLC activity through synergistic actions of \( \text{Go}_q \) and \( \text{G}\beta\gamma \)-subunits derived from \( \text{Go}_q \). The most straightforward interpretation of this is that \( \text{Go}_q \) and \( \text{G}\beta\gamma \)-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 cross talk have been described (for review, see Werry et al., 2003). Further investigation of these is required to define whether they are able to contribute to cross talk under this or any other example of cross talk.

References


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