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, Gα₁-q/i-coupled P2Y₂ nucleotide receptors with UTP reveals an intracellular Ca²⁺ response to activation of recombinant, Gα₁-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 Gβγ-scavenger Gα₁ (transducin α subunit), indicating the involvement of Gα₃ and Gβγ 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 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, Gα₁-q/i-coupled receptors can markedly enhance the Ca²⁺ signaling of simultaneously activated Gα₁1-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., 1999; Hirono et al., 2001) and peripheral tissues (Shah et al., 1999; Hirono et al., 2001) and peripheral tissues (Shah et al., 1999; Hirono et al., 2001).

ABBREVIATIONS: GPCR, G protein-coupled receptor; HEK, human embryonic kidney; HEK-CXCR2, human embryonic kidney cell with stable expression of recombinant human CXCR2; CXCR2, CXC chemokine receptor 2 or IL-8 receptor B; PTX, pertussis toxin; PLC, phospholipase C; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; AM, acetoxymethyl ester; IL-8, interleukin-8; 2-APB, 2-aminoethoxydiphenyl borane; RGS, regulator of G protein signaling; BSS, balanced salts solution; [Ca²⁺], intracellular Ca²⁺ concentration; GFP, green fluorescent protein; InsPₓ, inositol phosphates; PI 3-kinase, phosphatidylinositol 3-kinase; PI 4-kinase, phosphatidylinositol 4-kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; DMSO, dimethyl sulfoxide; PP, protein phosphatase; U73122, 1-[6-([17beta-3-methoxyestra-1,3,5(10)-trien-17-yl) amino]hexyl]-1H-pyrrole-2,5-dione; U73343, 1-[6-([17beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl]-2,5-pyrolinedione.
Materials and Methods

Materials. Cell culture reagents were obtained from Invitrogen (Paisley, Scotland). Cell culture plastics were obtained from Nalgene (Paisley, Scotland). Fluo-3/AM was from TEF Labs (Austin, TX). Cell culture plastics were obtained from Nalgene (Paisley, Scotland). 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 transduction reagent was from Novagen (through CN Biosciences, Nottingham, UK), and pTracer expression vector was supplied by Invitrogen (Inchannah, Scotland). Fura-2 acetoxymethyl ester (fura-2/AM), UTP, thapsigargin, ryanodine, and caffeine were obtained from Sigma Chemical (Poole, Dorset, UK). Fluo-3/AM was from TEF Labs (Austin, TX). Interleukin-8 (IL-8) was supplied by R&D Systems (Abingdon, UK). Staurosporine, 2-aminoethyl diphenyl borane (2-APB), cyclopiazonic acid, A, and okadaic acid were from Calbiochem (through CN Biosciences, Nottingham, UK). All other reagents were of analytical grade and were obtained from Sigma Chemical or Fisher Scientific (Loughborough, UK).

Cloned cDNA encoding either the bovine transducin Gαi1 subunit (Gαi1) or human Gαi2-specific regulator of G protein signaling 2 (RGS2) were kind gifts from Prof. G. Milligan (University of Glasgow, Glasgow, UK) and Dr. C. Doupnik (University of South Florida, Tampa, FL), respectively.

Assay buffer used in all experiments was a balanced salts solution (BSS) composed of 130 mM NaCl, 5.4 mM KCl, 16 mM NaHCO3, 1.3 mM NaH2PO4, 0.8 mM MgCl2, 1.8 mM CaCl2, 10 mM HEPES, and 5.5 mM d-glucose, pH 7.4.

Cell Culture: HEK-CXCR2 Cell Line. The HEK cell line expressing recombinant human CXCR2 at approximately 50,000 sites/cell was generated and selected as described previously (Werry et al., 2002). This cell line (HEK-CXCR2) was maintained in Dulbecco's modified Eagle's medium (containing 25 mM d-glucose, 4 mM L-alanyl-l-glutamine, and 1 mM sodium pyruvate) supplemented with 10% fetal calf serum, 1% nonessential amino acids, 50 μg/ml gentamicin, and 400 μg/ml G418 at 37°C in a 5% CO2 humidified atmosphere.

Generation of HEK-CXCR2 Cells Expressing Either Gαq or RGS2. The coding sequence for Gαq1 or Gαi1 was ligated into pTracer using EcoRI and Xhol restriction sites. The cDNA encoding RGS2 was ligated into pTracer using BstXI restriction sites. HEK-CXCR2 cells were transfected with one of these pTracer constructs or were control-transfected (using pTracer containing neither Gαq1 nor RGS2 cDNA) as a control. Transfection was performed using Genejuice transfection reagent according to the manufacturer's instructions. Nonclonally selected cell lines containing either Gαq1, 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 Gαi1-transfected cells.

Measurement of the Intracellular Ca2+ Concentration ([Ca2+]i). 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 (fluor-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 (fluor-3) or above 510 nm (fura-2) were detected by a charge-coupled device camera at a rate of 0.75 frames/s (fluor-3) or 0.5 frames/s (fura-2) and converted into on-screen images by UltraVIEW imaging software (PerkinElmer Life Sciences). Fluoro-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 UTP 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 5 μCi/ml [3H]myo-inositol. After washing and preincubation (20 min, 37°C) with BSS containing 10 μM 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 ion exchange columns (8% cross linkage, 100–200 dry mesh; Sigma 1 × 8-200) by adding the sample to the column, and washing firstly with water and then with 25 mM ammonium formate. [3H]Inositol phosphates ([3H]InoP) were eluted from the col-
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-

![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, floo-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 floo-3 fluorescence in a small population of cells (<20) as an index of \([Ca^{2+}]_i\). Arrow A indicates the point at which some of the inhibitors were added (see text and other figures for details). b, floo-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 floo-3 fluorescence in a small population of cells (<20) as an index of \([Ca^{2+}]_i\). 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.

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
protocol and Ca\textsuperscript{2+} determined by unpaired Student’s t test. Responses to caffeine stimulation of P2Y\textsubscript{2} Nucleotide Receptors and CXCR2. Responses after Coaddition of UTP and 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 U73343) 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 [\textsuperscript{3H}]InsP\textsubscript{3} (Fig. 3). 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\textsuperscript{2+} Responses after Coaddition of UTP and IL-8 Are Inhibited by 2-APB.** To examine the involvement of Ins(1,4,5)P\textsubscript{3} receptors in the potentiated response to CXCR2 activation, the effects of the putative Ins(1,4,5)P\textsubscript{3} 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 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)P\textsubscript{3} 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)P\textsubscript{3}.

The role of PLC was also explored using the putative PLC inhibitor U73122. After store drainage, a 5-min incubation with 10 µM U73122 (from point A, Fig. 1) reduced the Ca\textsuperscript{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 U73343) 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 [\textsuperscript{3H}]InsP\textsubscript{3} (Fig. 3). 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.

### Inositol Phosphate Generation Is Enhanced by Co-stimulation of P2Y\textsubscript{2} Nucleotide Receptors and CXCR2.

Agonist-mediated accumulation of [\textsuperscript{3H}]InsP\textsubscript{x} against a Li\textsuperscript{+} block of inositol monophosphatase was determined as an index of PLC activity. HEK-CXCR2 cells loaded with [\textsuperscript{3H}]myo-inositol were stimulated with the indicated agonist additions for varying durations (range 0–30 min). IL-8 alone evoked little or no accumulation of [\textsuperscript{3H}]InsP\textsubscript{x} (Fig. 3). In contrast, UTP caused an accumulation of [\textsuperscript{3H}]InsP\textsubscript{x} to a maximum of 1.6 ± 0.09-fold of basal, whereas coaddition of ryanodine receptors because it inhibited the Ca\textsuperscript{2+} elevation seen after stimulation with 10 mM caffeine (Fig. 2c).

**Fig. 2.** The Ca\textsuperscript{2+} store accessed by IL-8 in the presence of UTP is thapsigargin-sensitive (a), but is not ryanodine-sensitive (b). a, HEK-CXCR2 cells were incubated with or without 2 µM thapsigargin for 5 min before assay. Cells were then stimulated with a single addition of either 100 µM UTP or a coaddition of 100 µM UTP and 10 nM IL-8. Using a Ca\textsuperscript{2+} imaging system, changes in fluo-3 fluorescence were measured as an index of [Ca\textsuperscript{2+}]. Responses are expressed as a percentage of the maximal response to 100 µM UTP in the absence of thapsigargin pretreatment. Data are mean ± S.E.M., n = 4, with *P < 0.05 and **P < 0.01 determined by unpaired Student’s t test. b, using the store depletion protocol and Ca\textsuperscript{2+} imaging (Fig. 1), cells were incubated with or without 30 µM ryanodine after store depletion in the absence of extracellular Ca\textsuperscript{2+} (point A, Fig. 1) before stimulating with a coaddition of 100 µM UTP and 10 nM IL-8. Responses to this coaddition were measured as changes in fluo-3 fluorescence and are expressed as a percentage of the maximal response to 100 µM 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 10 nM caffeine in the absence or presence of 30 µM ryanodine (with a 5-min preincubation). Responses to caffeine stimulation are expressed as fluo-3 fluorescence. Data are mean ± S.E.M., n = 4, with *P < 0.05 determined by unpaired Student’s t test. The role of PLC was also explored using the putative PLC inhibitor U73122. After store drainage, a 5-min incubation with 10 µM U73122 (from point A, Fig. 1) reduced the Ca\textsuperscript{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 U73343) 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 [\textsuperscript{3H}]InsP\textsubscript{x} (Fig. 3). 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\textsuperscript{2+} Responses after Coaddition of UTP and IL-8 Are Inhibited by 2-APB.** To examine the involvement of Ins(1,4,5)P\textsubscript{3} receptors in the potentiated response to CXCR2 activation, the effects of the putative Ins(1,4,5)P\textsubscript{3} 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.
these circumstances, 2-APB markedly reduced the Ca\(^{2+}\) responses to 100 \(\mu M\) UTP (Fig. 4b). After Ca\(^{2+}\) store depletion using 100 \(\mu M\) UTP (Fig. 1), cells were exposed to either 100 \(\mu M\) 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 \(\mu M\) 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 Ca\(^{2+}\) response, but this was significantly reduced in the presence of 2-APB (Fig. 4a). Neither agonist alone elevated \([\text{Ca}^{2+}]_i\) 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 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 Ca\(^{2+}\) responses to 10 nM IL-8 when IL-8 was added with either 3 \(\mu M\) UTP (42 \(\pm\) 3 versus 38 \(\pm\) 5% in controls, where responses are expressed as a percentage of the response to addition of 100 \(\mu M\) UTP alone in naive cells) or 100 \(\mu M\) UTP (56 \(\pm\) 8 versus 65 \(\pm\) 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 10 \(\mu M\) to inhibit PI 4-kinase activity and limit the supply of PIP\(_2\) (Nakanishi et al., 1995; Willars et al., 1998). A 20-min preincubation with 10 \(\mu M\) wortmannin had no effect on the response to IL-8 when this agonist was added after 150-s prestimulation with 100 \(\mu M\) UTP (Fig. 5a). When the prestimulation with UTP was extended to 7 min in the absence of wortmannin, the Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 PIP\(_2\) 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 PIP\(_2\) 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 \(\mu M\) wortmannin. Responses to these stimulations with UTP (in the presence of extracellular Ca\(^{2+}\)) 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 G\(\alpha_q\)-coupled muscarinic M\(_3\) receptor (thus further depleting the cellular PIP\(_2\) pool but without sustained activation and potential desensitization of the P2Y\(_{12}\) nucleotide receptors) (Fig. 5b).

**Incremental Ca\(^{2+}\) Release Occurs in HEK-CXCR2 Cells.** Cells were repetitively stimulated (in the absence of extracellular Ca\(^{2+}\), 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\((1,4,5)P_3\) to investigate whether incremental increases in Ins\((1,4,5)P_3\) resulted in a corresponding fractional release of Ca\(^{2+}\) from intracellular stores. Stimulation with a train of 20-s pulses of 1 \(\mu M\) UTP (with 20-s perfusion of nominally Ca\(^{2+}\)-free buffer separating each) caused progressively di-

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**Fig. 4.** Antagonism of Ins\((1,4,5)P_3\) receptors with 2-APB inhibits potentiation. a, after depletion of the UTP-sensitive Ca\(^{2+}\) store (Fig. 1), cells were incubated with or without 2-APB (100 \(\mu M\); 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 \(\mu M\) UTP and 10 nM IL-8, using fluo-3 fluorescence as an index of \([\text{Ca}^{2+}]_i\). Data are expressed as a percentage of the initial response to 100 \(\mu M\) UTP, mean \(\pm\) 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 \(\mu M\) UTP by perfusion for 30 s, followed by washout for 90 s with buffer containing Ca\(^{2+}\) to allow refilling of intracellular Ca\(^{2+}\) stores. Cells were then restimulated twice more with 100 \(\mu M\) UTP, first in the absence of 2-APB (to demonstrate reproducibility of responses) and subsequently after a 5-min incubation with 100 \(\mu M\) 2-APB (during which time extracellular Ca\(^{2+}\) was present to facilitate store refilling). Data are expressed and analyzed as in main figure; \(n = 4\) (***, \(P < 0.001\)).
To assess the participation of 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).

To test the involvement of Gβγ subunits in the potentiated response to CXCR2, HEK-CXCR2 cells were transfected with the Gβγ-scavenger Gα11. Expression of Gα11 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 Gαq/o- and Gα11-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.
of variance that gave $P_{\text{maximal}}$ (100 nM UTP) with approximately 100 nM UTP (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., $n = 5$, 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 P2Y$_2$ nucleotide receptors and CXCR2 requires costimulation 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 P2Y$_2$ nucleotide receptors to potentiate Ca$^{2+}$ signaling by CXCR2 is dependent upon the concentration of UTP with an EC$_{50}$ 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 Ca$^{2+}$ response is dependent upon a thapsigargin-sensitive intracellular Ca$^{2+}$ store but independent of ryanodine receptors, suggesting that this cross talk may be Ins(1,4,5)P$_3$-dependent. Inhibition of the potentiated Ca$^{2+}$ response by the putative Ins(1,4,5)P$_3$ receptor antagonist 2-APB supports this conclusion. Although 2-APB can

have nonselective effects on Ca$^{2+}$ handling, particularly the block of Ca$^{2+}$ channels other than Ins(1,4,5)P$_3$ 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 [H]InsP$_{1}$ during costimulation with UTP and IL-8 (compared with UTP alone) indicates that potentiated PLC activity is associated with coactivation of P2Y$_2$ nucleotide receptors and CXCR2. It is of interest that after longer stimulation with UTP in the presence of 10 μM wortmannin (to reduce PIP$_2$ levels in the plasma membrane), the Ca$^{2+}$ responses to receptor coactivation were reduced. This confirms that PIP$_2$ is required, presumably for the generation of Ins(1,4,5)P$_3$.

If levels of PIP$_{2}$ are rate-limiting for PLC activity, then an increase in its supply could account for enhanced PLC activity and Ca$^{2+}$ signaling. In this respect, an increase in PIP$_2$ levels mediated via PTX-sensitive G proteins enhances muscarinic receptor-mediated Ca$^{2+}$ signaling in HEK cells (Schmidt et al., 1996). Thus, an increased supply of PIP$_2$ in response to activation of CXCR2 could result in enhanced P2Y$_2$ nucleotide receptor-mediated Ins(1,4,5)P$_3$ generation. PI 4-kinase may limit the supply of PIP$_2$ (Willars et al., 1998), and we therefore determined the effect on the cross
talk 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 Ca\textsuperscript{2+} responses to coaddition after a relatively short prestimulation with UTP. However, other phosphoinositide kinases may play a role in the maintenance of PIP\textsubscript{2} levels and thus a role of, for example, phosphatidylinositol 4-phosphate 5-kinase, cannot be excluded.

In the absence of Ca\textsuperscript{2+} store refilling, consecutive increases in the concentration of UTP caused further release of intracellular Ca\textsuperscript{2+}. This is consistent with quantal Ca\textsuperscript{2+} release (Bootman, 1994) or incremental detection (Meyer and Stryer, 1990). Thus, the intracellular Ca\textsuperscript{2+} store seems to be functionally divided into fractions that are released incrementally according to the concentration of Ins(1,4,5)P\textsubscript{3}. The presence of incremental Ca\textsuperscript{2+} release in these cells suggests that other mechanisms such as agonist-dependent shifting of Ca\textsuperscript{2+} between stores (Short and Taylor, 2000) and sensitization of the Ins(1,4,5)P\textsubscript{3} receptor (Tovey et al., 2003) are not required to mediate cross talk, resulting in enhanced Ca\textsuperscript{2+} mobilization when PLC activity and Ins(1,4,5)P\textsubscript{3} generation are potentiated. It is also possible that Ca\textsuperscript{2+} is released from stores that are accessed according to the locality of Ins(1,4,5)P\textsubscript{3} generation. However, at the resolution of our imaging equipment, we were unable to distinguish any spatial differences in the Ca\textsuperscript{2+} signaling mediated by either UTP alone or a coaddition of UTP and IL-8.

Cross talk between CXCR2 and P2Y\textsubscript{2} nucleotide receptors is abolished by PTX treatment (Werry et al., 2002). Given that CXCR2 is coupled to G\textsubscript{o}, this may explain the PTX sensitivity of the cross talk, although there remains the possibility that coupling of P2Y\textsubscript{2} nucleotide receptors to G\textsubscript{o} may be important. However, inhibition of cross talk by the G\textsubscript{o}-specific RGS protein RGS2 indicates a role for G\textsubscript{o}. One possible mechanism is that coincident receptor stimulation enhances activation of G\textsubscript{o} 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\textsubscript{o} directly in cell membranes using the binding of \textsuperscript{35}Sguanosine 5’-O-(3-thio)triphosphate and specific immunoprecipitation. However, this was not possible as UTP competed effectively with the radiolabeled compound for binding to G\textsubscript{o}.

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 \textsuperscript{3}HInsP\textsubscript{3} 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 P2Y\textsubscript{2} 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 P2Y\textsubscript{2} 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 Ca\textsuperscript{2+} responses, suggesting that P2Y\textsubscript{2} nucleotide receptor dephosphorylation and any associated resensitization is not the mechanism of cross talk.

Inhibition of potentiated Ca\textsuperscript{2+} signaling by expression of G\textsubscript{o}, indicates a role for G\textsubscript{o} 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 Ca\textsuperscript{2+} response is not, however, a simple consequence of pooling G\textsubscript{o} subunits from two coactivated receptor populations. Thus, although G\textsubscript{o}-coupled \beta_{3}-adrenoceptors are able to elevate [Ca\textsuperscript{2+}]\textsubscript{i}, if P2Y\textsubscript{2} nucleotide receptors are also activated in these cells (Werry et al., 2002), coactivation of CXCR2 and \beta_{3}-adrenoceptors did not influence [Ca\textsuperscript{2+}]\textsubscript{i}.
The requirement for both Goq and Gb3 may be at distinct sites. Alternatively, they could converge to enhance PLC activity directly. The Gb3-sensitive isoforms of PLC (β1–5) have distinct binding sites for Goq and Gb3, and stimulation of PLC by PTX-insensitive Gq proteins and Gb3 can be additive (Smrcka and Sternweis, 1993) or even synergistic (Zhu and Birnbaumer, 1996), providing a mechanism for receptor cross talk. Indeed, Goq may prime PLCβ to subsequent activation by Gb3 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 after Goq binding that relieves a steric hindrance to Gb3 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 Goq and Gb3.

This study demonstrates that cross talk between P2Y2 nucleotide receptors and CXCR2 results in the release of Ca2+ from a thapsigargin-sensitive, In1(1,4,5)P3-dependent intracellular store. Furthermore, cross talk results in the potentiation of PLC activity, and our data suggest that the enhanced generation of In1(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 Goq and Gb3 subunits derived from Goq. The most straightforward interpretation of this is that Goq and Gb3 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 determine whether they are able to contribute to cross talk under this or any other example of cross talk.

References


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