Role of G-protein $\beta\gamma$ subunits in the augmentation of P2Y$_2$ (P$_{2U}$) receptor-stimulated responses by neuropeptide Y Y$_1$ G$_{i/o}$-coupled receptors

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Neuropeptide Y (NPY) significantly potentiates the constrictor actions of noradrenaline and ATP on blood vessels via a pertussis toxin (PTX)-sensitive mechanism involving $G_{i/o}$ ($\alpha\beta\gamma$) protein subunits ($G_{i/o}$, GTP-binding proteins sensitive to PTX). In Chinese hamster ovary K1 (CHO K1) cells expressing specific receptors for these neurotransmitters, stimulation of $G_{i/o}$ protein-coupled receptors for NPY and other neurotransmitters can augment the $G_{q/11}$-coupled ($G_{q/11}$, GTP-binding proteins insensitive to PTX) α_{1B} adrenoceptor- or ATP receptor-induced arachidonic acid (AA) release and inositol phosphate (IP) production (early events which may precede vasoconstriction). In this study, we have assessed the role of $G\beta\gamma$ subunits in the synergistic interaction between $G_{i/o}$- (NPY Y_1, 5-hydroxytryptamine 5-HT$_{1B}$, adenosine A_1) and $G_{q/11}$- [ATP P2Y$_2$ (P$_{2U}$)]-coupled receptors on AA release by using the specific abilities of regions of the β-adrenergic receptor kinase (βARK1 residues 495–689) and the transducin α subunit to associate with G-protein $\beta\gamma$ subunit dimers and to act as $G\beta\gamma$ subunit scavengers. Transient expression of βARK1(495–689) in CHO K1 cells heterologously expressing NPY Y_1 receptors had no significant effect on the PTX-insensitive ability of ATP to stimulate AA release. Stimulation of NPY Y_1 receptors (as well as the endogenous 5-hydroxytryptamine 5-HT$_{1B}$ receptor and the transiently expressed human adenosine A_1 receptor) resulted in a PTX-sensitive augmentation of ATP-stimulated AA release, which was inhibited by expression of both $G\beta\gamma$ subunit scavengers. Expression of βARK1(495–689) similarly inhibited NPY Y_1 receptor augmentation of ATP-stimulated IP production (a measure of phospholipase C activity), a step thought to precede the NPY Y_1 receptor-augmented protein kinase C-dependent AA release previously observed in these cells. These experiments demonstrate that $G\beta\gamma$ subunits, as inhibited by two different $G\beta\gamma$ scavengers, significantly contribute to the synergistic interaction between NPY Y_1 $G_{i/o}$- and $G_{q/11}$-coupled receptor activity, and are required for the augmentation of IP production and AA release observed in this model cell system.

INTRODUCTION

Many neurotransmitters, often found co-localized in the same neurons, interact with specific G-protein-coupled receptors, which activate associated heterotrimeric G-proteins ($\alpha\beta\gamma$) to result in a range of direct and synergistic cellular changes. For example, neuropeptide Y (NPY), an abundant peptide neuromodulator that plays a major role in the control of cardiovascular function and vascular tone (as reviewed in [1,2]), is found co-localized with noradrenaline and ATP in sympathetic nerves. NPY has been shown to potentiate the constrictor effects of several agents including noradrenaline, angiotensin II, histamine, prostaglandin F2α, endothelin and ATP, acting on both vascular and non-vascular smooth muscle from human, dog, rabbit and guinea-pig tissues [3–10]. The post-junctional actions of NPY are, in most systems, a result of NPY Y_1 receptor stimulation, as has been demonstrated using Y_1 receptor-specific peptide agonists and antagonists and Y_1 receptor-specific antisense oligonucleotides [11–13]. The cloning of the human Y_1 receptor has confirmed the ability of this receptor to stimulate G-protein-dependent [pertussis toxin (PTX)-sensitive] increases in cytosolic Ca^{2+} concentrations, inositol phosphates (IP) and protein kinase C (PKC) activation when expressed in Chinese hamster ovary K1 (CHO K1) cells [14,15]. In this cell model of receptor action, NPY Y_1 receptor stimulation results in marked augmentation of $G_{q/11}$-coupled receptor-mediated IP production ($G_{q/11}$, GTP-binding proteins insensitive to PTX, PKC activation and arachidonic acid (AA) release [14].

$G_{i/o}$ protein-coupled receptor augmentation ($G_{i/o}$, GTP-binding proteins sensitive to PTX) of $G_{q/11}$ protein-coupled receptor function appears to be a general property of a number of neurotransmitter receptors (including those responsive to noradrenaline, adenosine, 5-hydroxytryptamine and acetylcholine) that have been shown to augment the constrictor actions of both ATP and noradrenaline. When transfected into CHO K1 cells, muscarinic m2- and m4-, α_2-adrenergic-, dopamine D_2- and adenosine A_1 receptors also augment AA release and in some cases IP production, resulting from $G_{q/11}$ protein-coupled α_{1B}-adrenergic receptor or P2Y$_2$ (P$_{2U}$) receptor activation in CHO K1 cells [14,16,17]. In the absence of conditional activation of phospholipase A_2 (PLA_2) by $G_{q/11}$ protein-coupled receptors, however, stimulation of these $G_{i/o}$ receptors alone is without effect on AA release. In the case of NPY responses, we have shown that NPY Y_1 receptor-mediated augmentation of a $G_{q/11}$ protein-coupled receptor stimulation of AA release is sensitive to inhibition by PTX, suggesting a role for $G_{i/o}$ heterotrimeric proteins [14].

The mechanisms underlying this type of receptor synergy are thought to involve enhanced activation of specific isoforms of intracellular signalling enzymes, including phospholipase C (PLC), PLA_2 and PKC by direct activation of G-proteins or more downstream modulation by kinase activity and calcium concentrations. The more recent observations of $G\beta\gamma$-sensitive PLC and PLA_2 isoforms [18–21] and the $\beta\gamma$ subunit involvement in $G_{i/o}$ protein-coupled receptor effects on PLC activity [22], suggest an important role for $\beta\gamma$ subunits in receptor signalling.

Abbreviations used: NPY, neuropeptide Y; PYY, peptide YY; IP, inositol phosphate; AA, arachidonic acid; G-protein, heterotrimeric signal-transducing GTP-binding protein; $G_{i/o}$, GTP-binding proteins sensitive to PTX; $G_{q/11}$, GTP-binding proteins insensitive to PTX; βARK1, β-adrenergic receptor kinase; PLC, phospholipase C; PLA_2, phospholipase A_2; CHO K1, Chinese hamster ovary K1 cell line; PKC, protein kinase C; PTX, pertussis toxin; CPA, N^6-cyclopentyladenosine; SPAP, secreted placental alkaline phosphatase; MAPK, mitogen-activated protein kinase.

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Furthermore, it has been demonstrated that Gα protein subunits act synergistically with βγ subunits to regulate different βγ-sensitive isoforms of adenylate cyclase [23,24] and PLC [25]. In the present study, we have investigated the role of Gβγ subunits in the augmentation of AA release and IP production by $G_{i/o}$ proteins, using the release of AA as a measure of receptor synergy and the ability of scavengers of Gβγ subunits to inhibit the proposed involvement of Gβγ subunits.

EXPERIMENTAL

Materials

ATP disodium salt, 5-hydroxytryptamine creatinine sulphate complex, N^6-cyclopentyladenosine (CPA) and Hank's balanced salt solution were obtained from Sigma (Poole, Dorset, U.K.). Peptides (PYY and NPY) were obtained from Novabiochem. ^{125}I-labelled PYY and [^{3}H]*myo*-inositol were from NEN/DuPont and [^{3}H]AA was from Amersham. PTX (purified from *Bordetella pertussis*; Wellcome 28 strain) was from Speywood. The β-adrenergic receptor kinase (βARK1) minigene construct βARK1(495–689)/pRK5 expression vector and βARK specific antisera were kindly provided by Dr. R. J. Lefkowitz. The human transducin α subunit and adenosine A_1 receptor cDNAs were purchased from the American Type Culture Collection and specific affinity-purified rabbit polyclonal antibody against α transducin [$G_{\alpha t1}$ (K-20); cat. sc-389] was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell culture and transfection

CHO K1 (American Type Culture Collection CCL 61) cells were previously stably transfected using a modified calcium phosphate method with the human NPY Y_1 receptor cDNA [15] in the pcDNA Neo vector (Invitrogen) and the hamster α_{1B}-adrenergic receptor cDNA in a pMT2 vector (cell line designated here as Y1R-CHO.2), and maintained as described [14]. Stably transfected cells or wild-type CHO K1 cells were transiently transfected using a modified calcium phosphate method with pZeoLacZ (Invitrogen), and at least 50 % of cells expressed observable β-galactosidase activity. Y1R-CHO.2 cells were transiently transfected with the βARK1 minigene construct βARK1(495–689)/pRK5 [22], containing residues Gly^{495} to Leu^{689}, which encode the βγ subunit binding region. Alternatively, cells were transfected with the human transducin α subunit cDNA, which had been subcloned into the *Eco*RI restriction site of the pcDNA3 expression vector (Invitrogen). The control pRK5 vector was prepared from the βARK/pRK5 construct by removal of the insert with *Eco*RI and *Hin*dIII digestion, and ligation of an oligonucleotide dimer with *Eco*RI and *Hin*dIII termini to form an 'empty' vector, according to standard methods. The human adenosine A_1 receptor cDNA was subcloned into the *Eco*R1 site of the pcDNA3 vector. DNA for transfection was prepared using the Promega DNA maxipreparation kit (Promega). Transient expression efficiency at 48 h post-transfection was monitored by co-transfection with 1 μg of secreted placental alkaline phosphatase (SPAP) cDNA in a cytomegalovirus-promoter vector (kindly provided by Glaxo Wellcome). SPAP secreted into the medium was measured (after inactivation of endogenous alkaline phosphatases by heating to 65 °C for 15 min) by assay of 10 μl of supernatant in 200 μl of 5 mM *p*-nitrophenol phosphate in DEA buffer (1 M diethanolamine/280 mM NaCl/0.5 mM $MgCl_2$, pH 9.85) for 30 min to 1 h, then the absorbance was read at 405 nm. SPAP activity was calculated according to [26]. DNA preparations were standardized to give maximal levels of SPAP expression when co-transfected with the βARK/pRK5 and transducin α/pcDNA3 vectors.

Western blotting

Cell membranes and cytosol fractions (10–20 μg) were prepared as described from cells at 48 h post-transfection [27], except that cells were lysed by freezing before preparation. Samples were then run on a 12 % (w/v) acrylamide gel, transferred to nitrocellulose, and blotted with either βARK-specific antisera at 1:1000 dilution or α transducin-specific affinity-purified rabbit polyclonal antibody. Resultant bands were detected with the Amersham enhanced chemiluminescence detection reagent and exposure to autoradiographic film.

Receptor binding assay

Transiently transfected Y1R-CHO.2 cells (48 h post-transfection) were tested for the ability to bind PYY or NPY. Transfected cells [$(5–8) \times 10^5$/well] were plated in 24-well tissue culture plates for 2 h, washed once in the assay buffer [Hank's balanced salt solution with 20 mM Hepes, pH 7.4, and 0.1 % (v/v) BSA], then incubated in 0.25 ml of assay buffer in the presence of 0.05 nM ^{125}I-labelled PYY and increasing concentrations of PYY for 1 h at 25 °C. After two washes with assay buffer, cells were removed by addition of 0.2 ml of 0.5 M NaOH and 50 μl collected for protein concentration determination. Radioactivity associated with the cells was counted in a gamma counter. Non-specific binding was assessed with 1 μM NPY. K_d and B_{max} values were calculated using PRISM v1.09 (GraphPad Software, San Diego, CA, U.S.A.).

Measurement of [^{3}H]AA release

Transiently transfected cells harvested 48 h post-transfection (5×10^4/ml) were plated in 48-well tissue culture plates for 2 h, then labelled with [^{3}H]AA (1.6 μCi/ml = 60 kBq/ml) for 3 h at 37 °C. After washing twice with Hank's balanced salt solution containing 20 mM Hepes (HB) and 0.2 % BSA, cells were incubated in 0.125 ml of HB/0.2 % BSA for 10 min at 37 °C, and then various agents in 0.125 ml were added for 15 min at 37 °C. In some experiments, cells were incubated with PTX for 3 h at 100 ng/ml during labelling. Supernatants (0.2 ml) were removed to a fresh tube and 4 ml of Packard Emulsifier Scinitillator Plus liquid-scintillation fluid was added, and the samples counted for 2 min in an LKB 1214 RACKBETA liquid-scintillation counter. Release of AA was previously confirmed by TLC [14]. In addition, it has been reported that cytoplasmic PLA_2 and not diacylglycerol is primarily responsible for released AA in these cells [28,29]. Levels of significance were assessed with the Student's *t* test.

Measurement of [^{3}H]IP production

Transiently transfected cells, harvested 48 h post-transfection [$(5–8) \times 10^5$/well], were labelled for 6 h with [^{3}H]*myo*-inositol (74 Bq/ml) in 24-well plates in inositol-free Dulbecco's modified Eagle's medium with 0.1 % (v/v) fetal-calf serum. Cells were then washed in HB buffer, incubated for 30 min in buffer containing 20 mM LiCl, then stimulated for 15 min with various agonists. Incubations were terminated, and total [^{3}H]inositol phospholipids were isolated by Dowex anion-exchange chromatography, as described [30].

RESULTS

NPY Y_1 receptor augmentation of ATP-stimulated AA release

In [^{3}H]AA-labelled CHO K1 cells, stably expressing the human NPY Y_1 receptor (Y1R-CHO.2 cells), stimulation of endogenous $P2Y_2$ (P_{2U}) purinoceptors [31] with ATP (10 μM) resulted in an increase in [^{3}H]AA release (Table 1), which was insensitive to PTX (100 ng/ml) treatment. At this concentration, ATP has previously been shown to maximally stimulate AA release [16], calcium mobilization [31] and IP [32] accumulation in CHO K1 cells. Stimulation of Y1R-CHO.2 with PYY (100 nM) had no effect on AA release alone (Table 1), although at such concentrations, PYY can maximally stimulate increases in cytosolic calcium in this cell line [14,15]. However, in the presence of 10 μM ATP, PYY was able to augment the $P2Y_2$-receptor-stimulated AA release (Table 1). This PYY-mediated augmentation of ATP responses was shown to be sensitive to PTX treatment (Table 1). These data are consistent with previous studies of NPY Y_1 receptor-mediated augmentation of α_{1B} adrenoceptor-stimulated AA release [14].

The augmentation via NPY Y_1 receptors was not confined to overexpressed $G_{i/o}$-coupled receptors in transfected cells, since co-stimulation of the endogenous 5-hydroxytryptamine 5-HT_{1B} receptor (previously described in CHO K1 cells and reported to be expressed at such low receptor numbers as not to be detected by radioligand binding studies [33]), also potentiated ATP-stimulated AA release in a PTX-sensitive fashion (Table 1). As for NPY, 5-hydroxytryptamine alone (1 μM) had no effect on AA release (Table 1), even though these concentrations resulted in maximal stimulation of increases in cytosolic calcium and maximal inhibition of forskolin-stimulated cAMP accumulation [33].

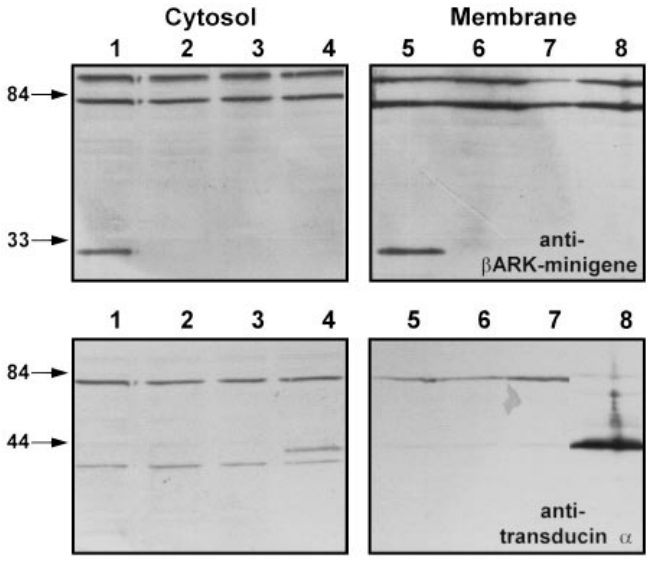

Figure 1 Western blot analysis of βARK1(495–689) minigene and transducin α subunit expression in transfected cells

Forty-eight hours after transient transfection with βARK1/pRK5 (lanes 1, 5) or transducin α/pcDNA3 (lanes 4, 8), or the control vectors pRK5 (lanes 2, 6) or pcDNA3 (lanes 3, 7), cells were harvested and cytosolic (lanes 1–4) and membrane (lanes 5–8) fractions prepared as described. Samples (10 μg of protein) were run on 12.5% (w/v) acrylamide gels and transferred to nitrocellulose. Blotting of filters with antisera specific for the βARK1(495–689) minigene (top panels) revealed an approx. 27 kDa specific band, and with antisera specific for the human transducin α subunit (bottom panels) revealed an approx. 39 kDa specific band. Non-specific bands were evident with both antisera. Positions of standard molecular-mass markers of 33, 44 and 84 kDa are shown on the left.

Table 1 Effect of NPY Y_1 and 5-HT_{1B} receptor stimulation on ATP-mediated increases of [^{3}H]AA release from CHO K1 cells stably expressing NPY Y_1 receptors

Forty-eight hours after transfection with the control vector pRK5, Y1R-CHO.2 cells were stimulated (A) with PYY (100 nM), ATP(10 μM), or both, or (B) with 5-hydroxytryptamine (5-HT; 1 μM), ATP (10 μM), or both, for 15 min and [^{3}H]AA release measured. Transiently transfected cells were also pretreated for 3 h with PTX (100 ng/ml) and then assayed. Data are percentages of basal responses (basal = 100%) and are means ± S.E.M. of four experiments in triplicate. Responses from control cells stimulated with PYY + ATP or 5HT + ATP were significantly different from cells stimulated similarly and treated with PTX (Student's *t* test, $^*P < 0.01$).

(A)

	[^{3}H]AA release (%)	
Agonist	Control	PTX-treated
PYY	100 ± 4	109 ± 11
ATP	220 ± 27	184 ± 22
PYY + ATP	303 ± 20	199 ± 23*

(B)

	[^{3}H]AA release (%)	
Agonist	Control	PTX-treated
5HT	99 ± 4	108 ± 8
ATP	167 ± 9	166 ± 12
5HT + ATP	253 ± 14	174 ± 20*

Inhibition of $G_{i/o}$-coupled receptor augmentation of AA release by expression of G$\beta\gamma$ subunit scavengers

The C-terminal fragment of βARK1 has been shown previously to bind $\beta\gamma$ subunits and to inhibit $G_{i/o}$ $\beta\gamma$ coupled receptor effects on PLC and type II adenylate cyclase activity [22,34,35]. In order to scavenge G$\beta\gamma$ subunits, we transiently expressed a construct containing the residues Gly^{495} to Leu^{689} of βARK1 (βARK1/pRK5) in Y1R-CHO.2 cells. Expression of this protein fragment was confirmed by Western blotting using a βARK-specific antisera, and shown in unstimulated cells to be expressed in both the cytosol and the membrane fractions (Figure 1). In Y1R-CHO.2 cells transiently transfected with βARK1(495–689), no significant decrease in ATP-stimulated AA release was observed. PYY alone had no direct effect in cells transfected either with the control vector pRK5 or with the βARK1/pRK5 vector (Table 2). In Y1R-CHO.2 cells transiently transfected with βARK1(495–689), the NPY Y_1 receptor-mediated augmentation of ATP-induced AA release was significantly reduced (Table 2A).

Since the specificity of βARK1 for various combinations of the different β and γ subunits has not yet been fully characterized, but is suggested to have the highest affinity for the $\beta2\gamma2$ dimer [36], we determined the effects of an additional $\beta\gamma$ scavenger, the human transducin α subunit, on AA release [23]. Cells transiently transfected with transducin α were slightly less responsive to ATP than the control cells (Table 2). However, there was a further inhibition of the augmentation to PYY (Table 2A).

The inhibition did not appear to be due to a decrease in receptor expression levels, as the ATP effects were not greatly reduced and the levels of NPY receptors did not differ sig-

Table 2 Effect of transient expression of βARK1(495–689) minigene or transducin α subunit on NPY Y_1 receptor and 5-HT_{1B} receptor augmentation of ATP-mediated [^{3}H]AA release

Forty-eight hours after transient transfection of Y1R-CHO.2 cells with 5–10 μg of various DNA constructs, [^{3}H]AA release was measured in response to PYY (100 nM), ATP (10 μM), or both (A), or in response to 5-hydroxytryptamine (5-HT; 1 μM), ATP (10 μM), or both (B). Data are percentages of basal responses (basal = 100%) and are means ± S.E.M. of n experiments in triplicate. In (A), responses of control cells treated with PYY and ATP were significantly different from cells treated with ATP alone (Student's t test, $^*P < 0.0025$) and from cells transfected with βARK1/pRK5 or transducin α/pcDNA3 and stimulated with PYY and ATP (Student's t test, $^{**}P < 0.0025$). In (B), the equivalent comparisons gave $^*P < 0.0025$ for control cells, $^{**}P < 0.05$ for βARK1/pRK5-transfected cells, and $^{***}P < 0.005$ for transducin α/pcDNA3-transfected cells. Basal d.p.m. values did not differ between control vector and βARK1/pRK5 or control vector and transducin α/pcDNA3 transfected cells.

(A)

	[^{3}H]AA release (%)			
	pRK5 ($n = 6$)	βARK1/pRK5 ($n = 6$)	pcDNA3 ($n = 4$)	Transducin α ($n = 4$)
PYY	104 ± 5	108 ± 4	95 ± 4	103 ± 4
ATP	199 ± 7	193 ± 10	232 ± 12	202 ± 7
PYY + ATP	287 ± 10*	219 ± 15**	333 ± 16*	258 ± 7**

(B)

	[^{3}H]AA release (%)			
	pRK5 ($n = 5$)	βARK1/pRK5 ($n = 5$)	pcDNA3 ($n = 4$)	Transducin α ($n = 4$)
5HT	113 ± 7	102 ± 4	102 ± 3	105 ± 3
ATP	201 ± 8	203 ± 15	232 ± 12	198 ± 8
5HT + ATP	306 ± 22*	253 ± 19**	329 ± 18***	249 ± 10***

Table 3 Effect of transient co-expression of βARK1(495–689) minigene or transducin α subunit and adenosine A_1 receptor cDNA on augmentation of ATP-stimulated [^{3}H]AA release

CHO K1 cells were transiently transfected with 1 μg of adenosine A_1 receptor cDNA/pcDNA3 (A1R/pcDNA3) and 9 μg of one of the following: control pcDNA3 vector ($n = 5$), βARK1/pRK5 ($n = 6$) or transducin α/pcDNA3 ($n = 3$). [^{3}H]AA release was measured in response to CPA (1 μM), ATP (10 μM) or both. Data are percentages of basal responses (basal = 100%) and are means ± S.E.M. of n experiments in triplicate. Responses of control cells treated with CPA and ATP were significantly different from cells treated with ATP alone (Student's t test, $^*P < 0.0005$), and from cells co-transfected with the receptor and either βARK1/pRK5 or transducin α/pcDNA3, and stimulated with CPA and ATP ($^{**}P < 0.0025$).

	[^{3}H]AA release (%)		
	Control ($n = 5$)	βARK1/pRK5 ($n = 6$)	Transducin α/pcDNA3 ($n = 3$)
CPA	119 ± 4	95 ± 4	105 ± 3
ATP	228 ± 10	198 ± 13	209 ± 15
CPA + ATP	319 ± 16*	222 ± 19**	230 ± 16**

Table 4 Effect of transient expression of βARK1(495–689) minigene on NPY Y_1 receptor augmentation of ATP-stimulated [^{3}H]IP production

Y1R-CHO.2 cells were transiently transfected with 5–10 μg of either the control pRK5 vector or the βARK1(495–689) minigene vector βARK1/pRK5. [^{3}H]IP production was measured, as described, in response to PYY (100 nM), ATP (10 μM) or both. Data are percentages of basal responses (basal = 100%) and are means ± S.E.M. of five experiments in triplicate. Basal d.p.m. values did not differ between pRK5 and βARK1/pRK5 transfected cells. Responses of control cells treated with PYY and ATP were significantly different from cells treated with ATP alone (Student's t test, $^*P < 0.005$), or from cells transfected with βARK1/pRK5 and stimulated with PYY and ATP ($^{**}P < 0.01$).

	[^{3}H]IP production (%)	
	Control	βARK1/pRK5
PYY	109 ± 4	102 ± 4
ATP	231 ± 17	210 ± 17
PYY + ATP	323 ± 18*	251 ± 20**

nificantly between the control vector transfected and βARK1(495–689) or transducin α subunit transfected cells. NPY receptor levels and calculated K_d values of control vector transfected cells ($3.34 \times 10^4 \pm 0.42$ receptors/cell or 0.34 ± 0.05 pmol/mg of protein; $K_d = 1.52 \pm 0.39$ nM; $n = 7$), as determined by receptor binding with radiolabelled PYY, did not differ significantly from βARK/pRK5 transfected cells ($3.92 \times 10^4 \pm 0.67$ receptors/cell or 0.42 ± 0.05 pmol/mg; $K_d = 1.86 \pm 0.59$; $n = 5$) or from transducin α/pcDNA3 transfected cells ($3.84 \times 10^4 \pm 0.75$ receptors/cell or 0.45 ± 0.07 pmol/mg; $K_d = 1.99 \pm 0.35$ nM; $n = 5$).

These effects of Gβγ subunit scavengers are also observed on $G_{i/o}$-coupled receptors responsive to 5-hydroxytryptamine (5-HT_{1B} receptor) and adenosine (A_1 receptor). The $5HT_{1B}$-mediated augmentation of ATP-stimulated AA release was significantly inhibited by expression of either βARK1 (495–689) or transducin α subunit (Table 2). To assess whether Gβγ scavengers could maximally inhibit responses in cells transiently co-transfected with receptor cDNA and G βγ scavenger cDNA, wild-type CHO K1 cells were co-transfected with the human adenosine A_1 receptor cDNA subcloned into pcDNA3 and equivalent amounts of βARK1 (495–689) or transducin α subunit cDNA or control vector. Stimulation of these transiently transfected cells with maximally effective ([^{3}H]IP accumulation and calcium mobilization) [32,37] concentrations of CPA (1 μM) alone had no significant effect on AA release (1.08 ± 0.08-fold increase). Co-stimulation of cells with both CPA (1 μM) and ATP (10 μM) resulted in similar augmentation of AA release, which was markedly attenuated in cells expressing βARK1 (495–689) or transducin α subunit (Table 3).

Inhibition of NPY Y_1 receptor augmentation of IP production by expression of Gβγ subunit scavengers

NPY Y_1 receptor stimulation has previously been shown to augment adrenoceptor-stimulated IP production in CHO K1 cells [14]. Activation of PLC results in production of IP and diacylglycerol, an activator of some PKC isoforms. Since we have previously shown that NPY augmentation of adrenoceptor-stimulated AA release was dependent on PKC activation, and might indicate action at PLC, we assessed the effects of Gβγ subunit expression on ATP-stimulated IP accumulation in Y1R-CHO.2 cells. ATP (10 μM) alone stimulated increases in IP production (Table 4). Responses to PYY alone, although observable in some experiments, were small; this was expected since PYY responses were previously shown to be more apparent when IPs were separated by the more sensitive technique of anion-exchange HPLC [14]. Co-stimulation with both PYY and ATP resulted in a significant synergy of IP production, similar to that observed on AA release (see Tables 1 and 2). Transient

expression of βARK1 (495–689) reduced the PYY-mediated synergism (Table 4), implying a role for G$\beta\gamma$ subunits in the NPY Y_1R-mediated augmentation of $G_{q/11}$-receptor-stimulated PLC activity, as well as AA release. Notably we have observed that G$\beta\gamma$ subunits can stimulate PLC activity in CHO K1 cell membranes (results not shown) in a similar manner to that previously reported in the hamster smooth muscle DDT_1-MF2 cells [27].

DISCUSSION

We have shown here that the PTX-sensitive effects of NPY Y_1 receptor stimulation on AA release are dependent upon conditional activation of a $G_{q/11}$-coupled receptor (in this case the $P2Y_2$ purinoceptor), as the NPY Y_1 receptor agonist PYY alone had no effect. This was not due to heterologous expression of the NPY Y_1 receptor in CHO K1 cells, as stimulation of endogenous 5-hydroxytryptamine $5HT_{1B}$ receptors, which are expressed at low levels, also had no effect on AA release alone, but did produce a similar augmentation of $P2Y_2$ purinoceptor-mediated responses. This observation of receptor synergy may be a general mechanism of $G_{i/o}$ protein-coupled receptor cross-talk, as it can be extended to the adenosine A_1 receptor, as well as a number of other neurotransmitter receptors previously reported [14,16,17]. We report here that this augmentation is dependent on G$\beta\gamma$ subunits, since transient expression of two different G$\beta\gamma$ subunit scavengers inhibits the augmentation of ATP-stimulated AA release produced by agonists of these three different $G_{i/o}$-coupled receptor systems. However, in cells expressing either G$\beta\gamma$ subunit scavengers [or βARK1(495–689) minigene and transducin α subunit], the $G_{q/11}$-coupled receptor remained tightly coupled to the stimulation of AA release and IP production. Our results suggest that βARK(495–689) and transducin α subunit share similar specificity for the different β and γ subunit isoforms, as they were both effective inhibitors. The mechanism of inhibition is assumed to be a scavenging action of $\beta\gamma$ subunits to form an inactive complex. In these studies, AA release was initially used as a measure of the amplified response upon receptor co-stimulation, since no direct $G_{i/o}$-coupled receptor effects on AA release were observed. However, we have also demonstrated that NPY Y_1 receptor augmentation of ATP-stimulated IP production is inhibited by expression of G$\beta\gamma$ scavengers, suggesting that G$\beta\gamma$ subunits may act upstream of PLA_2 activation (and subsequent AA release) to inhibit PLC activity and subsequent effects on PLA_2 activation. The involvement of G$\beta\gamma$ subunits in such augmentation suggests a major role for this subunit dimer in synergistic receptor actions involved in the regulation of PLA_2 activity (as measured by AA release) and PLC activity (as measured by IP production).

G$\beta\gamma$ subunits have been implicated in the regulation of a wide range of signalling proteins. Direct effects of G$\beta\gamma$ subunits on PLC β2, PLC β3, adenylate cyclase I, voltage-dependent N-type Ca^{2+} channels and cardiac K^+ atrial channels have been observed [19–21,38]. In contrast, the influence of G$\beta\gamma$ subunits on adenylate cyclases II and IV requires the conditional activation of a Gsα subunit [23]. The data obtained in the present study suggest that the effect of G$\beta\gamma$ subunits, derived from PTX-sensitive G-proteins, is more consistent with this second category of response, where there is a requirement for $G_{q/11}$ protein-coupled activation of PLA_2/AA release to result in an amplification of the AA response.

The sites of G$\beta\gamma$ subunit action, leading to augmentation of $G_{q/11}$-coupled receptor-stimulated AA release, are most probably upstream of PLA_2 activation, since $G_{i/o}$-coupled receptor activation alone has no direct effect on AA release. PKC and mitogen-activated protein kinase (MAPK) are potential candidates for such upstream modulators, as both enzymes have been implicated in the activation of PLA_2 [29,39]. In addition, stimulation of both PLC (and subsequent PKC activity) and MAPK by $G_{i/o}$-coupled receptors has been shown to be sensitive to inhibition by G$\beta\gamma$ scavengers [22,40] and would thus be inhibited in the studies reported here. The most likely explanation for our results is that G$\beta\gamma$ subunits (from PTX-sensitive $G_{i/o}$ proteins) stimulate $\beta\gamma$-sensitive PLC isoforms to liberate IP and diacylglycerol, an endogenous activator of PKC, to result in a potentiation of $G_{q/11}$-coupled-receptor actions. This hypothesis is supported by the observations that (i) $G_{i/o}$-coupled receptors (for NPY and adenosine A_1-selective agonists) directly stimulate IP production and augment $G_{q/11}$-coupled receptors (ATP or α_{1B}-adrenoceptor)-stimulated PLC activity and AA release ([14,32,33] and this report), (ii) $G_{i/o}$-coupled receptor augmentation of $G_{q/11}$-coupled receptor-mediated AA release is PKC-dependent, since such augmentation is sensitive to depletion of PKC by prolonged pretreatment with phorbol ester [14,32] and (iii) $G_{i/o}$-coupled receptor augmentation of both PLC activity and AA release is sensitive to expression of G$\beta\gamma$ scavengers (Tables 1–4). Although $G_{i/o}$-coupled receptor-stimulated MAPK activity can be inhibited by expression of G$\beta\gamma$ scavengers, this pathway is not dependent on PKC activity, and appears to be distinct from $G_{i/o}$-coupled receptor effects on PLC [40,41]. However, we cannot exclude a possible role for MAPK activity further downstream, since PKC has been shown to activate MAPK in some cases [42–44]. Interestingly, G_o α subunits have been suggested recently to mediate a PKC-dependent activation of MAPK activity [45]. However, since this pathway is not inhibited by expression of G$\beta\gamma$ scavengers, it is unlikely that such a mechanism contributes greatly to the G$\beta\gamma$-dependent effects of $G_{i/o}$-coupled receptors observed in our studies.

The release of AA and its metabolites precedes a number of physiological events thought to contribute to constriction. Extracellular release of AA and metabolites such as prostacyclin may result in cell-to-cell communication, amplifying a signal in a tissue. The intracellular actions of AA have also been documented, and shown to be implicated in the inhibition of myosin light-chain phosphatase and sensitization of smooth-muscle-cell myofilaments to calcium [46,47], in the modulation of G-protein-gated cardiac potassium channels acting on a G-protein in a receptor-independent fashion, which may involve G$\beta\gamma$ [38], and in the inhibition of (Na^+/K^+)ATPase [48]. Agents that can augment AA release, such as 5-hydroxytryptamine and NPY, have been shown to sensitize myofilaments via PLA_2 activation [49]. The ability of NPY to potentiate AA production, to potentiate angiotensin II-induced phosphorylation of myosin light chain in aortic smooth-muscle cells [50], and to cause increases in cytosolic calcium [15], together may result in increased sensitivity to local concentrations of calcium, allowing for more potent vasoconstriction, mediated by the co-stimulation of NPY and G-protein-coupled receptors for ATP, noradrenaline and angiotensin II.

In conclusion, we have demonstrated that NPY Y_1, 5-hydroxytryptamine $5\text{-}HT_{1B}$ and adenosine A_1 receptor stimulation can all result in augmentation of PLA_2 activity (as measured by AA release). The fact that these $G_{i/o}$-coupled receptors only influence AA levels when a $G_{q/11}$-coupled receptor is also activated, points to the requirement for a conditional activation, similar to that observed with certain adenylate cyclase isoforms. However, it remains to be established whether this receptor synergy generally reflects an interaction at the level of PLA_2 itself, or whether, as in the cells reported here, it is more consistent with 'cross-talk' interactions occurring earlier in the signal-transduction cascade,

and the need to achieve a certain threshold of response. Our data provide strong evidence that $G_{i/o}$ $\beta\gamma$ subunits are involved in this amplification of ATP-stimulated AA release and IP accumulation, and suggest that $G_{i/o}$ $\beta\gamma$ subunits act at $\beta\gamma$-sensitive isoforms of PLC to mediate NPY Y_1 receptor augmentation of IP production and subsequent AA release. The involvement of $G\beta\gamma$ subunits in such receptor synergy further suggests that the level of proteins in cells (e.g. βARK1) that possess pleckstrin homology $\beta\gamma$-binding domains [38] may have additional influences on cell signalling by determining to what extent these amplifying interactions are triggered.

This work was supported by the Wellcome Trust (Grant References 043029 and 046755) and Medical Research Council (G9405616MB). L.A.S. is the recipient of a Wellcome Trust Career Development Research Fellowship. We gratefully acknowledge the technical contribution of Jayson Bispham, and thank Dr. T.J. Biden for useful discussions and manuscript review and Dr. R. J. Lefkowitz for the contribution of the βARK1(495–689) minigene construct and specific antisera.

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Received 28 April 1997/11 July 1997; accepted 18 July 1997