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Involvement of G-protein βγ subunits in coupling the adenosine A_1 receptor to phospholipase C in transfected CHO cells

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Abstract

In transfected Chinese hamster ovary (CHO-A_1) cells the human adenosine A_1 receptor directly stimulates pertussis toxin-sensitive increases in inositol phosphate production and potentiates (synergistically) the inositol phosphate responses mediated by G_q-coupled $P2Y_2$ purinoceptor and CCK_A receptors. In the present study we have investigated the role of Gβγ subunits in mediating adenosine A_1 receptor effects on phospholipase C activation (both direct and synergistic) by transiently transfecting CHO-A_1 cells with a scavenger of Gβγ subunits: the C-terminus of β-adrenoceptor kinase 1 (βark1 residues 495–689). [^{3}H]inositol phosphate responses to the selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA; 1 μM) were inhibited (41 ± 1%) in CHO-A_1 cells transiently transfected with the Gβγ scavenger, βark1 (495–689). Expression of βark1 (495–689) protein was confirmed by Western blotting. In contrast, adenosine A_1 receptor-mediated inhibition of forskolin stimulated [^{3}H]cyclic AMP accumulation was unaffected by transient expression of βark1 (495–689). βark1 (495–689) expression had no significant effect on the [^{3}H]inositol phosphate responses produced by activation of the endogenous $P2Y_2$ purinoceptor (100 μM UTP; 92 ± 0.8% of control). [^{3}H]inositol phosphate accumulation in response to adenosine A_1 receptor activation was also attenuated in CHO-K1 cells co-transfected with the βark1 (495–689) minigene (59 ± 4% inhibition of control response to 1 μM CPA). Finally, transient expression of βark1 (495–689) in CHO-A_1 cells inhibited the augmentation of [^{3}H]inositol phosphate responses resulting from co-activation of adenosine A_1 receptors and $P2Y_2$ purinoceptors. These experiments indicate that Gβγ subunits are involved in the direct coupling the adenosine A_1 receptor to phospholipase C and that they also participate in the augmentation of $P2Y_2$ purinoceptor-mediated [^{3}H]inositol phosphate responses by the adenosine A_1 receptor.

Keywords: Adenosine A_1 receptor; Gβγ subunit; Phospholipase C; Chinese hamster ovary (CHO) cell

1. Introduction

Inositol lipid-specific phospholipase C isoenzymes hydrolyse phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) to generate the intracellular second messengers inositol 1,4,5 trisphosphate and 1,2-diacylglycerol (Lee and Rhee, 1995). To date, three distinct families of phospholipase C have been identified, phospholipase C-β, phospholipase C-γ and phospholipase C-δ, which differ markedly in their modes of regulation (Rhee and Bae, 1997). The β isoforms of phospholipase C (phospholipase C-β1-4) are regulated by receptors which couple to heterotrimeric G-proteins (Exton, 1996). For example, receptors coupling to the G_q-class of G-proteins (G_q, G_{11}, G_{14}, G_{15} and G_{16}) activate the β isoforms of phospholipase C via their respective (subunits, whereas the activation of phospholipase C-β through receptors which couple to the G_i/G_o family of inhibitory G-proteins (G_{i1}, G_{i2}, G_{i3} and G_o) has been reported to involve βγ subunits derived from these G_i/G_o proteins (Rhee, 1994; Exton, 1996; Rhee and Bae, 1997).

The adenosine A_1 receptor belongs to the seven transmembrane G-protein-coupled receptor superfamily and couples to the pertussis toxin-sensitive family of inhibitory G_i/G_o proteins (Olah and Stiles, 1995). The signal transduction pathways generally associated with this 'inhibitory' receptor include the inhibition of adenylyl cyclase activity, the closing of voltage sensitive Ca^{2+} channels and the opening of K^+ channels (Olah and Stiles, 1995).

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Adenosine A_1 receptor activation can also stimulate pertussis toxin-sensitive increases in inositol phospholipid hydrolysis and Ca^{2+} mobilisation (measures of phospholipase C activation) in a wide variety of cell types (Gerwins and Fredholm, 1992; Dickenson and Hill, 1993a; Iredale et al., 1994; Peakman and Hill, 1995). The sensitivity to pertussis toxin suggests that G_i/G_o proteins are involved in coupling the adenosine A_1 receptor to phospholipase C. Furthermore, in addition to directly activating phospholipase C, the adenosine A_1 receptor also potentiates the phospholipase C responses mediated by a range of G_q-coupled receptors in DDT_1MF-2 cells, FRTL-5 cells and RINm5F cells (Okajima et al., 1989a,b; Nazarea et al., 1991; Sho et al., 1991; Gerwins and Fredholm, 1992; Biden and Browne, 1993; Dickenson and Hill, 1993b). However, the molecular mechanism(s) by which the adenosine A_1 receptor directly activates phospholipase C and/or augments phospholipase C activation induced by G_q-coupled receptors are poorly understood (Dickenson and Hill, 1994).

In our previous studies we have investigated human adenosine A_1 receptor-mediated phospholipase C signalling in stably transfected Chinese hamster ovary (CHO-A_1) cells (Townsend-Nicholson and Shine, 1992). These studies have revealed that the transfected adenosine A_1 receptor stimulates pertussis toxin-sensitive increases in inositol phosphate accumulation and augments the inositol phosphate responses triggered via several endogenous G_q coupled receptors, for example, CCKA receptors, $P2Y_2$ purinoceptors and thrombin receptors (Megson et al., 1995; Dickenson and Hill, 1996, 1997). We and others have speculated that Gβγ subunits are involved in the direct coupling of the adenosine A_1 receptor to phospholipase C and in the augmentation of G_q-coupled receptor phospholipase C responses (Gerwins and Fredholm, 1992; Dickenson and Hill, 1996). In this study we have investigated the role of Gβγ subunits in mediating adenosine A_1 receptor effects on phospholipase C activation (both direct and synergistic) by transiently transfecting CHO-A_1 cells with a cellular scavenger of Gβγ subunits, namely the carboxyl-terminus of β-adrenoceptor kinase 1 (βark1 residues 495–689; Koch et al., 1994).

2. Materials and methods

2.1. cDNAs and expression vectors

Human adenosine A_1 receptor cDNA was purchased from the American Type Culture Collection and subcloned into the *Not*I/*Apa*I site of the expression vector pcDNA3 (Invitrogen). The pRK5 plasmid containing the carboxyl-terminal polypeptide of the β-adrenoceptor kinase 1 (termed the βark minigene) was a generous gift from Robert Lefkowitz. The βark minigene insert was removed from pRK5 using *Eco*RI and *Xba*I digestion and ligated into the *Eco*RI/*Xba*I site of pcDNA3.

2.2. Cell culture and cDNA transfection

Chinese hamster ovary cells transfected with the human adenosine A_1 receptor (CHO-A_1 cells) were a generous gift from Dr. Andrea Townsend-Nicholson and Professor John Shine, Garvan Institute, Sydney, Australia. Parent CHO-K1 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). CHO cells were routinely cultured in Dulbecco's modified Eagles medium (DMEM)/nutrient F12 (1:1) supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere and subcultured using trypsin (0.05% w/v)/EDTA (0.02% w/v). CHO cells were seeded at 2.6×10^6 cells/75 cm^2 flask 24 h prior to transient transfection using Lipofectamine (Life Technologies) according to the manufacture's instructions. Cells were washed once with Opti-MEM (Life Technologies) and then incubated for 5 h at 37°C in 9.2 ml of Opti-MEM containing a total of 40 μg of plasmid DNA and 50 μl of Lipofectamine. After 5 h the transfection mixture was replaced with 20 ml of normal growth medium and cells cultured for a further 24 h.

2.3. Accumulation of [^{3}H]inositol phosphates

Following 24 h transfection, cells from one 75 cm^2 flask were split into 24-well cluster dishes (Costar) and labelled with [^{3}H]*myo*-inositol (37 kBq/well) for 18 h in 500 μl/well inositol-free DMEM containing 1% (v/v) foetal calf serum. [^{3}H]inositol labelled cells were then washed once with 1 ml/well Hanks/HEPES buffer pH 7.4 and incubated at 37°C for 30 min in the presence of 20 mM LiCl (290 μl/well). Agonists were then added in 10 μl of medium and the incubation continued for 40 min (unless otherwise stated) at 37°C. Incubations were terminated by aspiration of the incubation medium and the addition of 900 μl cold (−20°C) methanol/0.12 M HCl (1:1 v/v). Cells were left a minimum of 2 h at −20°C before isolation of total [^{3}H]inositol phosphates in the supernatant of the disrupted cell monolayers by anion exchange chromatography. 800 μl aliquots of the supernatant were neutralised by the addition of 135 μl 0.5 M NaOH, 1 ml 25 mM Tris–HCl (pH 7.0) and 3.1 ml distilled water and added to columns of Dowex 1 anion exchange resin (X8, 100–200 mesh, chloride form). [^{3}H]inositol and [^{3}H]glycerophosphoinositol were removed with 20 ml of distilled water and 10 ml 25 mM ammonium formate, respectively. Total [^{3}H]inositol phosphates were then eluted with 3 ml of 1 M HCl and the columns regenerated with 10 ml 1 M HCl followed by 20 ml distilled water. Radioactivity was quantified by scintillation counting in the gel phase (scintillator plus, Packard).

2.4. Measurement of [^{3}H]cyclic AMP accumulation

Following 24 h transfection, cells from one 75 cm^2 flask were split and cultured for a further 18 h in 24-well cluster dishes before loading for 2 h at 37°C with 500 μl of Hanks/HEPES buffer (pH 7.4) containing [^{3}H]adenine (37 kBq/well). [^{3}H]adenine-labelled cells were washed once and then incubated in 1 ml/well Hanks/HEPES buffer containing the cyclic AMP phosphodiesterase inhibitor, rolipram (10 μM) for 15 min at 37°C. Agonists were added (in 10 μl of medium) 5 min prior to the incubation with 3 μM forskolin (10 min). Incubations were terminated by the addition of 50 μl concentrated HCl. [^{3}H]cyclic AMP was isolated by sequential Dowex-alumina chromatography as previously described (Donaldson et al., 1988). After elution, the levels of [^{3}H]cyclic AMP were determined by liquid scintillation counting.

2.5. Whole cell [^{3}H]DPCPX binding

Levels of expression of adenosine A_1-receptor in transient transfections were determined by measuring the specific binding of the A_1-receptor antagonist 8-cyclopentyl-[^{3}H]1,3-dipropylxanthine ([^{3}H]DPCPX) to intact CHO cells. Following 24 h transfection, cells from one 75 cm^2 flask were split and cultured for 18 h in 6-well cluster dishes. Cells were washed once and then incubated in 1 ml/well Hanks/HEPES buffer, pH 7.4, containing adenosine deaminase (1 unit/ml) in the presence (non-specific binding) or absence (total binding) of 5 mM theophylline for 30 min at 37°C. [^{3}H]DPCPX (final concentration 3 nM) was added in 10 μl of Hanks/HEPES buffer containing 0.05% Triton X-100 and incubated for 1 h at 37°C. Incubations were then terminated by aspiration of the incubation medium followed by one wash using 1 ml/well Hanks/HEPES and the addition of 0.5 ml/well of 0.5 M NaOH. Cells were then incubated for 10 min at 57°C before transferring the lysates to scintillation vial inserts containing 4 ml of Emulsifier-Safe scintillator (Packard) added. Levels of [^{3}H]DPCPX binding were determined by liquid scintillation counting.

2.6. Western blot analysis

Transfected cells (36 h) from 75 cm^2 flasks were solubilised in 3 ml of lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, pH 7.4) containing 1% (v/v) IGEPAL CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM Na_3VO_4, 1 mM NaF, 1 mM benzamidine, 0.1 mM phenylmethylsulphonylfluoride, 10 μg/ml leupeptin and 5 μg/ml aprotinin. After solubilization (4°C for 20 min) cell lysates were centrifuged at $10\,000 \times g$ for 10 min to remove any insoluble material. Protein determination were made using the method of Lowry et al. (1951) using bovine serum albumin as the standard and samples stored at −20°C until required.

Protein samples were separated by Sodium Dodecyl Sulphate/Polyacrylamide Gel Electrophoresis (SDS/PAGE; 10% acrylamide gel) using Bio-Rad Mini-Protean II system (1 h at 200 V). Proteins were transferred to nitrocellulose membranes using a Bio-Rad Trans-Blot system (1 h at 100 V in 25 mM Tris, 192 mM glycine and 20% MeOH). Following transfer, the membranes were washed with phosphate buffered saline (PBS) and blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in PBS. Blots were then incubated overnight at 4°C with primary antibodies in 5% (w/v) skimmed milk powder dissolved in PBS-Tween 20 (0.5 vol.%). The primary antibody was removed and the blot extensively washed with PBS/Tween 20. Blots were then incubated for 2 h at room temperature with the secondary antibody (swine anti-rabbit IgG coupled to horseradish peroxidase) at 1:1000 dilution in 5% (w/v) skimmed milk powder dissolved in PBS/Tween 20. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Enhanced Chemiluminescence detection system (Amersham).

2.7. Cell membrane preparation

Cells from 16 confluent 175 cm^2 flasks (sufficient to produce approximately 600 μg of membrane protein) were detached mechanically (using a cell scraper) into a Ca^{2+}/Mg^{2+} free phosphate buffered saline solution (80 mM Na_2HPO_4 100 mM NaCl, pH 7.5). After centrifugation (150 g for 5 min) cells were resuspended in 10 ml of ice-cold hypotonic Tris buffer (20 mM Tris/HCl, 2 nM EGTA, pH 7.4) which contained the following protease inhibitors 2 μg/ml soybean trypsin inhibitor, 3 mM benzamidine, 1 μM pepstatin, 1 μM leupeptin, 100 μM phenylmethylsulphonyl fluoride and 100 nM tosyl lysine chloromethyl ketone. Cells were then homogenised using a glass teflon homogeniser (approximately 20 strokes) and centrifuged at $500 \times g$ for 10 min to remove unbroken cells and nuclei. The supernatant was removed and centrifuged at $36\,000 \times g$ for 30 min. Following centrifugation, the membrane pellet was resuspended in hypotonic buffer to a final protein concentration of 4–6 mg/ml and stored at −80°C until required.

2.8. Phospholipase C assay

Phospholipase C activity in membrane fractions from CHO cells was assayed using exogenous radiolabelled substrate. Mixed phospholipid vesicles containing phosphatidylethanolamine (0.7 μl/assay tube from 10 mg/ml stock solution in $CHCl_3$), PtdIns(4,5)P_2 (1.1 μl/assay tube from 1 mg/ml stock solution in $CHCl_3$) and [^{3}H]PtdIns(4,5)P_2 (0.37 kBq/assay tube) were prepared by drying the lipids under a stream of N_2 and resuspending them in an appropriate volume (20 μl/assay tube) of

reaction mixture buffer (87.5 mM Tris/maleate, pH 7.0, 17.5 mM LiCl, 17.5 mM 2,3 bisphosphoglycerate, 5.25 mM EGTA, 8.75 mM $MgCl_2$ and 0.087% (mass/vol) sodium deoxycholate) followed by sonication for 30 min. Assays were performed for 30 min at 25°C in a total volume of 35 μl which consisted of 5 μl membrane or soluble protein (usually 10 μg of protein in 20 mM Tris/HCl buffer and protease inhibitors); 20 μl lipid substrate mixture; 5 μl βγ subunits (in 10 mM Tris/HCl, pH 7.5, 6 mM $MgCl_2$, 1 mM dithiothreitol, 20 vol.% glycerol, 100 μl phenylmethylsulphonyl fluoride) and 5 μl of $CaCl_2$ to give the indicated free Ca^{2+} concentrations (calculated using the computer programme EqCal). The reaction was stopped by adding 175 μl of $CHCl_3/CH_3OH/$ concentrated HCl (500:500:3, by vol), vortexing and adding 50 μl of 1 M HCl containing 5 mM EGTA. Phase separation was accelerated by centrifugation for 1 min in an Eppendorf microcentrifuge. A 100 μl aliquot of the upper aqueous phase (containing tritiated products) was removed and radioactivity quantified by counting in the gel phase (scintillator 299, Packard).

2.9. Data analysis

pEC_{50} ($-\log EC_{50}$; concentration of drug producing 50% of the maximal response) values were obtained by computer assisted curve fitting by use of the computer programme Prism (GraphPAD, CA, USA). Statistical significance was determined by Student's unpaired *t* test ($P < 0.05$ was considered statistically significant). All data are presented as mean ± S.E.M. The *n* in the text refers to the number of separate experiments.

2.10. Chemicals

[2-^{3}H]*myo*-inositol and [2,8-^{3}H]adenine were supplied by Amersham International (Aylesbury, Bucks). 8-cyclopentyl-[^{3}H]1,3-dipropylxanthine ([^{3}H]DPCPX) and inositol-2-[^{3}H]phosphatidyl-inositol 4,5-biphosphate were from New England Nuclear (Stevenage, Hertfordshire, UK). 2,3-biphosphoglycerate, phosphatidylethanolamine, soybean trypsin inhibitor, benzamidine, pepstatin, aprotinin, leupeptin, phenylmethylsulphonyl fluoride, tosyl lysine chloromethyl ketone, N^6-cyclopentyladenosine (CPA) and forskolin were from Sigma Chemical (Poole, Dorset, UK). Lipofectamine reagent, Opti-MEM and geneticin (G-418) were from Gibco BRL (Life Technologies). Dulbecco's modified Eagles Medium/Nutrient Mix F-12 (1:1) and foetal calf serum were from Sigma Chemical. Specific rabbit polyclonal antibodies to rat brain phospholipase C β1, human phospholipase C β2 and rat brain phospholipase C β3 were purchased from Santa Cruz Biotechnology, CA, 95060, USA. βark minigene specific antisera was kindly provided by Dr. R. Lefkowitz. βγ subunits from bovine rod outer segments were a generous gift from Dr. P. Gierschik. All other chemicals were of analytical grade.

3. Results

3.1. Effect of βark minigene expression on adenosine A_1 receptor-mediated inositol phosphate production

In order to investigate the role of Gβγ subunits in mediating adenosine A_1 receptor effects on phospholipase C activation (both direct and synergistic) we transiently transfected CHO cells with the pleckstrin homology domain of β-adrenoceptor kinase 1 (residues 495–689) which functions as a Gβγ scavenger (Koch et al., 1994). Expression of βark1-(495–689) was confirmed by Western blotting using specific antisera (Fig. 1).

The effect of βark1-(495–689) expression on adenosine A_1 receptor-mediated inhibition of [^{3}H]cyclic AMP accumulation and stimulation of [^{3}H]inositol phosphate production was initially explored in CHO cells stably transfected with the human adenosine A_1 receptor cDNA (CHO-A_1 cells; Townsend-Nicholson and Shine, 1992). As shown in Fig. 2a, transient expression of βark1-(495–689) significantly inhibited the accumulation of [^{3}H]inositol phosphates elicited by the selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA). For example, the [^{3}H]inositol phosphate response to 1 μM CPA was inhibited by $41.4 \pm 1.2\%$ ($n = 3$; $P < 0.05$) relative to the response obtained with 1 μM CPA in cells transfected with the control pcDNA3 vector. The pEC_{50} for CPA did not differ significantly between the control vector transfected ($pEC_{50} = 8.07 \pm 0.03$; $n = 3$) and βark1 (495–689) transfected ($pEC_{50} = 7.81 \pm 0.09$; $n = 3$) cells. βark1-

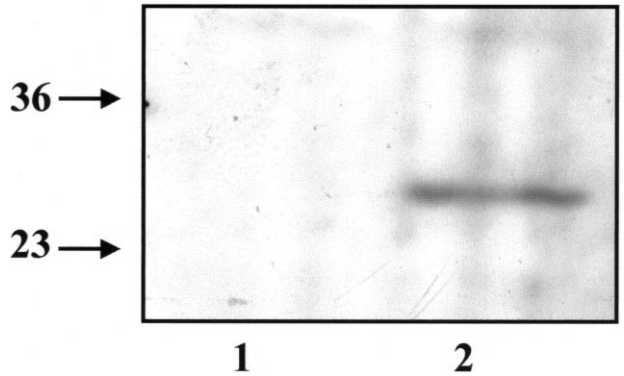

Fig. 1. Expression of βark1-(495–689) minigene in transfected CHO cells. Cell lysates (20 μg protein) from CHO-A_1 cells transfected with pcDNA3/βark1-(495–689) or empty expression vector were resolved by SDS-PAGE and transferred to nitrocellulose membranes before being probed with antisera specific for the βark1-(495–689) minigene (1:5000 dilution). A band representing the βark1-(495–689) minigene (circa 27 kDa; lane 2) was obtained indicating the expression of βark1 minigene protein in CHO cells. Lane 1 represents CHO cells transfected with control vector alone. Positions of standard molecular-mass markers of 36 and 23 kDa are shown on the left.

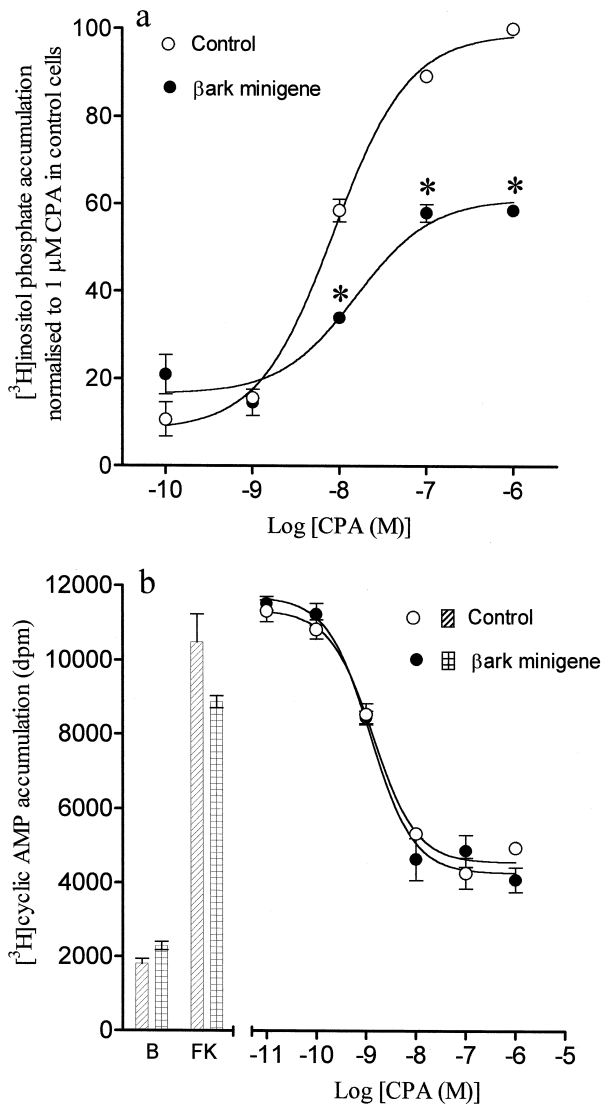

Fig. 2. Effect of transient expression of βark1-(495–689) minigene on adenosine A_1 receptor-mediated accumulation of [^{3}H]inositol phosphates and inhibition of forskolin-stimulated [^{3}H]cyclic AMP accumulation in CHO-A_1 cells. Concentration–response curves for CPA-mediated [^{3}H]inositol phosphate accumulation (panel a) and inhibition of forskolin stimulated [^{3}H]cyclic AMP accumulation (panel b) in CHO-A_1 cells transiently transfected with 40 μg pcDNA3 (control cells) or 40 μg pcDNA3/βark1-(495–689). In (a) data are expressed as a percentage of the response to1 μM CPA in control cells.Values represent mean ± S.E.M. obtained from three independent experiments each performed in triplicate. ∗Significant ($P < 0.05$, Student's *t*-test) difference from response to CPA obtained in control cells. In (b) cells were pre-stimulated for 5 min with the various concentrations of CPA before stimulating with 3 μM forskolin for 10 min in the continued presence of agonist. The histograms show basal (B) and forskolin stimulated [^{3}H]cyclic AMP accumulation (FK). Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in two further experiments.

(495–689) acts as a specific cellular inhibitor of Gβγ-dependent signalling and therefore phospholipase C responses mediated via G_q-coupled receptors should be unaffected. The specificity of βark1-(495–689) in our experiments was confirmed by measuring [^{3}H]inositol phosphate accumulation in response to activation of the G_q-linked $P2Y_2$ purinoceptor (Iredale and Hill, 1994; Megson et al., 1995). [^{3}H]inositol phosphate accumulation in response to UTP (100 μM) was not affected by expression of the

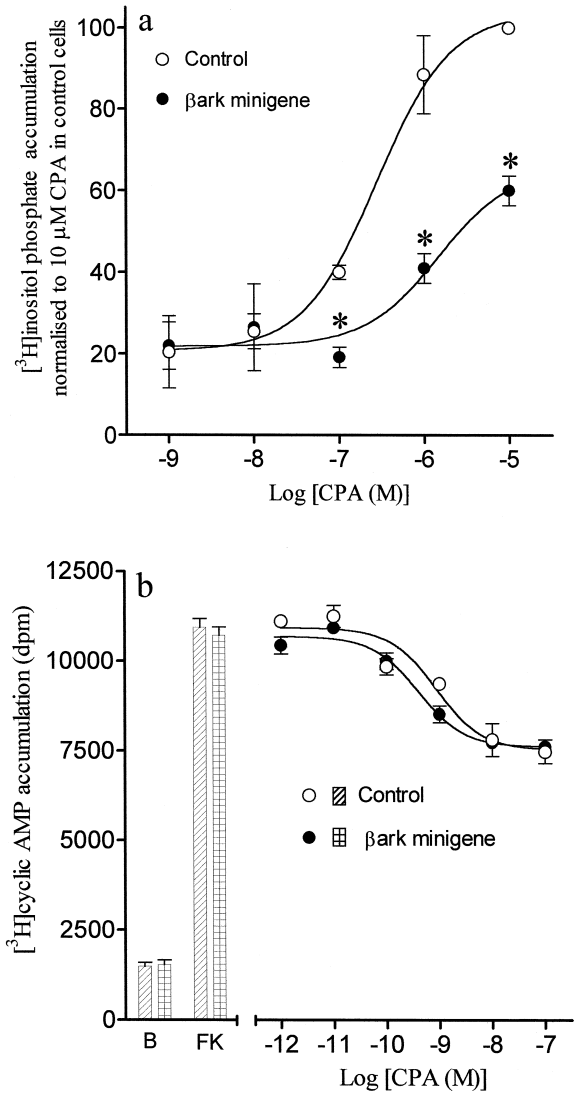

Fig. 3. Effect of βark1-(495–689) minigene expression on adenosine A_1 receptor-mediated accumulation of [^{3}H]inositol phosphates and inhibition of forskolin-stimulated [^{3}H]cyclic AMP accumulation in co-transfected CHO-K1 cells. Concentration–response curves for CPA-mediated [^{3}H]inositol phosphate accumulation (panel a) and inhibition of forskolin stimulated [^{3}H]cyclic AMP accumulation (panel b) in CHO-K1 cells transiently co-transfected with plasmid (pcDNA3) DNA containing human adenosine A_1 receptor (5 μg) and either pcDNA3 alone (35 μg; control cells) or 35 μg pcDNA3/βark1-(495–689). In (a) data are expressed as a percentage of the response to 1 μM CPA in control cells.Values represent mean (S.E.M. obtained from four independent experiments each performed in triplicate. ∗Significant ($P < 0.05$, Student's *t*-test) difference from response to CPA obtained in control cells. In (b) cells were pre-stimulated for 5 min with the various concentrations of CPA before stimulating with 3 μM forskolin for 10 min in the continued presence of agonist. The histograms show basal (B) and forskolin stimulated [^{3}H]cyclic AMP accumulation (FK). Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in three further experiments.

βark1-(495–689) minigene (92.5 ± 7.9% of control UTP response; $n = 3$).

The specificity of βark1-(495–689) expression was examined further by investigating its effects on adenosine A_1 receptor-mediated inhibition of forskolin stimulated [^{3}H]cyclic AMP accumulation. As shown in Fig. 2b, CPA-induced inhibition of adenylyl cyclase (a $G_i\alpha$-mediated event) was unaffected by transient expression of βark1-(495–689). In these experiments, the maximally effective concentration of CPA (1 μM) inhibited forskolin (3 μM) responses by 62 ± 6% ($n = 3$) in βark1 (495–689) transfected cells and 59 ± 5% ($n = 3$) in control cells (transfected with vector alone). Furthermore, the pEC_{50} for CPA did not differ significantly between the control vector transfected ($pEC_{50} = 8.95 \pm 0.07$; $n = 3$) and βark1-(495–689) transfected (pEC_{50} 8.92 ± 0.05; $n = 3$) cells. These results demonstrate that expression of the Gβγ scavenger βark1-(495–689) in CHO-A_1 cells specifically inhibits adenosine A_1 receptor-induced phospholipase C activation.

We also assessed the effects of βark1 (495–689) minigene expression on adenosine A_1-receptor cell signalling in CHO-K1 cells transiently co-transfected with human adenosine A_1 receptor cDNA and either pcDNA3 vector (control cells) or pcDNA3 βark1-(495–689) DNA. The level of adenosine A_1-receptor expression in transient transfections was determined by specific binding of the A_1-receptor antagonist [^{3}H]DPCPX. Specific adenosine A_1 receptor expression (in fmol receptor/mg membrane protein) was 69 ± 9 ($n = 4$) and 76 ± 8 ($n = 4$) in control and βark1-(495–689) expressing cells, respectively. CPA elicited concentration-dependent increases in the accumulation of [^{3}H]inositol phosphates ($pEC_{50} = 6.43 \pm 0.22$; $n = 4$) in CHO-K1 cells transiently transfected with the human adenosine A_1 receptor cDNA (Fig. 3a). Co-expression of βark1-(495–689) significantly inhibited CPA-induced [^{3}H]inositol phosphate responses (59 ± 3.6% inhibition of the response to 1 μM CPA; $n = 4$; $P < 0.05$). In these experiments the pEC_{50} for CPA was 5.60 ± 0.29 ($n = 4$). The response to UTP (100 μM), in these co-transfected CHO-K1 cells, was not affected by expression of the βark1-(495–689) minigene (134 ± 15% of control UTP response; $n = 4$).

Finally, as shown in Fig. 3b, CPA-mediated inhibition of adenylyl cyclase was unaffected by co-expression of βark1-(495–689). The maximally effective concentration of CPA (1 μM) inhibited forskolin (3 μM) responses by 25 ± 6% ($n = 4$) in βark1-(495–689) co-transfected cells and 28 ± 5% ($n = 4$) in pcDNA3 vector control cells. Furthermore, the pEC_{50} for CPA did not differ significantly between cells co-transfected with either pcDNA3 vector ($pEC_{50} = 9.88 \pm 0.34$; $n = 4$) or pcDNA3-βark1-(495–689) DNA ($pEC_{50} = 8.95 \pm 0.23$; $n = 4$).

3.2. Effect of βark minigene expression on adenosine A_1 receptor-mediated augmentation of $P2Y_2$ purinoceptor stimulated inositol phosphate production

Adenosine A_1 receptor stimulation has previously been shown to potentiate $P2Y_2$ purinoceptor-stimulated [^{3}H]inositol phosphate accumulation in CHO-A_1 cells (Megson et al., 1995). In this study we have investigated whether the enhancement of $P2Y_2$ purinoceptor-mediated phospholipase C signalling by the A_1 receptor involves Gβγ subunits. Co-stimulation of CHO-A_1 cells with CPA (1 μM) and either 1, 10 or 100 μM UTP produced synergistic increases in [^{3}H]inositol phosphate accumulation, similar to those observed previously (Megson et al., 1995; Table 1). Transient expression of βark1-(495–689) significantly reduced the adenosine A_1 receptor-mediated augmentation of UTP-induced [^{3}H]inositol phosphate responses (Table 1). In these experiments the responses to UTP alone were unaffected by transient expression of βark1-(495–689). These data suggest a role for Gβγ subunits in the adenosine A_1 receptor-mediated augmenta-

Table 1
Effect of transient expression of βark1-(495–689) minigene on adenosine A_1 receptor-mediated augmentation of UTP-stimulated [^{3}H]inositol phosphate accumulation

	pcDNA3	pcDNA3/βark1-(495–689)
100 μM UTP	100	103 ± 10
10 μM UTP	109 ± 6	117 ± 11
1 μM UTP	48 ± 4	50 ± 6
1 μM CPA	46 ± 3	27 ± 2a
100 μM UTP and 1 μM CPA	271 ± 14 (146 ± 3)	176 ± 9[a] (130 ± 10)
10 μM UTP and 1 μM CPA	287 ± 9 (155 ± 7)	195 ± 12[a] (144 ± 11)
1 μM UTP and 1 μM CPA	159 ± 8 (94 ± 5)	69 ± 5[a] (77 ± 6)

CHO-A_1 cells were transiently transfected with 40 μg pcDNA3 (control cells) or 40 μg pcDNA3/βark1-(495–689). [^{3}H]inositol phosphate accumulation was measured in response to UTP alone (100 μM, 10 μM and 1 μM), CPA alone (1 μM) or a combination of UTP and CPA. Data are expressed as a percentage of the response to 100 μM UTP in control cells (pcDNA3 alone). Values represent mean ± S.E.M. obtained from four independent experiments each performed in triplicate.

[a]Significantly ($P < 0.05$, Student's *t*-test) different from control responses. The values in parenthesis represent the predicted additive responses to CPA and UTP and were calculated by adding the response obtained with CPA alone (i.e., 46 ± 3%) to that obtained with the appropriate concentration of UTP alone (i.e., 10 μM = 109 ± 6% and therefore the predicted additive response would be 155 ± 7%).

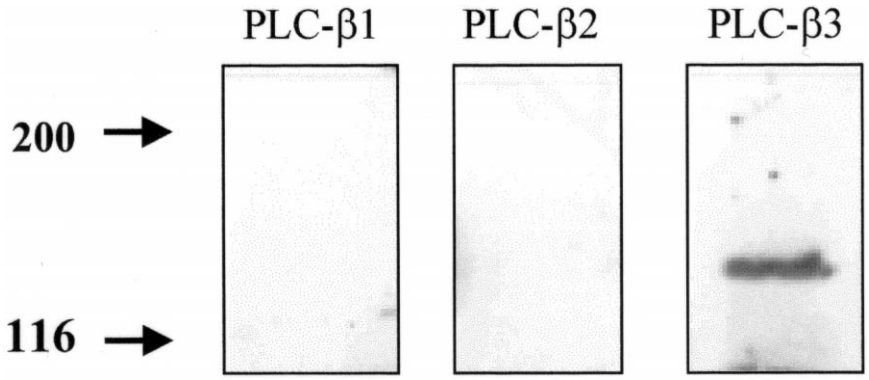

Fig. 4. Western blot analysis of phospholipase C β isoforms expressed in CHO cells. Cell lysates (50 μg protein) were resolved by SDS-PAGE and transferred to nitrocellulose membranes before being probed with specific rabbit polyclonal antibodies to rat brain phospholipase C β1 (1:1000 dilution), human phospholipase C β2 (1:1000 dilution) and rat brain phospholipase C β3 (1:1000 dilution). Positions of standard molecular mass markers of 200 and 116 kDa are shown on the left.

tion of $P2Y_2$ purinoceptor-stimulated [^{3}H]inositol phosphate responses.

3.3. βγ-dependent stimulation of phospholipase C

The data presented so far indicates that transient expression of the Gβγ scavenger, βark1-(495–689), can attenuate adenosine A_1 receptor-mediated phospholipase C signaling in CHO-A_1 cells. These results suggest that CHO cells express phospholipase C isoforms that are sensitive to activation by Gβγ subunits (namely phospholipase C isoforms β1, β2 and β3). Therefore, we screened CHO cells for the presence of phospholipase C β isoforms and determined whether purified Gβγ subunits stimulate phospholipase C activity in CHO cell membranes. Western blot analysis using specific phispholipase C β isoform antibodies revealed the presence of phospholipse C β3 in cell lysates derived from CHO cells (Fig. 4). Furthermore, as shown in Fig. 5, Gβγ subunits purified from bovine retinal transducin produced significant increases in phospholipase C activity in CHO-A_1 cell membranes.

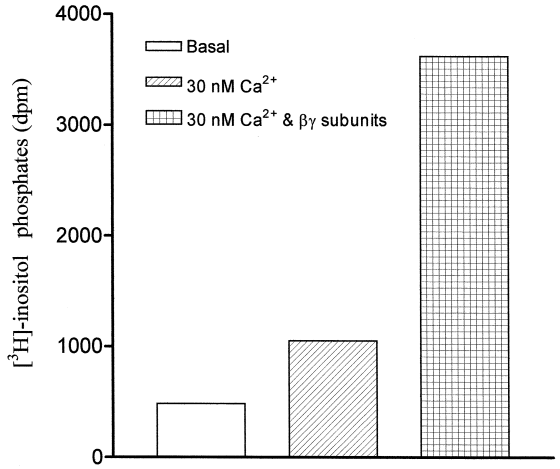

Fig. 5. Stimulation of phospholipase C by G protein βγ subunits in CHO-A_1 cells. Membrane protein (10 μg/assay) was incubated in the presence of 30 nM free Ca^{2+} or 30 nM free Ca^{2+} and 1.9 μM βγ subunits. Data represent the mean ± S.E.M. of three independent experiments each measured in duplicate.

4. Discussion

The βark1 (495–689) minigene has been used effectively by numerous research groups to identify the involvement of Gβγ subunits in mediating intracellular cell signalling responses (Koch et al., 1994; Herrlich et al., 1996; Dorn et al., 1997). In the present study we have used the βark1 (495–689) minigene to investigate the role of Gβγ subunits in mediating human adenosine A_1 receptor stimulation of phospholipase C in transfected CHO cells. In our previous studies we have demonstrated that the transfected human adenosine A_1 receptor stimulates pertussis toxin-sensitive increases in [^{3}H]inositol phosphate accumulation and augments the [^{3}H]inositol phosphate responses elicited by several endogenous Gq-coupled receptors (Megson et al., 1995; Dickenson and Hill, 1996, 1997). In this study we have shown that transient expression of the βark1 (495–689) minigene (Gβγ scavenger) markedly reduced adenosine A_1 receptor-mediated phospholipase C activation in CHO-A_1 cells. These data strongly support our notion that βγ subunits are involved in the direct coupling of the adenosine A_1 receptor to phospholipase C in CHO-A_1 cells (Dickenson and Hill, 1996). In marked contrast, adenosine A_1 receptor-mediated inhibition of adenylyl cyclase and $P2Y_2$ purinoceptor stimulation of phospholipase C (presumably via G_q) were not affected by βark1 (495–689) expression. The lack of effect of the βark1 minigene construct on adenosine A_1 receptor-mediated inhibition of adenylyl cyclase suggests the exclusive involvement of $G_i\alpha$ subunits. It is known that type I adenylyl cyclase is sensitive to inhibition by Gβγ subunits, however, this isoform is expressed only in neurons (Sunahara et al.,

1996). Furthermore, it has been reported that cellular expression of the βark1 minigene construct did not affect G_i-coupled receptor-mediated inhibition of adenylyl cyclase type 1 expressed in HEK 293 cells (Nielson et al., 1996).

It seems likely that the direct activation of phospholipase C by the adenosine A_1 receptor will involve the β isoforms of phospholipase C, which are sensitive to βγ subunits (Rhee, 1994; Exton, 1996; Rhee and Bae, 1997). In support of this, we have shown that CHO cell express the β3 isoform of phospholipase C and that CHO cell membranes contain phospholipase C activity which can be directly stimulated by purified βγ subunits.

A notable feature of the data presented in this study is that the EC_{50} for CPA shifted to approximately 10-fold higher concentrations in cells transiently co-transfected with both the adenosine A_1 receptor and the βark1 minigene construct (see Fig. 3a). In contrast, there was no difference in the EC_{50} for CPA in cells stably expressing the adenosine A_1 receptor and then transiently transfected with the βark1 minigene construct (see Fig. 2a). It is important to note, the approximately only 50% of CHO cells are transfected under the conditions used in this study (based on observable β-galactosidase activity). In cells stably expressing the adenosine A_1 receptor which are then transiently transfected with the βark1 minigene construct it is likely that only 50% of cells will actually express the βark1 fragment. This may explain why there was no significant difference between the EC_{50} values for CPA (pEC_{50}'s of 8.07 compared to 7.81). In contrast, the transient co-transfection of CHO cells will produce a larger proportion of cells expressing both the adenosine A_1 receptor and the βark1 minigene construct. This approach should remove the problem of measuring responses to CPA in cells that do not express the βark1 minigene construct (as is the case in cells stably expressing the adenosine A_1 receptor and then transient transfected with the βark1 minigene construct) and probably explains the observed shift in the EC_{50} value for CPA. Furthermore, the EC_{50} for CPA-induced inositol phosphate accumulation (in the absence of the βark1 minigene construct) differed between CHO cells stably ($pEC_{50} = 8.07$) and transiently transfected ($pEC_{50} = 6.43$) with the adenosine A_1 receptor. This difference in the EC_{50} value for CPA may reflect the difference in adenosine A_1 receptor number between the stably transfected (200 fmol/mg of protein; Iredale et al., 1994) and transiently transfected cells (circa 70 fmol/mg of protein).

In addition to the direct effect of adenosine A_1 receptor activation on [^{3}H]inositol phosphate accumulation in CHO-A_1 cells we have previously demonstrated that adenosine A_1 receptor stimulation can augment the [^{3}H]inositol phosphate responses triggered by thrombin, $P2Y_2$ purinoceptors and CCK_A receptors in these cells (Megson et al., 1995; Dickenson and Hill, 1996; Dickenson and Hill, 1997). Furthermore, we have postulated that βγ subunits released from G_i/G_o proteins are responsible for the synergistic increases in [^{3}H]inositol phosphate accumulation in these cells. In the present study we have shown for the first time that the synergistic interaction between CPA and UTP stimulated [^{3}H]inositol phosphate accumulation can be attenuated by transient expression of the Gβγ scavenger, βark1 (495–689). These data provide evidence for the involvement of βγ subunits in mediating the synergistic activation of phospholipase C by the adenosine A_1 receptor and the $P2Y_2$ purinoceptor in CHO-A_1 cells. The synergistic stimulation of phospholipase C responses by G_i and G_q-protein coupled receptors has been observed in a variety of cells including FRTL-5 thyroid cells, NG108-15 cells and DDT1MF-2 cells (Okajima et al., 1989a,b; Nazarea et al., 1991; Sho et al., 1991; Gerwins and Fredholm, 1992; Biden and Browne, 1993; Dickenson and Hill, 1993b). Hence, the mechanism(s) underlying the synergy observed in these cells may also involve βγ subunits. However, exactly how βγ subunits modulate the activity of phospholipase C (leading to synergistic increases in [^{3}H]inositol phosphate accumulation) is still unclear.

The β isoforms of phospholipase C (phospholipase C-β1-3) are regulated by the α subunits belonging to the G_q class of G-proteins (predominantly $G\alpha_q$ and $G\alpha_{11}$) and by G-protein βγ subunits (Exton, 1996; Rhee and Bae, 1997). Therefore, one possible mechanism may involve the dual regulation of phospholipase C β isoforms by $G\alpha_q$ and Gβγ subunits. Indeed, evidence in the literature indicates that the β isoforms of phospholipase C are sensitive to co-activation by G-protein α and βγ subunits. For example, $G\alpha_q$ and Gβγ synergistically activated purified rat brain phospholipase C-β3 (Smrcka and Sternweis, 1993) and co-expression of $G\alpha_{16}$ and $G\beta_1\gamma_1$ in COS-7 cells synergistically activated recombinant phospholipase C-β2 (Wu et al., 1993). Furthermore, βγ subunits potentiated $P2Y_2$ purinoceptor activation of phospholipase C in turkey erythrocyte membrane preparations (Boyer et al., 1989). Therefore, dual activation of phospholipase C-β isoforms by G_q and Gβγ subunits may account for synergistic stimulation of phospholipase C responses by G_i and G_q-protein coupled receptors.

In conclusion, we have shown for the first time that Gβγ subunits are involved in the direct coupling of the human adenosine A_1 receptor to phospholipase C in CHO-A_1 cells. Furthermore, our data suggest that G_i/G_o protein derived βγ subunits are also involved in the adenosine A_1 receptor-mediated potentiation of G_q-coupled receptor stimulated phospholipase C responses.

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