0026-895X/01/5906-1523–1532$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 59:1523–1532, 2001

Vol. 59, No. 6
781/908121
Printed in U.S.A.

ADP-Ribosylation Factor-Dependent Phospholipase D Activation by VPAC Receptors and a PAC_1 Receptor Splice Variant

DEREK A. MCCULLOCH,[1] EVE M. LUTZ,[2] MELANIE S. JOHNSON, DEREK N. ROBERTSON, CHRIS J. MACKENZIE,[3] PAMELA J. HOLLAND, and RORY MITCHELL

Medical Research Council Membrane and Adapter Proteins Co-operative Group, Membrane Biology Group, Department of Biomedical Sciences, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh, United Kingdom

Received December 27, 2000; accepted February 28, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The $VPAC_1$ and $VPAC_2$ receptors for vasoactive intestinal polypeptide and the PAC_1 receptor for pituitary adenylate cyclase-activating polypeptide are members of a subfamily of G protein-coupled receptors (GPCRs). We recently reported that phospholipase D (PLD) activation by members of the rhodopsin group of GPCRs occurs by at least two routes, one of which seems to involve the small G protein ADP-ribosylation factor (ARF) and its physical association with GPCRs. Here we report that rat VPAC and PAC_1 receptors can also stimulate PLD (albeit less potently than adenylate cyclase) in transfected cells and also in cells where they are natively expressed. PLD responses of the VPAC receptors and the hop1 spice variant of the PAC_1 receptor but not its null form are sensitive to brefeldin A (BFA), an inhibitor of GTP exchange at ARF. The presence of the hop1 cassette in the rat PAC_1 receptor facilitates PLD activation in the absence of marked changes in ligand binding, receptor internalization, and adenylate cyclase activation, with some reduction in phospholipase C activation. Both $VPAC_2$ and $PAC_{1\text{-hop1}}$ (but not $PAC_{1\text{-null}}$) receptors were shown to associate with immunoprecipitates directed against native or epitope-tagged ARF. A chimeric construct of the $VPAC_2$ receptor body with intracellular loop 3 (i3) of the $PAC_{1\text{-null}}$ receptor mediated BFA-insensitive activation of PLD, whereas the response of the corresponding $PAC_{1\text{-hop1}}$ construct was BFA-sensitive. Motifs in i3 of the $PAC_{1\text{-hop1}}$ receptor may act as critical determinants of coupling to ARF-dependent PLD activation by contributing to the GPCR:ARF interface.

G protein-coupled receptors (GPCRs) have been classified into a number of different families, according to functional criteria or to sequence homology (Probst et al., 1992; Ji et al., 1998). One of the distinct families of GPCRs is that for large peptide hormones such as secretin, parathyroid hormone, glucagon, and glucagon-like peptide 1 (GLP-1). These receptors, which include the $VPAC_1$ and $VPAC_2$ receptors (for VIP/PACAP) and the PAC_1 receptor (selective for PACAP) retain the architecture of seven transmembrane helices and the general principles of signal transduction common to all GPCRs (Segre and Goldring, 1993; Harmar and Lutz, 1994; Arimura and Shioda, 1995; Donnelly, 1997). The rat $VPAC_1$ and $VPAC_2$ receptors were cloned by Ishihara et al. (1992) and Lutz et al. (1993) and were shown to activate adenylate cyclase (AC) and thereby raise intracellular cAMP levels (Ishihara et al., 1992; Lutz et al., 1993). In some studies the $VPAC_1$ and $VPAC_2$ receptors have also been shown to elicit small inositol phosphate responses (MacKenzie et al., 1996; van Rampelbergh et al., 1997). The closely related PAC_1 receptor (Arimura and Shioda, 1995) was cloned independently by six different laboratories (Hashimoto et al., 1993; Hosoya et al., 1993; Morrow et al., 1993; Pisegna and Wank, 1993; Spengler et al., 1993; Svoboda et al., 1993). The PAC_1 receptor couples to the activation of AC and phospholipase C (PLC) (Hashimoto et al., 1993; Hosoya et al., 1993; Morrow et al., 1993; Pisegna and Wank, 1993; Spengler et al., 1993; Svoboda et al., 1993), and exists in at least six splice variants, a short form, $PAC_{1\text{-null}}$, and five variants

This work was supported by the Medical Research Council (United Kingdom).

[1] Present address: Kennedy Institute of Rheumatology, 1 Aspenlea Rd., Hammersmith, London, UK.

[2] Present address: Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow, UK.

[3] Present address: Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, UK.

ABBREVIATIONS: GPCR, G protein-coupled receptor; GLP-1, glucagon-like peptide 1; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; AC, adenylate cyclase; PLC, phospholipase C; i3, intracellular loop 3; PLD, phospholipase D; CHO, Chinese hamster ovary; NCS, newborn calf serum; tm, transmembrane domain; PCR, polymerase chain reaction; BSA, bovine serum albumin; EBSS, Earle's balanced salt solution; InsP, inositol phosphate; PtdBut, phosphatidylbutanol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; PEG polyethylene glycol 8000; HA, hemagglutinin; PBS, phosphate-buffered saline; BFA, brefeldin A.

with extra amino acid inserts in intracellular loop 3 (i3), including the $PAC_{1\text{-}hop1}$ variant investigated here (Spengler et al., 1993). Splice variants of both the rat and human PAC_1 receptors may activate PLC with differing efficiency (Spengler et al., 1993; Pisegna and Wank, 1996). The widespread importance of i3 in the coupling of GPCRs to guanine nucleotide-binding (G) proteins has been well established by many studies involving mutant and chimeric receptor constructs (Wess, 1997).

The activation of phospholipase D (PLD) has been implicated in many key physiological processes (Exton, 1997) but has been little investigated for receptors of the secretin/parathyroid hormone receptor family. We report for the first time the stimulation of PLD by the $VPAC_1$, $VPAC_2$, $PAC_{1\text{-}null}$, and $PAC_{1\text{-}hop1}$ receptors at nanomolar concentrations of VIP/PACAP that could be physiologically relevant. Similar responses to those in transfected cells are seen in cells natively expressing $VPAC_2$ and PAC_1 receptors. Furthermore, we provide evidence that the hop-1 splicing insert in i3 of the PAC_1 receptor selectively facilitates receptor coupling to ARF-dependent PLD activation, but not other signaling pathways, and enables coimmunoprecipitation of the receptor with ARF (which could also be observed with the $VPAC_2$ receptor).

Experimental Procedures

Materials

All tissue culture media, including animal serum, geneticin, penicillin, and streptomycin were obtained from Life Technologies, Irvine, UK. Radiochemicals; [^{125}NaI], ^{125}I-PACAP-27, myo-[2-^{3}H]inositol, and [9,10-^{3}H]palmitic acid were obtained from PerkinElmer Life Science Products, Dreiech, Germany. All peptides were from Novabiochem, Nottingham, UK, and all biochemicals were from Sigma, Poole, UK, unless otherwise stated. Any reagents with relatively lower aqueous solubility (such as brefeldin A or U 73122) were added to cell signaling assays from solutions in dimethylformamide. Corresponding additions of vehicle were made to control and test wells and were limited to a concentration of 0.3% (at which level no effects could be detected on any of the responses).

Generation of Stable Chinese Hamster Ovary (CHO) Cell Lines

cDNAs encoding the rat $VPAC_1$, $VPAC_2$, $PAC_{1\text{-}null}$, and $PAC_{1\text{-}hop1}$ receptors were introduced into the expression vector pcDNA1, containing the neomycin resistance gene (Invitrogen BV, Groningen, The Netherlands). CHO cells were transfected with the receptor plasmids using lipofectamine (Life Technologies). Forty-eight hours after transfection, geneticin (500 μg/ml) was added to cells grown in Ham's F-12 nutrient media with 10% NCS, 100 U/ml penicillin, and 100 μg/ml streptomycin to select for cells expressing constructs. Clonal cells were picked and growth was continued for 1 month in geneticin-containing media. Clonal lines expressing the different receptors were selected for their ability to stimulate cAMP production in response to VIP, PACAP, and other VIP-like peptides. The expression of the mRNA for the various receptors was also confirmed by Northern analysis (data not shown).

Construction of Chimeric Receptors

Chimeric receptors were made by replacement of the i3 domain of the rat $VPAC_2$ receptor with the i3 domains from either the short form or the hop-1 form of the rat PAC_1 receptor. Exchange sites were within transmembrane domain (tm) 5 and tm7 (Fig. 4). This was achieved using cDNAs encoding the rat $VPAC_2$ receptor (R4, pBluescript) and the null (R7b, pBluescript) and hop1 (R7/9.1, pBluescript) splice variants of the rat PAC_1 receptor. The first domain exchange was made by using a conserved restriction (*Hin*cII) site in the region of the cDNAs encoding the fifth transmembrane region of the $VPAC_2$ and PAC_1 receptors. After digestion with *Hin*cII, the appropriate cDNA fragments were gel purified and then ligated with T4 DNA ligase (Promega, Southampton, UK). These were inserted into pBluescript for selection of appropriate clones by sequence analysis of the domain exchange region. The second domain exchange within tm7 was made by overlap extension polymerase chain reaction (PCR) mutagenesis (Huang et al., 1995). The reaction was heated to 95°C for 5 min and then maintained at 80°C while adding 2.5 U of Pfu (*Pyrococcus furiosus*) polymerase (Stratagene, Amsterdam, The Netherlands), after which the reaction was put through 30 cycles with denaturing at 94°C (1 min), annealing at 57°C (1 min), and extension at 72°C (3 min). After the first round of PCR, 10-μl samples were analyzed by electrophoresis. The remaining PCR reactions were purified by extracting with the Wizard cDNA purification system (Promega), and then in the second round of PCR amplification 1-μl volumes of each appropriate extract were mixed and amplified using the flanking pBluescript primers under the same conditions as the first round of amplification. These were ligated into pBluescript for selection of appropriate clones by sequence analysis and then inserted into the expression vector pcDNA1 for functional expression in COS 7 cells.

Cell Culture and Transient Transfection of Receptor cDNAs

CHO cell lines stably expressing the $VPAC_1$, $VPAC_2$, $PAC_{1\text{-}null}$, and $PAC_{1\text{-}hop1}$ receptors were grown in 80-cm^2 flasks in Ham's modified F-12 medium containing 10% NCS, 300 μg/ml geneticin, 100 U/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C. GH_3 and αT3-1 cells were cultured as described previously (Johnson et al., 2000). Confluent cultures were trypsinized and seeded into 12-well cell culture plates for assay of PLC or PLD activity, or 24-well plates for assay of cAMP production. COS 7 cells were grown in 175-cm^2 flasks in Dulbecco's modified Eagle's medium containing 10% NCS, 100 U/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C. The cDNAs for the chimeric $VPAC_2/PAC_{1\text{-}null}$ and $VPAC_2/PAC_{1\text{-}hop1}$ (VP/4/7b/2.1c and VP/4/7/2.1c, pcDNA1) receptors were transfected into COS 7 cells using 30 μg of cDNA/6 $\times$ 10^6 cells and DEAE dextran (Promega) or FuGENE 6 (Roche Diagnostics Ltd., Lewes, UK) as described previously (Morrow et al., 1993; MacKenzie et al., 2001).

Whole-Cell Ligand Binding

The density and affinity of ligand binding sites in the $VPAC_1$ $VPAC_2$, $PAC_{1\text{-}null}$, and $PAC_{1\text{-}hop1}$ receptor-expressing cell lines were determined by nonlinear curve-fitting analysis of homologous displacement curves (Swillens, 1992). This method allows calculation of the number and affinity of binding sites in circumstances (such as with ligands iodinated in-house) where the precise ligand specific activity is not known and therefore Scatchard type analysis is not possible. Experiments were carried out at 37°C using intact cells in 12-well plates. This enabled assessment of both cell-surface association and internalization of ligand, reflecting the cellular disposition of the receptors under near-physiological conditions. Cells were incubated in 0.5 ml of Medium 199 with 0.2% BSA, 30 μg/ml bacitracin, and 10 μg/ml aprotinin, plus ^{125}I-helodermin (for $VPAC_1$/$VPAC_2$ receptors) or ^{125}I-PACAP-27 (for PAC_1 receptors), using 20,000 to 50,000 cpm/well. ^{125}I-Helodermin (approximately 770 Ci/mmol) was prepared by iodination using chloramine-T and purified by high-performance liquid chromatography according to methods described previously (Ogier et al., 1987). Increasing concentrations of unlabeled helodermin/PACAP-27 were also present as required. Nonspecific binding was defined with 300 nM unlabeled helodermin or PACAP-27, respectively. The plates were incubated at 37°C for 20 min unless otherwise indicated. The assay was terminated by aspiration of the medium and the cells were washed three times with 0.5

ml of ice-cold EBSS containing 0.1% BSA. Externally bound ligand was dissociated by a 5-min wash with 0.5 ml of an ice-cold acid strip solution (0.2 M acetic acid/0.5 M NaCl) (Slice et al., 1994). Internalized ligand was determined by solubilization of the cells after the acid strip wash using 1% Triton X-100 in 0.1 M NaOH. Protein content was determined using the bicinchoninic acid system (Pierce, Rockford, IL). After incubation of ^{125}I-PACAP-27 with $PAC_{1\text{-null}}$ receptor-containing CHO cells, the integrity of the ligand was assessed by reverse-phase chromatography on C18-silica (Waters Ltd., Watford, UK) using an H_2O/methanol gradient, containing 0.2% trifluoroacetic acid. After 30-min incubation at 37°C, 80 to 83% of the ligand in the extracellular medium and that released from the cells by hypotonic lysis in 10 mM formic acid still eluted as authentic ^{125}I-PACAP-27.

Determination of cAMP Production

CHO/COS 7 cells expressing the $VPAC_1$, $VPAC_2$, $PAC_{1\text{-null}}$, and $PAC_{1\text{-hop1}}$ receptors were preincubated for 10 min with the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (500 μM) and then stimulated with agonist (for 15 min). Intracellular cAMP levels were measured using a radioimmunoassay technique described previously (Morrow et al., 1993; Lutz et al., 1999).

Determination of [^{3}H]Inositol Phosphate Production

After 16-h labeling with 1 μCi/ml myo-[2-^{3}H]inositol (20 Ci/mmol), PLC activity in response to 30-min stimulation with agonist was monitored as formation of [^{3}H]inositol phosphate ([^{3}H]InsP) in the presence of 10 mM LiCl, as described previously (MacKenzie et al., 1996, 2001).

Measurement of [^{3}H]Phosphatidylbutanol ([^{3}H]PtdBut) Production

Cells in 12-well plates that had reached 80 to 100% confluence were placed in serum-free medium and labeled by incubation with 10 μCi/ml/well [9,10-^{3}H]palmitate (40 Ci/mmol) for 18 h before assay. PLD activity was monitored as the production of [^{3}H]PtdBut when cells were stimulated in the presence of 30 mM butan-1-ol (Mitchell et al., 1998). Before stimulation, cells were washed twice with MEM containing fatty acid-free BSA (1%), before replacement with minimal essential medium containing 0.5% BSA. The assay (30 min) was started with addition of agonist and terminated by aspiration of the medium and addition of 0.5 ml of ice-cold methanol. Cells were homogenized and samples transferred to 2-ml glass vials, before chloroform and H_2O were added to give a ratio of methanol/chloroform/H_2O of 1:1:0.8. Samples were vortexed and left for 15 min and then spun for 8 min in a low-speed centrifuge to separate the aqueous and organic layers. The upper aqueous layer was removed and an aliquot of the lower organic phase was evaporated under vacuum at 30°C in a centrifugal evaporator (Jouan, Nottingham, UK). Lipids were redissolved in 50 μl of chloroform/methanol (19:1) and separated by thin-layer chromatography on LK5D silica gel plates (Whatman, Maidstone, UK) using the upper phase of a mixture of 110 ml of ethyl acetate, 50 ml of 2,2,4-trimethylpentane, 20 ml of acetic acid, and 100 ml of water. The region of the thin-layer chromatography plate corresponding to [^{3}H]PtdBut, as determined by authentic standards, was scraped into vials and the radioactivity was quantified by liquid scintillation counting.

Receptor:Small G Protein Coimmunoprecipitation Studies

Native ARF. Experiments to assess association of native ARF with the $VPAC_2$ receptor were carried out using the $VPAC_2$ receptor-expressing CHO cell line. Cells in Dulbecco's modified Eagle's medium were incubated for 10 min with/without 10 nM VIP before washing in cold EBSS and solubilization (30 min at 4°C) in 20 mM sodium HEPES (pH 7.5) with 1 mM sodium orthovanadate, 1 mM NaF, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 2 μg/ml aprotinin, 4 μg/ml leupeptin, 2 μg/ml pepstatin A, 50 μg/ml soybean trypsin inhibitor plus 5 mM CHAPS, 0.1% sodium cholate, and 1 M NaCl. Extracts were diluted 1:1 with the same buffer lacking salt but including 20% glycerol. After preclearing with protein G-Sepharose, samples were centrifuged at 12,000*g* for 30 min. Aliquots of supernatant were retained for ligand binding by polyethylene glycol 8000 (PEG) precipitation (see below), whereas others were incubated (16 h rolling at 4°C) with sheep anti-$ARF1_{98\text{-}112}$ immunoglobulin at 10 μl/ml, with/without blocking peptide at 10 μg/ml (Mitchell et al., 1998) or nonimmune sheep IgG (3 μg/ml; Sigma) as control. Excess protein G-Sepharose was added to each tube and incubated with rolling for 3 h at 4°C before centrifugation (12,000*g* for 5 min). The pellet was washed twice with equivalent buffer before resuspension into similar buffer with the addition of (sonicated) phosphatidylcho-

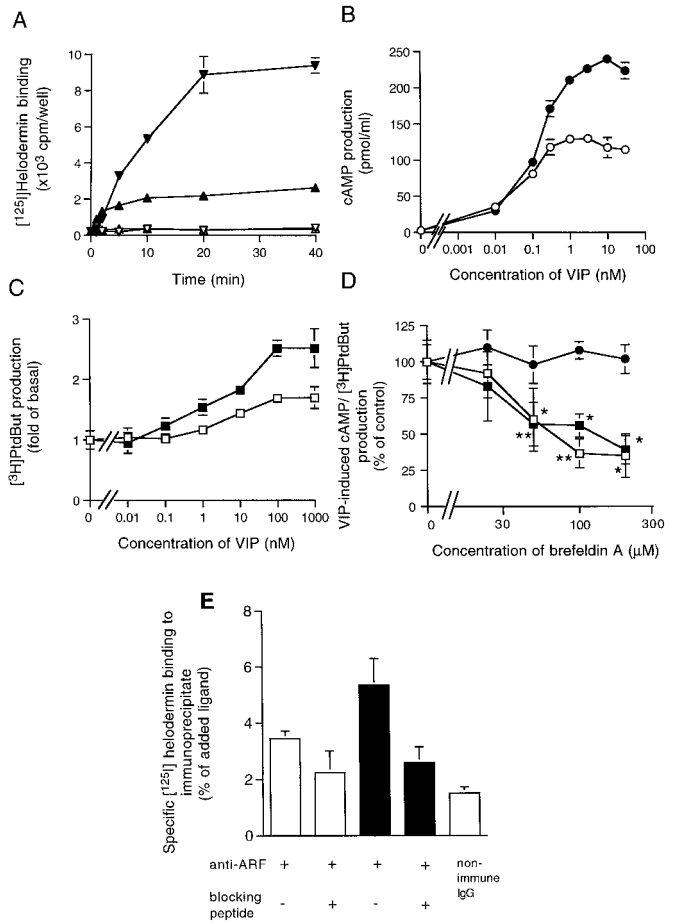

Fig. 1. Ligand binding and intracellular signaling from $VPAC_1$/$VPAC_2$ receptors. A, binding of ^{125}I-helodermin to intact $VPAC_2$ receptor-expressing CHO cells, carried out at 37°C. Cell-surface binding (dissociable by acid-salt wash) is shown as ▲ and ligand internalized to intracellular sites (released by detergent lysis of cells) is shown as ▼. Nonspecific binding (in the presence of 300 nM helodermin) is shown by corresponding open symbols. All values are means ± S.E.M. (n = 6–10). B, cAMP production responses of the $VPAC_1$ receptor-expressing CHO cell line are shown as ○ and for the $VPAC_2$ receptor line as ●. All values are means ± S.E.M. (n = 4–10). C, concentration-dependent [^{3}H]PtdBut production responses of the $VPAC_1$ receptor and $VPAC_2$ receptor-expressing CHO cell lines are shown as □ and ■, respectively. D, concentration-dependent inhibition by brefeldin A of [^{3}H]PtdBut production induced by 1 μM VIP in $VPAC_1$ (□) and $VPAC_2$ (■) receptor-expressing CHO cell lines and its lack of effect on cAMP production (●) in $VPAC_2$ receptor-expressing cells. Statistical significance of the inhibition was assessed by Mann-Whitney U test; $^*p < 0.05$, $^{**}p < 0.01$ (n = 6–8). E, specific ^{125}I-helodermin binding to ARF 1/3-directed immunoprecipitates in the $VPAC_2$ receptor-expressing CHO cell line. Solid columns show cells exposed to 100 nM VIP for 10 min before solubilization, whereas open columns show controls, not previously exposed to agonist. In some samples, excess antigenic peptide was supplied to block the antibody, as indicated.

TABLE 1
Characteristics of cell surface and internalized ^{125}I-helodermin binding in CHO cells expressing $VPAC_1$ and $VPAC_2$ receptors

	$VPAC_1$		$VPAC_2$	
	External	Internal	External	Internal
IC_{50} helodermin (nM)	1.02 ± 0.35	0.92 ± 0.17	3.05 ± 0.52	1.72 ± 0.24
B_{max} (pmol/mg protein)	0.07 ± 0.01	0.21 ± 0.02	1.23 ± 0.34	2.77 ± 0.61

line (Sigma) to 3 mg/ml. The suspension was aliquoted (100 μl) into tubes with 400 μl of 50 mM Tris-HCl (pH 7.4) containing 6.25 mM $MgCl_2$, 1% BSA, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.5 mg/ml bacitracin, 7% glycerol, and 2 mg/ml phosphatidylcholine. Approximately 80,000 cpm of ^{125}I-helodermin was added per tube, with/without 1 μM unlabeled helodermin to define nonspecific binding. Assays were incubated for 1 h on ice before the addition of 0.6 ml of 30% PEG and 0.1 ml of bovine γ-globulin (0.1%). After mixing and standing on ice for 15 min, the samples were centrifuged at 12,000*g* for 20 min, the supernatant was aspirated, and the tube tips were removed for gamma-counting of the pellets.

Epitope-Tagged ARF. COS 7 cells were cotransfected with expression plasmids encoding the $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptors and ARF1 with a carboxyl-terminal HA epitope tag (in pcDNA3 and pXS, respectively). Transfections were carried out with 8 μg of receptor plasmid DNA, 2 μg of ARF plasmid DNA, and 30 μl of FuGENE 6/175-cm^2 flask. Seventy-two hours later, quiescent cells were washed in cold EBSS before solubilization (1 h on ice) in standard phosphate-buffered saline (PBS) with the same protease- and phosphatase-inhibitors used for experiments with native ARF, plus 1% CHAPS. After preclearing with protein G-Sepharose, extracts were centrifuged at 12,000*g* for 30 min. Aliquots of supernatant were retained for binding and PEG precipitation, whereas others were incubated (16 h rolling at 4°C) with mouse monoclonal anti-HA IgG (clone 12CA5; Roche Diagnostics Ltd.) and/or control nonimmune mouse IgG to a total of 2 μg/ml. Excess protein G-Sepharose was added and incubated with rolling for 3 h at 4°C before centrifugation. The pellet was washed with solubilization buffer and then with PBS before resuspension into PBS. Samples were aliquoted (100 μl) into tubes with 400 μl of PBS and final concentrations of 10 mM $MgCl_2$ and 0.2% BSA. Approximately 11,000 cpm of ^{125}I-PACAP-27 was added per tube, with/without 100 nM PACAP-38 to define nonspecific binding. After incubation for 1 h on ice, assays were terminated, and PEG precipitated and harvested as described above.

Results

$VPAC_1$ and $VPAC_2$ Receptors. The cell-surface expression of ^{125}I-helodermin binding sites and the internalization of ligand were measured in selected CHO cell clones expressing $VPAC_1$ and $VPAC_2$ receptors (Fig. 1A; Table 1). Cell surface $VPAC_1$ and $VPAC_2$ receptors showed high affinity for helodermin (approximately 1–3 nM), as did the recognition sites from which ligand had subsequently become internalized (presumably receptors that had been present at the cell surface). The amount of accumulated ligand in intracellular stores was consistently greater than that remaining on the cell surface after incubations of more than 5 to 10 min. The time course of ^{125}I-helodermin association with cell-surface $VPAC_2$ receptors and its subsequent internalization are illustrated in Fig. 1A, showing rapid equilibration of cell-surface binding to a steady state by 10 min and continuing extensive internalization reaching a maximum by 20 min. The time course of ligand binding in the $VPAC_1$ receptor CHO clone was not studied in detail.

The ability of the $VPAC_1$ and $VPAC_2$ receptor CHO clones to show activation of AC, PLC, and PLD in response to agonists was assessed by cAMP, [^{3}H]InsP, and [^{3}H]PtdBut production assays (Fig. 1, B and C; Table 2). VIP elicited robust, concentration-dependent increases in cAMP production at $VPAC_1$ and $VPAC_2$ receptors (Fig. 1B). The potency of the responses was similarly high at both receptors (subnanomolar) and the maximal size of response was less in the $VPAC_1$ receptor CHO cells; probably reflecting the lower level of receptor expression. Neither the $VPAC_1$ nor $VPAC_2$ receptor CHO cell clones showed produced any detectable rise in [^{3}H]InsP production in response to VIP (0.3 nM–3 μM). Both $VPAC_1$ and $VPAC_2$ receptors did however mediate small but consistent [^{3}H]PtdBut responses when stimulated by VIP (Fig. 1C). The potency of these PLD responses was considerably lower than that for cAMP responses of the receptors, but still in the low nanomolar range (Table 2). As with the cAMP responses, the maximal PLD response of the $VPAC_1$ receptor was less than that of the $VPAC_2$ receptor.

To assess whether the PLD responses might be downstream of the activation of G_s or adenylate cyclase, [^{3}H]PtdBut production was measured in cells stimulated with 1 and 5 μg/ml cholera toxin (16 h) or 1 and 5 μM forskolin (during the 30-min assay). None of these stimuli caused any detectable increase in [^{3}H]PtdBut production, despite large control PLD responses to 1 μM phorbol 12,13-dibutyrate being recorded in each assay (data not shown).

Receptors can activate PLD through many routes (Exton, 1997), including pathways downstream of PLC activation or involving the small G proteins ARF and Rho (Mitchell et al., 1998). Because PLC activation was undetectable in response to VIP in these cell lines, it seems unlikely that this is important in bringing about the PLD responses observed. In PLD responses of rhodopsin family GPCRs, sensitivity to BFA, an inhibitor of some ARF GTP-exchange factors, correlates closely with the involvement of ARF 1/3 in these responses, apparently through direct association with relevant receptors (Mitchell et al., 1998). Figure 1D shows that BFA inhibited $VPAC_1$ and $VPAC_2$ receptor PLD responses with relatively high potency, yet was without effect on cAMP responses.

Coimmunoprecipitation experiments were carried out on solubilized extracts of the $VPAC_2$ receptor-containing CHO cells to assess any ARF:receptor interaction. ARF 1/3 was immunoprecipitated broadly as described previously (Mitchell et al., 1998) and the $VPAC_2$ receptor was detected by specific binding of ^{125}I-helodermin to the precipitated proteins because suitable high-affinity antibodies for the $VPAC_2$ receptor are not available. No specific binding was detected in wild-type CHO cells. Approximately 28% of the initial specific ^{125}I-helodermin binding could be solubilized and recovered as viable specific ligand binding sites from $VPAC_2$ receptor-CHO cells by the procedure used. Figure 1E shows that specific binding of ^{125}I-helodermin was associated with ARF immunoprecipitates and this was considerably reduced when the ARF antibody was blocked with excess of the antigenic peptide or when nonimmune IgG was substituted. Brief preincubation with the agonist VIP seemed to facilitate ARF:receptor association. The affinity of solubilized, and presumably also immunoprecipitated receptors for ligand was reduced so it is not possible to reliably calculate the proportion of the receptors engaging with ARF.

TABLE 2
cAMP, PLC, and PLD responses to VIP in CHO cells expressing $VPAC_1$ and $VPAC_2$ receptors

	$VPAC_1$		$VPAC_2$	
	EC_{50}	E_{max}	EC_{50}	E_{max}
	nM	*fold of basal*	*nM*	*fold of basal*
cAMP Production	0.065 ± 0.013	59 ± 1	0.123 ± 0.016	118 ± 3
[^{3}H]InsP production	nil at 3 μM		nil at 3 μM	
[^{3}H]PtdBut production	6.2 ± 2.5	1.83 ± 0.29	5.3 ± 1.2	2.79 ± 0.46

$PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ Receptors. Similar levels of cell-surface ^{125}I-PACAP-27 binding sites and amounts of subsequently internalized ligand were measured in CHO cell clones expressing $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptors (Fig. 2, A and B; Table 3). The accumulation of ligand in both compartments of $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptor CHO cells was inhibited with moderately high affinity (9–21 nM) by unlabeled PACAP-27 with no clear evidence for multiple components (mean Hill coefficient of 0.99 ± 0.11 from all experiments). The time courses for cell surface association of ^{125}I-PACAP-27 and for the internalization of ligand bound to receptors were generally similar between $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptors (Fig. 2, A and B) and to data from the $VPAC_2$ receptor (Fig. 1A). The steady-state accumulation of ^{125}I-PACAP-27 into the intracellular compartment seemed to be slightly greater for the $PAC_{1\text{-null}}$ receptor than for the $PAC_{1\text{-hop1}}$ receptor but neither this, nor slight differences in rates between the splice variants, were investigated further.

The cAMP, [^{3}H]InsP, and [^{3}H]PtdBut production responses of the $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptor CHO cells are shown in Figs. 2, C and D; 3, A and B; Table 4. Their receptors mediated robust cAMP responses of very high potency in response to PACAP-38. Both EC_{50} values and maximal responses were almost identical (Fig. 2C). Consistent with previous reports (Spengler et al., 1993; van Rampelbergh et al., 1997), both $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptors also showed clear [^{3}H]InsP responses to PACAP-38 (Fig. 2D). The EC_{50} values were similar (4–9 nM) but of considerably lower potency than the cAMP responses. The maximal evoked [^{3}H]InsP response of the $PAC_{1\text{-null}}$ receptor was consistently 2.5- to 3-fold greater than that of the $PAC_{1\text{-hop1}}$ receptor despite the very similar cAMP responses. Both $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ clones also produced consistent [^{3}H]PtdBut responses of similar potency to the [^{3}H]InsP responses (Fig. 3A). The maximal evoked PLD response of the $PAC_{1\text{-hop1}}$ receptor was consistently around 3-fold that of the $PAC_{1\text{-null}}$ receptor, in contrast to the pattern of their PLC responses. Time courses of these PLD responses showed no evidence of desensitization for at least 15 min (Fig. 3B). The [^{3}H]PtdBut response of the $PAC_{1\text{-hop1}}$ receptor was significantly inhibited by BFA (at concentrations of 50 μM and above), whereas that of the $PAC_{1\text{-null}}$ receptor was little affected; consistent with a greater involvement in the former response of an ARF-dependent pathway. cAMP and [^{3}H]InsP responses of the $PAC_{1\text{-hop1}}$ receptor were unaffected by BFA. Figure 3D shows that the [^{3}H]PtdBut response of the $PAC_{1\text{-null}}$ receptor was inhibited to a greater extent than that of the $PAC_{1\text{-hop1}}$ receptor by the PLC inhibitor U 73122; the converse of their sensitivity to BFA and consistent with a greater contribution of a PLC-dependent pathway to the $PAC_{1\text{-null}}$ receptor response.

$PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptors were cotransfected with ARF1-HA into COS 7 cells and receptor:small G protein coimmunoprecipitation was investigated in HA-directed immunoprecipitates. These were probed for specific ^{125}I-PACAP-27 binding sites because suitable high-affinity PAC_1 receptor antibodies are not available. The solubilization procedure extracted similar levels of specific ^{125}I-PACAP-27 binding sites from $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptor-expressing cells (i.e., approximately 100 fmol/ml extract), as assessed by PEG precipitation binding assays, but no detectable specific binding in extracts of untransfected cells. With extracts of $PAC_{1\text{-hop1}}$ but not $PAC_{1\text{-null}}$ receptor-expressing cells, increasing levels of anti-HA IgG in the immunoprecipitation gave a concentration-dependent increase in the amount of specific ^{125}I-PACAP-27 binding recovered in the precipitate (Fig. 3E). Under the present experimental conditions, prein-

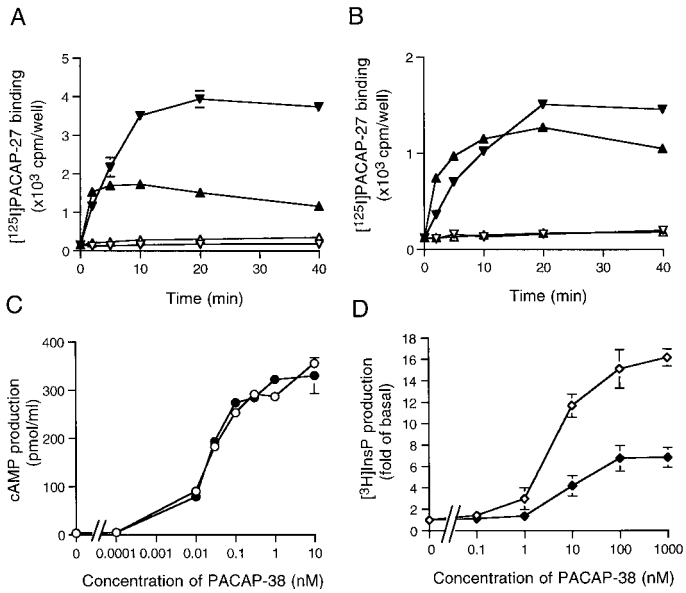

Fig. 2. Ligand binding and intracellular signaling responses of $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptors in CHO cells. A and B, ^{125}I-PACAP-27 binding to $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptors, respectively, in intact CHO cells at 37°C. Cell-surface binding (dissociable by acid-salt wash) is shown as ▲ and ligand internalized to intracellular sites (released by detergent lysis of cells) is shown as ▼. Nonspecific binding (in the presence of 300 nM PACAP-27) is shown by corresponding open symbols. All values are means ± S.E.M. (n = 6–12). C and D, cAMP and [^{3}H]InsP responses for $VPAC_1$ and $VPAC_2$ receptors (open and closed symbols, respectively), means ± S.E.M., n = 6–14.

TABLE 3
Characteristics of cell surface and internalized ^{125}I PACAP-27 binding in CHO cells expressing $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptors

	$PAC_{1\text{-null}}$		$PAC_{1\text{-hop1}}$	
	External	Internal	External	Internal
IC_{50} PACAP-27 (nM)	20.8 ± 3.2	10.8 ± 2.3	13.1 ± 5.8	9.2 ± 3.8
B_{max} (pmol/mg protein)	4.1 ± 0.5	7.9 ± 0.9	6.0 ± 0.5	4.5 ± 0.4

TABLE 4
cAMP, PLC, and PLD responses to PACAP-38 in CHO cells expressing $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptors

	$PAC_{1\text{-null}}$		$PAC_{1\text{-hop1}}$	
	EC_{50}	E_{max}	EC_{50}	E_{max}
	nM	*fold of basal*	*nM*	*fold of basal*
cAMP Production	0.027 ± 0.002	169 ± 4	0.022 ± 0.002	157 ± 18
[^{3}H]InsP production	4.7 ± 0.6	15.9 ± 0.3	8.9 ± 0.4	6.9 ± 0.1
[^{3}H]PtdBut production	1.7 ± 0.5	3.8 ± 0.2	3.0 ± 0.2	8.4 ± 0.1

cubation with agonist did not seem to be a prerequisite for this association. The overall levels of IgG were kept constant by substitution of nonimmune mouse IgG.

Chimeric $VPAC_2/PAC_1$ Receptors. To address the importance of the i3 sequence of the PAC_1 receptor splice variants in determining the route and extent of coupling to PLD, we constructed chimeric $VPAC_2/PAC_1$ receptors (Fig. 4). These contained the body of the $VPAC_2$ receptor with a segment from tm5 to tm7 (i.e., including the i3 domain) derived from either $PAC_{1\text{-null}}$ or $PAC_{1\text{-hop1}}$ receptor sequences. Thus, the only difference between these two constructs was the additional 28 amino acid hop-1 cassette in the $VPAC_2/PAC_{1\text{-hop1}}$ construct.

The chimeric receptors were transiently expressed in COS 7 cells for assessment of their signaling responses and transfected GLP-1 receptors were also studied for comparison. Both $VPAC_2/PAC_{1\text{-null}}$ and $VPAC_2/PAC_{1\text{-hop1}}$ receptor constructs mediated robust cAMP responses to VIP (mean maximal responses of 149- and 143-fold of basal and EC_{50} values of 0.8 ± 0.1 and 1.5 ± 0.2 nM, respectively; means ± S.E.M., $n = 6$; Fig. 5A). The potency of these responses was less than in CHO cells (as described previously for wild-type PAC_1 and $VPAC_2$ receptors in COS 7 cells (Lutz et al., 1999). This may relate to different complements of relevant cellular proteins in the two cell types. GLP-1 (7-36) amide potently activated cAMP production (in GLP-1 receptor-expressing cells only) with an EC_{50} value of 0.034 ± 0.014 nM (mean ± S.E.M., $n = 6$). [^{3}H]InsP responses of the receptors were not investigated. Both chimeric constructs, however, mediated clear and similar [^{3}H]PtdBut production responses to VIP (Fig. 5B). Although the EC_{50} values were greater than those for cAMP production, they were in the low nanomolar range (2.5 ± 1.7 and 5.6 ± 3.9 nM for the $VPAC_2/PAC_{1\text{-null}}$ and $VPAC_2/PAC_{1\text{-hop1}}$ constructs, respectively, means ± S.E.M., $n = 6$–10). The corresponding maximal response values were also similar; 3.2 ± 0.2- and 2.5 ± 0.4-fold of basal, respectively. The GLP-1 receptor mediated a [^{3}H]PtdBut response only at very high concentrations of GLP-1 (7-36)

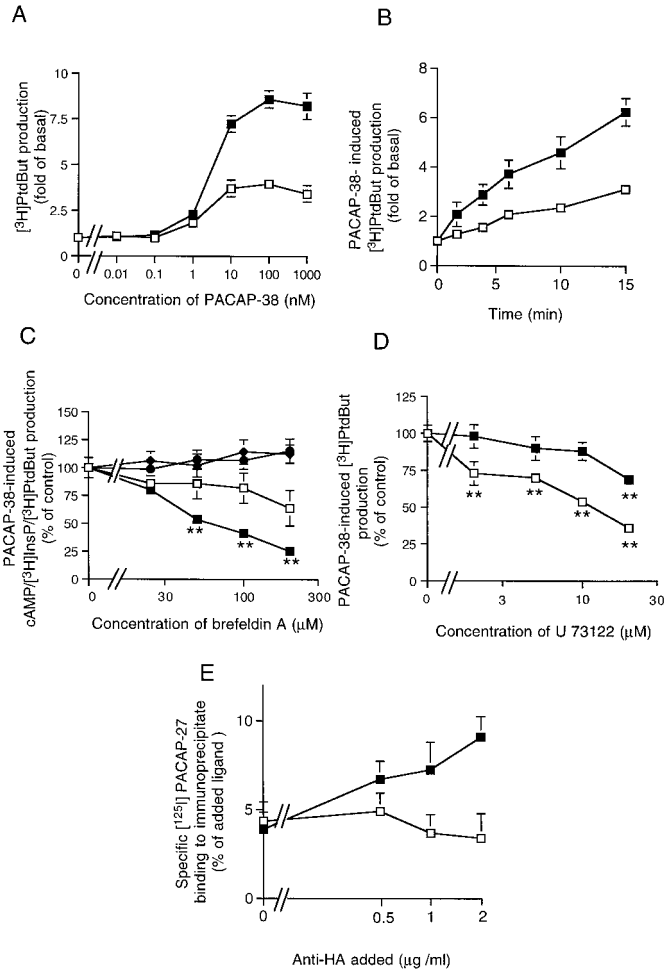

Fig. 3. [^{3}H]PtdBut responses of $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop-1}}$ receptors and coimmunoprecipitation with ARF1-HA. A and B, concentration-dependence and time course of [^{3}H]PtdBut production in response to PACAP-38 (100 nM in B) at $VPAC_1$ (□) and $VPAC_2$ (■) receptors (means ± S.E.M., $n = 5$–8). C, inhibitory effect of the ARF inhibitor brefeldin A on 100 nM PACAP-38-induced [^{3}H]PtdBut responses of the $PAC_{1\text{-hop1}}$ (■) but not the $PAC_{1\text{-null}}$ receptor (□), and the absence of inhibition of cAMP (●) or [^{3}H]InsP (♦) responses from the $PAC_{1\text{-hop1}}$ receptor (means ± S.E.M., $n = 6$–8; $^{**}p < 0.01$ by Mann-Whitney U test). D, effects of the PLC inhibitor, U 73122 on corresponding [^{3}H]PtdBut responses of the $PAC_{1\text{-null}}$ receptor (□) but to a much lesser degree on the $PAC_{1\text{-hop1}}$ receptor (■); means ± S.E.M., $n = 6$–8. $^{**}p < 0.01$ by Mann-Whitney U test. E, recovery of specific ^{125}I-PACAP-27 binding sites in HA-directed immunoprecipitates from COS 7 cells cotransfected with ARF1-HA and with either the $PAC_{1\text{-null}}$ (□) or the $PAC_{1\text{-hop1}}$ (■) receptor. Values are means ± S.E.M., $n = 6$.

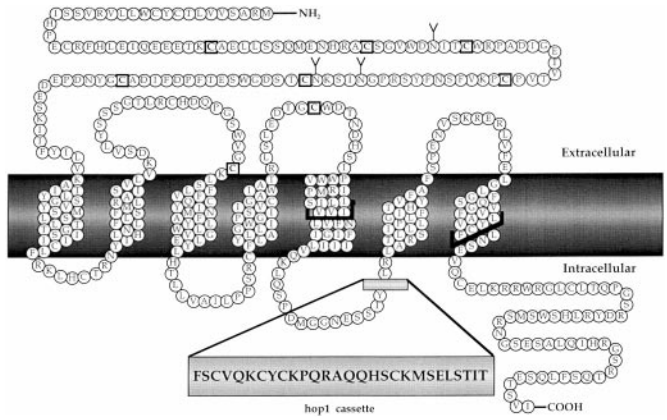

Fig. 4. Chimeric receptor constructs of the rat $VPAC_2$ receptor, containing intracellular loop 3 of the rat PAC_1-null receptor or the hop1 splice variant. The predicted arrangement of transmembrane domains is shown, as are conserved cysteines (boxed) and *N*-glycosylation sites (Y). The splicing position of the PAC_1 receptor hop1 cassette is indicated by the shaded bar. Exchange sites for the chimeric constructs are indicated by the black bars in transmembrane domains 5 and 7.

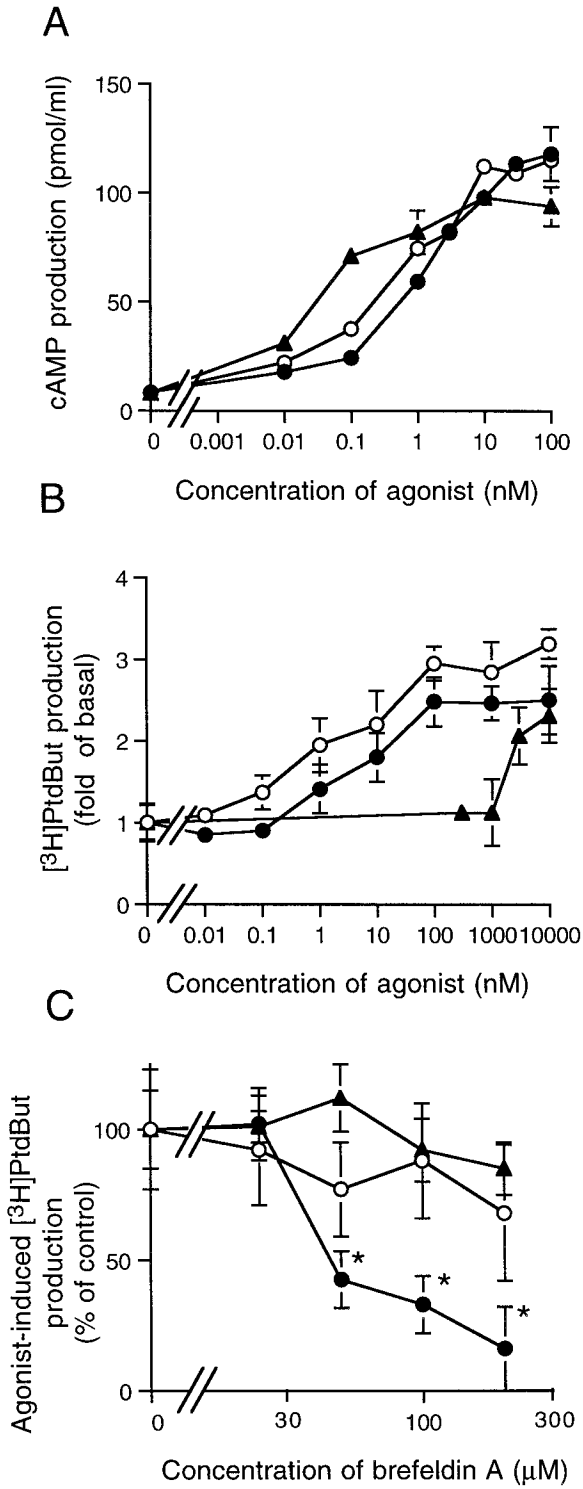

Fig. 5. cAMP and [^{3}H]PtdBut production responses of chimeric $VPAC_2$ receptors with intracellular loop 3 derived from $PAC_{1\text{-null}}$ or $PAC_{1\text{-hop1}}$ receptors. Receptors were transiently expressed in COS 7 cells. Responses to VIP of the $VPAC_2/PAC_{1\text{-null}}$ receptor construct are shown as ○ and those of the $VPAC_2/PAC_{1\text{-hop1}}$ receptor construct as ●. In cells transfected with the GLP-1 receptor, responses to GLP-1 (7–36) amide are shown as ▲. All values are means ± S.E.M. (n = 5–16). A and B, concentration dependence of agonist-induced cAMP and [^{3}H]PtdBut production, respectively. C, concentration-dependence of brefeldin A inhibition of the [^{3}H]PtdBut responses at the $PAC_{1\text{-hop1}}$ receptor (●) but not at the $PAC_{1\text{-null}}$ receptor (○) (induced by 1 μM VIP), or at the GLP-1 receptor (▲) (induced by 10 μM GLP-1 (7-36) amide), n = 6. Statistical significance of the inhibition was assessed by Mann-Whitney U test; *$p < 0.05$.

amide, with an EC_{50} value of 0.98 ± 0.22 μM (mean ± S.E.M., n = 6) (i.e., greater than 10,000-fold less potent than the receptor's cAMP response). When the [^{3}H]PtdBut responses of the receptors were challenged with BFA, only that of the $VPAC_2/PAC_{1\text{-hop1}}$ construct was significantly inhibited at BFA concentrations of 50 μM and above (Fig. 5C). This is consistent with the idea that transfer of the $PAC_{1\text{-null}}$ or $PAC_{1\text{-hop1}}$ i3 domains into the body of the $VPAC_2$ receptor had respectively conferred ARF-independent and ARF-dependent coupling to PLD activation.

Native $VPAC_2$ and PAC_1 Receptors. To assess whether similar mechanisms for PLD activation might occur in native cells, we examined responses of the $VPAC_2$ receptor in GH_3 cells and the PAC_1 receptor in αT3-1 cells. In each case these are the only members of the VPAC/PAC receptor family expressed, and the predominant form of the PAC_1 receptor in αT3-1 cells is known to be the hop-1 splice variant (Rawlings et al., 1995; MacKenzie et al., 2001). In GH_3 cells, VIP elicited a robust cAMP response with an EC_{50} value of 1.9 ± 0.4 nM and a maximal response 7.6 ± 0.4-fold of basal. A smaller PLD response was also seen, with an EC_{50} value of 30.6 ±11.2 nM and maximal response of 2.6 ± 0.1-fold of basal (means ± S.E.M., n = 6) (i.e., only around 12-fold lower potency than the cAMP response) (Fig. 6A). In αT3-1 cells, PACAP-38 elicited cAMP production with an EC_{50} value of 0.19 ± 0.13 nM and maximal response 12.1 ± 0.5-fold of basal. PLD was also clearly activated with an EC_{50} value of 6.7 ± 1.0 nM and a maximal response 3.2 ± 0.1-fold of basal (means ± S.E.M., n = 6) (i.e., approximately 35-fold lower potency than the cAMP response but still within the low nanomolar range of ligand concentration) (Fig. 6B). Both PLD responses were sensitive to BFA with mean IC_{50} values of 64 μM for the $VPAC_2$ receptor in GH_3 cells and 65 μM for the PAC_1 receptor in αT3-1 cells (Fig. 6C), concurring with the properties observed for $VPAC_2$ and $PAC_{1\text{-hop1}}$ receptors in transfected cells.

Discussion

Like other GPCRs in the secretin/parathyroid hormone receptor family (Probst et al., 1992; Segre and Goldring, 1993; Harmar and Lutz, 1994; Donnelly, 1997), the PAC_1 and VPAC receptors increase cellular cAMP levels, presumably via coupling to Gs. $VPAC_1$, $VPAC_2$, and PAC_1 receptors expressed here in CHO cells mediate robust increases in cellular cAMP levels but in addition can lead to the activation of PLD (and in some cases, PLC). cAMP signals occur at higher potency than phospholipase activation in all the receptors studied here, but substantial phospholipase activation still occurs at low nanomolar concentrations that are potentially relevant in a physiological context.

Neither the potency nor maximal response of cAMP production was altered by the presence of the hop1 cassette in i3 of the PAC_1 receptor (compared with the $PAC_{1\text{-null}}$ form). Similar numbers of receptors were expressed both at the cell surface and in pools internalized from the cell surface in the $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ CHO cells, matching their similar cAMP responses. In the $VPAC_1$ receptor CHO cells, receptor expression was lower than that in $VPAC_2$ and $PAC_{1\text{-null}}$ or $PAC_{1\text{-hop1}}$ cells and the maximum but not the potency of the cAMP response was diminished.

PAC_1 receptors activate PLC (through a pertussis toxin-in-

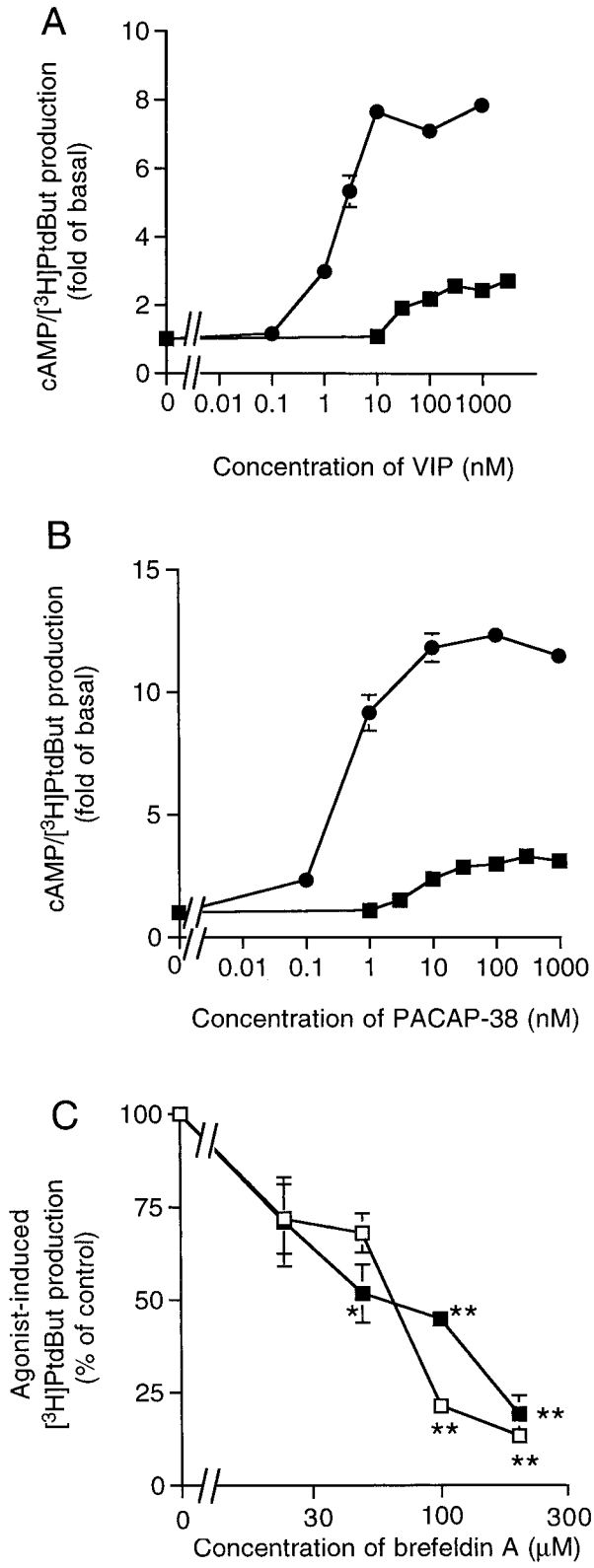

Fig. 6. cAMP and [^{3}H]PtdBut production responses of $VPAC_2$ and PAC_1 receptors natively expressed in GH_3 and αT3-1 cells. A and B, agonist-evoked responses of GH_3 and αT3-1 cells, respectively, with ● illustrating the cAMP production and ■ the [^{3}H]PtdBut responses. C, inhibition by brefeldin A of [^{3}H]PtdBut responses to 1 μM VIP in GH_3 cells (□) and to 100 nM PACAP-38 in αT3-1 cells (■). Statistical significance of inhibition was assessed by Mann-Whitney U test, means ± S.E.M., $n = 6–8$, $^*p < 0.05$; $^{**}p < 0.01$).

sensitive route) in a variety of cell types (Deutsch and Sun, 1992; Spengler et al., 1993; Schomerus et al., 1994; Pantaloni et al., 1996; Pisegna and Wank, 1996; van Rampelbergh et al., 1997). Rat $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptors expressed in LLC-PK1 cells activate PLC with similar potency (Spengler et al., 1993) although other splice variants, the hip form and an N-terminally deleted form show reduced, and facilitated, potency of PLC activation, respectively (Spengler et al., 1993; Pantaloni et al., 1996). Human $PAC_{1\text{-null}}$ and $PAC_{1\text{-SV-2}}$ (equivalent to hop1) receptors expressed in NIH/3T3 cells display similar potencies of PLC activation but the $PAC_{1\text{-SV-2}}$ variant gave a greater maximal response when expressed at similar levels of total receptors per cell (Pisegna and Wank, 1996). In the present study, the PLC response of the rat $PAC_{1\text{-null}}$ receptor in CHO cells was greater than that of the $PAC_{1\text{-hop1}}$ variant for similar levels of receptor expression. Both species differences and host cell differences may contribute to the disparity.

In contrast, $VPAC_1$ and $VPAC_2$ receptors are less well known to activate PLC. Both receptors can elicit a modest PLC response (which is partly sensitive to pertussis toxin) when expressed in COS 7 cells (MacKenzie et al., 1996, 2001). In addition, another group reported a 1.5-fold increase in phosphoinositide hydrolysis in CHO cells expressing the $VPAC_1$ receptor (van Rampelbergh et al., 1997). However, the receptor density in their stable clones was 20 pmol/mg protein compared with the expression of only of 0.06 ± 0.01 pmol $VPAC_1$ receptor/mg protein here. In the present experiments, neither $VPAC_1$ nor $VPAC_2$ receptor CHO cell clones demonstrated detectable [^{3}H]InsP responses to agonists.

The activation of PLD by members of the secretin/parathyroid hormone receptor family has been little studied, although it has been reported in the case of glucagon, calcitonin, and parathyroid hormone receptors (Pittner and Fain, 1991; Friedman et al., 1999; Naro et al., 1998). This is the first report of PLD activation by the $VPAC_1$, $VPAC_2$, $PAC_{1\text{-null}}$, and $PAC_{1\text{-hop1}}$ receptors. Unlike the PLD responses of many rhodopsin family GPCRs there was no evidence for rapid desensitization, but a variety of factors, including assay conditions, cellular context, and receptor type could contribute to this and the issue was not further investigated here. Both $VPAC_1$ and $VPAC_2$ receptors mediated modest PLD responses to VIP (with a lower maximal response for the $VPAC_1$ receptor, matching its lower level of expression). Similar nanomolar potencies were seen at both receptors although these were much weaker than the effects on cAMP production. PLD activation could not be mimicked by activators of Gs or adenylate cyclase and occurred in the absence of any detectable PLC responses, suggesting that activation of PLD did not occur downstream of either of these pathways. A lack of concurrent PLC activation was also seen with the glucagon receptor (Pittner and Fain, 1991), whereas PLD activation by the parathyroid hormone receptor was unaffected after the inhibition of PLC activity by U 73122 (Friedman et al., 1999). Instead, both $VPAC_1$ and $VPAC_2$ receptor PLD responses were inhibited with relatively high potency by the ARF inhibitor BFA, whereas cAMP responses of the $VPAC_2$ receptor, for example, were unaffected. These data are consistent with the physical association between VPAC receptors and the small G protein ARF demonstrated in Fig. 1E (a link that could potentially provide a basis for facilitated ARF-dependent PLD activation). Both $PAC_{1\text{-null}}$ and $PAC_{1\text{-hop1}}$ receptors also displayed PLD responses and although these were again of lower potency than cAMP responses, they were of similar (or greater)

potency than the PLC responses of the receptors. The PLD response of the $PAC_{1\text{-null}}$ receptor (but not the hop1 variant) was inhibited by low concentrations of the PLC inhibitor U 73122, suggesting that it may result substantially from PLC-dependent pathways. In contrast, the $PAC_{1\text{-hop1}}$ receptor displayed a much greater maximal PLD response that (unlike that of the null variant) was sensitive to BFA, whereas its cAMP and PLC responses were unaffected. This suggests that the presence of the hop1 cassette is critical in linking the PAC_1 receptor to an ARF-dependent route of PLD activation. Correspondingly, immunoprecipitation of epitope-tagged ARF1 resulted in coprecipitation of $PAC_{1\text{-hop1}}$ but not $PAC_{1\text{-null}}$ receptors (Fig. 3E).

Chimeric $VPAC_2/PAC_1$ receptors containing either $PAC_{1\text{-null}}$ or $PAC_{1\text{-hop1}}$ i3 domains were constructed to address whether BFA-sensitivity/insensitivity could be conferred just by an i3 domain swap. The chimeric $VPAC_2/PAC_1$ receptors with i3 domains from either $PAC_{1\text{-null}}$ or $PAC_{1\text{-hop1}}$ receptors showed no apparent difference in their cAMP responses. However, the $VPAC_2/PAC_{1\text{-null}}$ construct (just like the wild-type $PAC_{1\text{-null}}$ receptor) showed a PLD response insensitive to BFA, despite the main body of the construct, apart from i3, being of $VPAC_2$ (BFA-sensitive) origin. The $VPAC_2/PAC_{1\text{-hop1}}$ chimera retained BFA sensitivity, indicating that the i3 sequence of PAC_1 receptors is a critical determinant of coupling to ARF-dependent PLD activation. Some analogy can be drawn with the dopamine D_2 receptor, where i3 splice variants couple differentially to $G_{\alpha i2}$ (Guiramand et al., 1995) and the calcitonin receptor where i1 variants couple differentially to PLC but not to AC (Nussenzveig et al., 1994). Thus, the alternative splicing of receptors may allow a more subtle selection of signals and hence control of cellular activity to be achieved. Analogous behavior of natively expressed $VPAC_2$ and PAC_1 receptors was demonstrated using GH_3 and αT3-1 cell lines, respectively (Fig. 6). Modest but significant PLD responses to agonists were seen in each case with sensitivity to BFA (matching in the case of αT3-1 cells, their predominant expression of the hop-1 splice variant (Rawlings et al., 1995).

A number of studies have pointed to a role of amphipathic helical domains incorporating basic amino acids in the coupling of GPCRs to G proteins. Peptides derived from the α_2-adrenergic receptor activate $G_{i/o}$ in vitro, providing they possess basic amino acids spaced throughout the peptide and end with a BBxxB or BBxB (where x is any residue, and B is basic residue or in the last position either a basic or aromatic residue) (Ikezu et al., 1992; Wade et al., 1996). Groupings of basic and hydrophobic amino acids, situated in i3 of the muscarinic and the α_2-adrenergic receptors, have been implicated in interactions with G proteins (Burstein et al., 1998; Okamoto and Nishimoto, 1992; Wade et al., 1996; Wess, 1997). The $VPAC_2$ receptor contains a classical BBxxB motif at the i3/tm6 junction, whereas both the $VPAC_1$ and $PAC_{1\text{-hop1}}$ receptors contain a motif similar to that seen in the α_2-adrenergic receptor with spaced basic residues and a cluster of basic amino acids upstream of tm6. The presence of the hop-1 insert in the PAC_1 receptor provides the cluster of basic amino acids that completes a spaced basic residue motif. The $PAC_{1\text{-null}}$ receptor has no classical or spaced base motif present in its i3 and this may potentially underlie its minimal ARF-dependent coupling to PLD.

In summary, the VPAC and PAC_1 receptors can activate PLD and although this is at higher concentrations of agonist than those required to elicit cAMP production, they may still be physiologically relevant. There are marked differences in the mechanisms apparently used to bring about this activation, with both ARF-dependent routes and PLC-dependent routes being implicated in different cases. From data with i3 splice variants of the PAC_1 receptor and with chimeric receptor constructs incorporating i3 domain swaps it seems that the i3 structure of the PAC_1 receptor is a critical determinant of both its physical association with ARF1 and its ARF-dependent coupling to PLD.

Acknowledgments

Expression plasmids encoding ARF1-HA and the rat GLP-1 receptor were generously provided by Julie Donaldson and Dan Donnelly, respectively. We are grateful to Linda Pooley for kind help with cAMP assays; to Christine Morrison, John Bennie, and Sheena Carroll for aspects of technical assistance; and to Marianne Eastwood for help in preparation of the manuscript.

References

Arimura A and Shioda S (1995) Pituitary adenylate-cyclase activating polypeptide (PACAP) and its receptors - neuroendocrine and endocrine interaction. *Front Neuroendocrinol* **16:**53–88.

Burstein ES, Spalding TA and Brann MR (1998) Structure/function relationships of a G-protein coupling pocket formed by the third intracellular loop of the m5 muscarinic receptor. *Biochemistry* **37:**4052–4058.

Deutsch PJ and Sun Y (1992) The 38-amino acid form of pituitary adenylate cyclase-activating polypeptide stimulates dual signaling cascades in PC12 cells and promotes neurite outgrowth. *J Biol Chem* **267:**5108–5113.

Donnelly D (1997) The arrangement of the transmembrane helices in the secretin receptor family of G-protein-coupled receptors. *FEBS Lett* **409:**431–436.

Exton JH (1997) New developments in phospholipase D. *J Biol Chem* **272:**15579–15582.

Friedman PA, Gesek FA, Morley P, Whitfield JF and Willick GE (1999) Cell-specific signaling and structure-activity relations of parathyroid hormone analogs in mouse kidney cells. *Endocrinology* **140:**301–309.

Guiramand J, Montmayeur J, Ceraline J, Bhatia M and Borrelli E (1995) Alternative splicing of the dopamine D_2 receptor directs specificity of coupling to G-proteins. *J Biol Chem* **270:**7354–7358.

Harmar T and Lutz E (1994) Multiple receptors for PACAP and VIP. *Trends Pharmacol Sci* **15:**97–99.

Hashimoto H, Ishihara T, Shigemoto R, Mori K and Nagata S (1993) Molecular-cloning and tissue distribution of a receptor for pituitary adenylate cyclase-activating polypeptide. *Neuron* **11:**333–342.

Hosoya M, Onda H, Ogi K, Masuda Y, Miyamoto Y, Ohtaki T, Okazaki H, Arimura A and Fujino M (1993) Molecular-cloning and functional expression of rat cDNAs encoding the receptor For pituitary adenylate-cyclase activating polypeptide (PACAP). *Biochem Biophys Res Commun* **194:**133–143.

Huang Z, Chen Y and Nissenson RA (1995) The cytoplasmic tail of the G protein-coupled receptor for parathyroid hormone and parathyroid hormone-related protein contains positive and negative signals for endocytosis. *J Biol Chem* **270:**151–156.

Ikezu T, Okamoto T, Ogata E and Nishimoto I (1992) Amino acids 356–372 constitute a Gi-activator sequence of the α2-adrenergic receptor and have a Phe substitute in the G protein-activator sequence motif. *FEBS Lett* **311:**29–32.

Ishihara T, Shigemoto R, Mori K, Takahashi K and Nagata S (1992) Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron* **8:**811–819.

Ji TH, Grossmann M and Ji I (1998) G Protein-coupled receptors. *J Biol Chem* **273:**17299–17302.

Johnson MS, Lutz EM, MacKenzie CJ, Wolbers B, Robertson DN, Holland PJ and Mitchell R (2000) Gonadotropin-releasing hormone receptor activation of extracellular signal-regulated kinase and tyrosine kinases in transfected GH_3 cells and in αT3–1 cells. *Endocrinology* **141:**3087–3097.

Lutz EM, MacKenzie CJ, Johnson MS, West K, Morrow JA, Harmar AJ and Mitchell R (1999) Domains determining agonist selectivity in chimaeric VIP_2 ($VPAC_2$)/PACAP (PAC_1) receptors. *Br J Pharmacol* **128:**934–940.

Lutz EM, Sheward WJ, West KM, Morrow JA, Fink G and Harmar AJ (1993) The VIP_2 receptor - molecular characterization of a cDNA-encoding a novel receptor for vasoactive-intestinal-peptide. *FEBS Lett* **334:**3–8.

MacKenzie CJ, Lutz EM, Johnson MS, Robertson DN, Holland PJ and Mitchell R (2001) Mechanisms of phospholipase C activation by the VIP/PACAP type 2 ($VPAC_2$) receptor. *Endocrinology* **142:**1209–1217.

MacKenzie CJ, Lutz EM, McCulloch DA, Mitchell R and Harmar AJ (1996) Phospholipase C activation by VIP_1 and VIP_2 receptors expressed in COS 7 cells involves a pertussis toxin-sensitive mechanism. *Ann NY Acad Sci* **805:**579–584.

Mitchell R, McCulloch D, Lutz E, Johnson M, MacKenzie C, Fennell M, Fink G, Zhou W and Sealfon SC (1998) Rhodopsin-family receptors associate with small G proteins to activate phospholipase D. *Nature (Lond)* **392:**411–414.

Morrow JA, Lutz EM, West KM, Fink G and Harmar AJ (1993) Molecular-cloning and expression of a cDNA-encoding a receptor for pituitary adenylate-cyclase activating polypeptide (PACAP). *FEBS Lett* **329:**99–105.

Naro F, Perez M, Migliaccio S, Galson DL, Orcel P, Teti A and Goldring SR (1998) Phospholipase D- and protein kinase C isoenzyme-dependent signal transduction pathways activated by the calcitonin receptor. *Endocrinology* **139:**3241–3248.

Nussenzveig DR, Thaw CN and Gershengorn MC (1994) Inhibition of inositol phosphate second messenger formation by intracellular loop one of a human calcitonin receptor. *J Biol Chem* **269:**28123–28129.

Ogier S-A, Mitchell R and Fink G (1987) Solubilization of a large molecular weight from of the rat LHRH receptor. *J Endocrinol* **115:**151–159.

Okamoto T and Nishimoto I (1992) Detection of G protein-activator regions in M4 subtype muscarinic, cholinergic, and α2-adrenergic receptors based upon characteristics in primary structure. *J Biol Chem* **267:**8342–8346.

Pantaloni C, Brabet P, Bilanges B, Dumuis A, Houssami S, Spengler D, Bockaert J and Journot L (1996) Alternative splicing in the N-terminal extracellular domain of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor modulates receptor selectivity and relative potencies of PACAP-27 and PACAP-38 in phospholipase C activation. *J Biol Chem* **271:**22146–22151.

Pisegna JR and Wank SA (1993) Molecular-cloning and functional expression of the pituitary adenylate cyclase-activating polypeptide type-I receptor. *Proc Natl Acad Sci USA* **90:**6345–6349.

Pisegna JR and Wank SA (1996) Cloning and characterization of the signal transduction of four splice variants of the human pituitary adenylate cyclase activating polypeptide receptor. *J Biol Chem* **271:**17267–17274.

Pittner RA and Fain JN (1991) Activation of membrane-protein kinase-C by glucagon and calcium mobilizing hormones in cultured rat hepatocytes—role of phosphatidylinositol and phosphatidylcholine hydrolysis. *Biochem J* **277:**371–378.

Probst WC, Snyder LA, Schister DI, Brosius J and Sealfon SC (1992) Sequence alignment of the G-protein coupled receptor superfamily. *DNA Cell Biol* **11:**1–20.

Rawlings SR, Puiz I, Schlegel W, Bockaert J and Journot L (1995) Differential expression of PACAP/VIP receptor subtypes in clonal pituitary somatotrophs and gonadotrophs. *Endocrinology* **136:**2088–2098.

Schomerus E, Poch A, Bunting R, Mason WT and McArdle CA (1994) Effects of pituitary adenylate cyclase-activating polypeptide in the pituitary - activation of 2 signal-transduction pathways in the gonadotrope-derived alpha-T3-1 cell-line. *Endocrinology* **134:**315–323.

Segre GV and Goldring SR (1993) Receptors For secretin, calcitonin, parathyroid-hormone (PTH)/PTH-related peptide, vasoactive-intestinal-peptide, glucagon-like peptide-1, growth hormone-releasing hormone, and glucagon belong to a newly discovered G-protein-linked receptor family. *Trends Endocrinol Metab* **4:**309–314.

Slice LW, Wong HC, Sternini C, Grady EF, Bunnett NW and Walsh JH (1994) The conserved NPXnY motif present in the gastrin-releasing peptide receptor is not a general sequestration sequence. *J Biol Chem* **269:**21755–21762.

Spengler D, Waeber C, Pantaloni C, Holsboer F, Bockaert J, Seeburg PH and Journot L (1993) Differential signal-transduction by 5 splice variants of the PACAP receptor. *Nature (Lond)* **365:**170–175.

Svoboda M, Tastenoy M, Ciccarelli E, Stievenart M and Christophe J (1993) Cloning of a splice variant of the pituitary adenylate cyclase-activating polypeptide (PACAP) type-I receptor. *Biochem Biophys Res Commun* **195:**881–888.

Swillens S (1992) How to estimate the total receptor concentration when the specific radioactivity of the ligand is unknown. *Trends Pharmacol Sci* **13:**430–434.

van Rampelbergh J, Poloczek P, Francoys I, Delporte C, Winand J, Robberecht P and Waelbroeck M (1997) The pituitary adenylate cyclase activating polypeptide (PACAP I) and VIP (PACAP II VIP_1) receptors stimulate inositol phosphate synthesis in transfected CHO cells through interaction with different G proteins. *Biochim Biophys Acta* **1357:**249–255.

Wade SM, Scribner MK, Dalman HM, Taylor JM and Neubig RR (1996) Structural requirements for G_o activation by receptor-derived peptides: activation and modulation domains of the α_2-adrenergic receptors i3c region. *Mol Pharmacol* **50:**351–358.

Wess J (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *FASEB J* **11:**346–354.

Send reprint requests to: Rory Mitchell, MRC Membrane and Adapter Proteins Co-operative Group, Membrane Biology Group, Department of Biomedical Sciences, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK. E-mail: rory.mitchell@ed.ac.uk