ADP-Ribosylation Factor-Dependent Phospholipase D Activation by VPAC Receptors and a PAC1 Receptor Splice Variant

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ABSTRACT
The VPAC1 and VPAC2 receptors for vasoactive intestinal polypeptide and the PAC1 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 PAC1 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 splice variant of the PAC1 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 PAC1 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. A chimeric construct of the VPAC2 receptor body with intracellular loop 3 (i3) of the PAC1-null receptor mediated BFA-insensitive activation of PLD, whereas the response of the corresponding PAC1-hop1 construct was BFA-sensitive. Motifs in i3 of the PAC1-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 VPAC1 and VPAC2 receptors (for VIP/PACAP) and the PAC1 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 VPAC1 and VPAC2 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 VPAC1 and VPAC2 receptors have also been shown to elicit small inositol phosphate responses (MacKenzie et al., 1996; van Rampelbergh et al., 1993). The closely related PAC1 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 PAC1 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, PAC1-null, and five variants

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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; NOS, 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 PAC1-hop1 variant investigated here (Spengler et al., 1993). Splice variants of both the rat and human PAC1 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 VPAC1, VPAC2, PAC1-null, and PAC1-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 VPAC2 and PAC1 receptors. Furthermore, we provide evidence that the hop-1 splicing insert in i3 of the PAC1 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 VPAC2 receptor).

**Experimental Procedures**

**Materials**

All tissue culture media, including animal serum, genicin, penicillin, and streptomycin were obtained from Life Technologies, Irvine, UK. Radiochemicals: [125I]NaI, [125I]-PACAP-27, myo-[2-3H]inositol, and [9,10-3H]palmitic acid were obtained from PerkinElmer Life Science Products, Dreieich, 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 VPAC1, VPAC2, PAC1-null, and PAC1-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 mg/ml streptomycin, in a humidified atmosphere of 5% CO2 and 95% O2 at 37°C). Cells were cultured in Medium 199 with 0.2% BSA, 30 µg/ml geneticin, 100 U/ml penicillin, and 100 µg/ml streptomycin. After incubated for a period of 2 weeks, the medium and the cells were washed three times with 0.5 tol, and [9,10-3H]palmitic acid were obtained from PerkinElmer Life Science Products, Dreieich, 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).

**Construction of Chimeric Receptors**

Chimeric receptors were made by replacement of the i3 domain of the rat VPAC2 receptor with the i3 domains from either the short or the hop-1 form of the rat PAC1 receptor. Exchange sites were within transmembrane domain (tm) 5 and tm7 (Fig. 4). This was achieved using cDNAs encoding the rat VPAC2 receptor (R4, pBlueScript) and the null (R7b, pBlueScript) and hop1 (R7/9.1, pBlueScript) splice variants of the rat PAC1 receptor. The first domain exchange was made by using a conserved restriction (HinClI) site in the region of the cDNAs encoding the fifth transmembrane region of the VPAC2 and PAC1 receptors. After digestion with HinClI, 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, 10–100 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 1–100 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 VPAC1, VPAC2, PAC1-null, and PAC1-hop1 receptors were grown in 80-cm2 flasks in Ham’s modified F-12 medium containing 10% NCS, 300 µg/ml genicin, 100 U/ml penicillin, and 100 µg/ml streptomycin, in a humidified atmosphere of 5% CO2 and 95% O2 at 37°C. The cDNAs for the chimeric VPAC2/PAC1-null and VPAC2/PAC1-hop1 (VP4/7b/2.1c and VP4/7b/2.1c, pcDNA1) receptors were transfected into COS 7 cells using 30 µg of cDNA/6 × 106 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 VPAC1, VPAC2, PAC1-null, and PAC1-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 125I-helodermin (for VPAC1/VPAC2 receptors) or 125I-PACAP-27 (for PAC1 receptors), using 20,000 to 50,000 cpm/well. DEAE Dextran (Promega) or FuGENE 6 (Roche Diagnostics Ltd., Lewes, UK) 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 unlabelled 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% 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 125I-helodermin (for VPAC1/VPAC2 receptors) or 125I-PACAP-27 (for PAC1 receptors), using 20,000 to 50,000 cpm/well.
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 125I-PACAP-27 with PAC1null receptor-containing CHO cells, the integrity of the ligand was assessed by reverse-phase chromatography on C18-silica (Waters Ltd., Watford, UK) using an H2O/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 125I-PACAP-27.

**Determination of cAMP Production**

CHO/COS 7 cells expressing the VPAC1, VPAC2, PAC1null, and PAC1free 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 radiomunnoassay technique described previously (Morrow et al., 1993; Lutz et al., 1999).

**Measurement of [3H]Phosphatidylbutanol ([3H]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 [2-3H]palmitate (40 Ci/mmol) for 18 h before assay. PLD activity was monitored as the production of [3H]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 H2O were added to give a ratio of methanol/chloroform/H2O 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 redisolved in 50 μl of chloroform/ethanol (1:1) 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, 0.5 ml of 2,2,4-trimethylpentane, 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 [3H]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 VPAC2 receptor were carried out using the VPAC2 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 12,000g 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-ARF198-112 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 (12,000g for 5 min). The pellet was washed twice with equivalent buffer before resuspension into similar buffer with the addition of (sonicated) phosphatidycho-
The time course of ligand binding in the VPAC1 receptor showed extensive internalization reaching a maximum by 20 min. Surface binding reached a steady state by 10 min and continued, as illustrated in Fig. 1A, showing rapid equilibration of cell-surface binding. After incubation for 1 h, assays were terminated, and PEG precipitated and harvested as described to define nonspecific binding. After incubation for 1 h, the samples were centrifuged at 12,000 rpm for 30 min. Aliquots of supernatant were precipitated with protein G-Sepharose, and the pellets were washed with solubilization buffer and phosphatase-inhibitors used for experiments with native ARF, plus 1% CHAPS. After preclearing with protein G-Sepharose, extracts were centrifuged at 12,000 rpm 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 of IgG/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 MgCl₂ and 0.2% BSA. Approximately 11,000 cpm of ¹²⁵I-helodermin was added per tube, with or 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 12,000g for 20 min, the supernatant was aspirated, and the tube tips were removed for gamma-counting of the pellets.

**Epitope-Tagged ARF**

From the COS 7 cells cotransfected with expression plasmids encoding the PAC₁ and PAC₂ 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-cm² 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 rpm 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 of IgG/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 MgCl₂ and 0.2% BSA. Approximately 11,000 cpm of ¹²⁵I-PACAP-27 was added per tube, with or without 100 nM PACAP-38 to define nonspecific binding. After incubation for 1 h on ice, the samples were terminated, and PEG precipitated and harvested as described above.

**Results**

**VPAC₁ and VPAC₂ Receptors.** The cell-surface expression of ¹²⁵I-helodermin binding sites and the internalization of ligand were measured in selected CHO cell clones expressing VPAC₁ and VPAC₂ receptors (Fig. 1A; Table 1). Cell surface VPAC₁ and VPAC₂ 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 ligand binding in the VPAC₁ receptor CHO clone was not studied in detail.

<table>
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<tr>
<th>IC₅₀ helodermin (nM)</th>
<th>Bmax (pmol/mg protein)</th>
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<tr>
<td>VPAC₁</td>
<td>VPAC₂</td>
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<tr>
<td>External</td>
<td>Internal</td>
</tr>
<tr>
<td>1.02 ± 0.35</td>
<td>3.05 ± 0.52</td>
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<tr>
<td>0.07 ± 0.01</td>
<td>1.23 ± 0.34</td>
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The ability of the VPAC₁ and VPAC₂ receptor CHO clones to show activation of AC, PLC, and PLD in response to agonists was assessed by cAMP, [³H]InsP, and [³H]PtdBut production assays (Fig. 1, B and C; Table 2). VIP elicited robust, concentration-dependent increases in cAMP production at VPAC₁ and VPAC₂ 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 VPAC₁ receptor CHO cells; probably reflecting the lower level of receptor expression. Neither the VPAC₁ nor VPAC₂ receptor CHO cell clones showed produced any detectable rise in [³H]InsP production in response to VIP (0.3 nM–3 μM). Both VPAC₁ and VPAC₂ receptors did however mediate small but consistent [³H]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 VPAC₁ receptor was less than that of the VPAC₂ receptor.

To assess whether the PLD responses might be downstream of the activation of Gβ or adenylyl cyclase, [³H]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 [³H]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 VPAC₁ and VPAC₂ receptor PLD responses with relatively high potency, yet was without effect on cAMP responses.

Coimmunoprecipitation experiments were carried out on solubilized extracts of the VPAC₂ 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 VPAC₂ receptor was detected by specific binding of ¹²⁵I-helodermin to the precipitated proteins because suitable high-affinity antibodies for the VPAC₂ receptor are not available. No specific binding was detected in wild-type CHO cells. Approximately 28% of the initial specific ¹²⁵I-helodermin binding could be solubilized and recovered as viable specific ligand binding sites from VPAC₂ receptor-CHO cells by the procedure used. Figure 1E shows that specific binding of ¹²⁵I-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.
PAC1-null and PAC1-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 PAC1-null and PAC1-hop1 receptors (Fig. 2, A and B; Table 3). The accumulation of ligand in both compartments of PAC1-null and PAC1-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 PAC1-null and PAC1-hop1 receptors (Fig. 2, A and B) and to data from the VPAC2 receptor (Fig. 1A). The steady-state accumulation of $^{125}$I-PACAP-27 into the intracellular compartment seemed to be slightly greater for the PAC1-null receptor than for the PAC1-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 PAC1-null and PAC1-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 Rampelberg et al., 1997), both PAC1-null and PAC1-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 PAC1-null receptor was consistently 2.5- to 3-fold greater than that of the PAC1-hop1 receptor despite the very similar cAMP responses. Both PAC1-null and PAC1-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 PAC1-hop1 receptor was consistently around 3-fold that of the PAC1-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 PAC1-hop1 receptor was significantly inhibited by BFA (at concentrations of 50 μM and above), whereas that of the PAC1-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 PAC1-hop1 receptor were unaffected by BFA. Figure 3D shows that the $[^{3}H]$PtdBut response of the PAC1-null receptor was inhibited to a greater extent than that of the PAC1-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 PAC1-null receptor response.

PAC1-null and PAC1-hop1 receptors were cotransfected with ARF1-HA into COS 7 cells and receptor:small G protein immunoprecipitates. These were probed for specific $^{125}$I-PACAP-27 binding from PAC1-null and PAC1-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 PAC1-hop1 but not PAC1-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-

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**Table 2**

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<th>VCAP$_{1}$</th>
<th>VPAC$_{2}$</th>
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<tr>
<td></td>
<td>EC$_{50}$</td>
<td>$E_{\text{max}}$</td>
</tr>
<tr>
<td>cAMP Production</td>
<td>nM</td>
<td>fold of basal</td>
</tr>
<tr>
<td>$[^{3}H]$InsP production</td>
<td>0.065 ± 0.013</td>
<td>59 ± 1</td>
</tr>
<tr>
<td>$[^{3}H]$PtdBut production</td>
<td>6.2 ± 2.5</td>
<td>1.83 ± 0.29</td>
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**Table 3**

Characteristics of cell surface and internalized $^{125}$I-PACAP-27 binding in CHO cells expressing PAC1-null and PAC1-hop1 receptors

<table>
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<tr>
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<th>PAC1-null</th>
<th>PAC1-hop1</th>
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<tbody>
<tr>
<td></td>
<td>External</td>
<td>Internal</td>
</tr>
<tr>
<td>IC$_{50}$ PACAP-27 (nM)</td>
<td>20.8 ± 3.2</td>
<td>10.8 ± 2.3</td>
</tr>
<tr>
<td>B$_{\text{max}}$ (pmol/mg protein)</td>
<td>4.1 ± 0.5</td>
<td>7.9 ± 0.9</td>
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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 VPAC2/PAC1 Receptors. To address the importance of the i3 sequence of the PAC1 receptor splice variants in determining the route and extent of coupling to PLD, we constructed chimeric VPAC2/PAC1 receptors (Fig. 4). These contained the body of the VPAC2 receptor with a segment from tm5 to tm7 (i.e., including the i3 domain) derived from either PAC1-null or PAC1-hop1 receptor sequences. Thus, the only difference between these two constructs was the additional 28 amino acid hop-1 cassette in the VPAC2/PAC1-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 VPAC2/PAC1-null and VPAC2/PAC1-hop1 receptor constructs mediated robust cAMP responses to VIP (mean maximal responses of 149- and 143-fold of basal and EC50 values of 0.8 ± 0.1 and 1.5 ± 0.2 nM, respectively; means ± S.E.M., n = 10; Fig. 5A). The potency of these responses was less than in CHO cells (as described previously for wild-type PAC1 and VPAC2 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 EC50 value of 0.034 ± 0.014 nM (mean ± S.E.M., n = 6). [3H]InsP responses of the receptors were not investigated. Both chimeric constructs, however, mediated clear and similar [3H]PtdBut production responses to VIP (Fig. 5B). Although the EC50 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 VPAC2/PAC1-null and VPAC2/PAC1-hop1 constructs, respectively; means ± S.E.M., n = 5-10). 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 [3H]PtdBut response only at very high concentrations of GLP-1 (7-36)
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 [3H]PtdBut responses of the receptors were challenged with BFA, only that of the VPAC_{2}/PAC_{1-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-null} or PAC_{1-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 GH3 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 GH3 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 GH3 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-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-null} 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-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-null} and PAC_{1-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-null} or PAC_{1-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-
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 PAC1-null and PAC1-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 PAC1-null and PAC1-SV-2 (equivalent to hop1) receptors expressed in NIH/3T3 cells display similar potencies of PLC activation but the PAC1-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 PAC1-null receptor in CHO cells was greater than that of the PAC1-hop1 variant for similar levels of receptor expression. Both species differences and host cell differences may contribute to the disparity.

In contrast, VPAC1 and VPAC2 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 VPAC1 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 0.06 ± 0.01 pmol VPAC1 receptor/mg protein here. In the present experiments, neither VPAC1 nor VPAC2 receptor CHO cell clones demonstrated detectable [3H]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 VPAC1, VPAC2, PAC1-null, and PAC1-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 VPAC1 and VPAC2 receptors mediated modest PLD responses to VIP (with a lower maximal response for the VPAC1 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 VPAC1 and VPAC2 receptor PLD responses were inhibited with relatively high potency by the ARF inhibitor BFA, whereas cAMP responses of the VPAC2 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 PAC1-null and PAC1-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 PAC1-null receptor (but not the hop1 variant) was inhibited by low concentrations of the PLC inhibitor U73122, suggesting that it may result substantially from PLC-dependent pathways. In contrast, the PAC1-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 PAC1 receptor to an ARF-dependent route of PLD activation. Correspondingly, immunoprecipitation of epitope-tagged ARF1 resulted in coprecipitation of PAC1-hop1 but not PAC1-null receptors (Fig. 3E).

Chimeric VPAC2/PAC1 receptors containing either PAC1-null or PAC1-hop1 i3 domains were constructed to address whether BFA-sensitivity/insensitivity could be conferred just by an i3 domain swap. The chimeric VPAC2/PAC1 receptors with i3 domains from either PAC1-null or PAC1-hop1 receptors showed no apparent difference in their cAMP responses. However, the VPAC2/PAC1-hop1 construct (just like the wildtype PAC1-hop1 receptor) showed a PLD response insensitive to BFA, despite the main body of the construct, apart from i3, being of VPAC2 (BFA-sensitive) origin. The VPAC2/PAC1-hop1 chimera retained BFA sensitivity, indicating that the i3 sequence of PAC1 receptors is a critical determinant of coupling to ARF-dependent PLD activation. Some analogy can be drawn with the dopamine D3 receptor, where i3 splice variants couple differentially to Gαi2 (Guirandam et al., 1995) and the calcitonin receptor where i1 variants couple differentially to PLC but not to AC (Nussenzevig 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 VPAC2 and PAC1 receptors was demonstrated using GH3 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 hop1 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 α2-adrenergic receptor activate Gαi2 in vitro, providing they possess basic amino acids spaced throughout the peptide and end with a BBxBX 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 α2-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 VPAC2 receptor contains a classical BBxB motif at the i3tm6 junction, whereas both the VPAC1 and PAC1-hop1 receptors contain a motif similar to that seen in the α2-adrenergic receptor with spaced basic residues and a cluster of basic amino acids up-stream of tm6. The presence of the hop1 insert in the PAC1 receptor provides the cluster of basic amino acids that completes a spaced basic residue motif. The PAC1-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 PAC1 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 of the receptor 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 PAC1 receptor and with chimeric receptor constructs incorporating i3 domain swaps it seems that the i3 structure of the PAC1 receptor is a critical determinant of both its physical association with ARF1 and its ARF-dependent coupling to PLD.

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