British Journal of Pharmacology (2006) 148, 1051–1059
www.nature.com/bjp

Pharmacological properties of Chinese hamster ovary cells coexpressing two vasoactive intestinal peptide receptors (hVPAC1 and hVPAC2)

*,[1]Ingrid Langer, [1]Nathalie Gaspard & [1]Patrick Robberecht

[1]Department of Biological Chemistry and Nutrition, School of Medicine, Université Libre de Bruxelles, 808 Route de Lennik, CP 611, Brussels B-1070, Belgium

1 In the light of recent findings that VPAC1 and VPAC2 receptors form homodimers and heterodimers, we have evaluated the function of these receptors coexpressed in the same cells, using whole-cell and membrane preparations. Cells expressing each receptor alone were used for comparison.

2 The study was performed on Chinese hamster ovary cells stably transfected with both human recombinant receptors and we compared receptor occupancy and adenylate cyclase activation by VIP, Ro 25-1553 – a VPAC2 selective agonist – and [K^{15},R^{16},L^{27}]VIP(1-7)/GRF(8-27) – a VPAC1 selective agonist – on membranes prepared from each cell line and on a mixture of membranes from cells expressing each receptor individually. We also studied receptor internalization induced by the three agonists on intact cells expressing both receptors alone or together by fluorescence-activated cell sorting using monoclonal antibodies and demonstrated by using co-immunoprecipitation that the two receptors did interact.

3 The results indicated that coexpression of the receptors did not modify the recognition of ligands, nor the capacity of the agonists to stimulate adenylate cyclase activity and, in intact cells, to induce internalization of the receptors.

4 As a consequence, the properties of the selective ligands that were established on cell lines expressing a single population of VIP receptors were valid on cells expressing both receptors. Furthermore, the recently demonstrated VPAC1/VPAC2 receptor heterodimerization did not affect the function of either receptor.
British Journal of Pharmacology (2006) **148,** 1051–1059. doi:10.1038/sj.bjp.0706816;
published online 19 June 2006

Keywords: Vasoactive intestinal peptide; VPAC1; VPAC2; coexpression; selective agonist; receptor internalization; cAMP

Abbreviations: CHO, Chinese hamster ovary; FACS, fluorescence-activated cell sorting; GPCR, G-protein-coupled receptors; VIP, vasoactive intestinal peptide

Introduction

Vasoactive intestinal polypeptide (VIP) is a neuropeptide that contributes to the regulation of intestinal secretion and motility, of exocrine and endocrine secretions, and to homeostasis of the immune system (Harmar *et al*., 1998; Delgado *et al*., 2004). The effects of VIP are mediated through interaction with two receptors named VPAC1 and VPAC2, members of the G-protein-coupled receptors B (GPCR-B) family. This family also includes the receptors for pituitary adenylate cyclase activating peptide, secretin, glucagon, glucagon-like peptides, calcitonin, parathyroid hormone and growth hormone releasing factor. Like all members of the GPCR-B family, VPAC1 and VPAC2 are preferentially coupled to $G\alpha_s$ proteins that stimulate adenylate cyclase activity and induce cAMP increase, although a coupling to the phospholipase C and the calcium/IP3 pathway through either $G\alpha_q$ or $G\alpha_i$ is also effective (Harmar *et al*., 1998; Langer *et al*., 2001).

Current paradigms of ligand binding, GPCR activation and signal transduction are based on the assumption that GPCRs exist and function as individual entities (Gether & Kobilka, 1998; Ballesteros *et al*., 2001; Pierce *et al*., 2002). However, several recent studies have demonstrated that the pharmacological behaviour of one receptor may be modified by the presence of other, structurally related or not, receptors. Besides the recognized postreceptor interactions, recent biochemical and biophysical studies indicate that, as do single transmembrane receptors, GPCRs also form oligomeric complexes either with themselves (homo-oligomerization) or with other receptors (hetero-oligomerization) that may affect their pharmacological profiles (Milligan, 2004; Terrillon & Bouvier, 2004; Prinster *et al*., 2005). If the concept of GPCR oligomerization is now accepted, the pharmacological and functional consequences of those complexes remain incompletely understood.

Recently, Harikumar *et al*. (2006) demonstrated by using biophysical methods that VPAC1 and VPAC2 receptors formed constitutive homo- and hetero-oligomers (Harikumar *et al*., 2006) but the pharmacological and functional consequences of those oligomerizations were not studied. On the other hand, two studies performed on the prostate cancer cell line LNCaP (Juarranz *et al*., 2001) and on adipocytes (Akesson

*Author for correspondence; E-mail: ilanger@ulb.ac.be

et al., 2005) demonstrated that those cells expressed both VPAC1 and VPAC2 receptors but differed in their pharmacological response with respect to the selective VPAC1 and VPAC2 agonists. LNCaP cells displayed a VPAC1-like phenotype (the VPAC2 selective agonist being inactive) whereas adipocytes displayed a VPAC2-like phenotype (the VPAC1 selective agonist being inactive). One possible explanation of these results might be the formation of inactive receptor dimers, neutralizing the receptor expressed at the lowest concentration and allowing only one receptor to be active.

With this background, we have studied the effect of coexpressing VPAC1 and VPAC2 receptors in Chinese hamster ovary (CHO) cells on ligand binding, adenylate cyclase activation, receptor internalization and trafficking, and compared the results to those obtained with membranes or cells expressing only one receptor type. Three agonists were used, the natural ligand VIP, and selective VPAC1 and VPAC2 agonists. We found that pharmacological properties of cells expressing both receptors were not different from those obtained when mixing cells expressing each receptor individually. Similarly, coexpression of VIP receptors did not modify receptor internalization and trafficking patterns following exposure to VIP or selective agonists. These results suggest that if hetero-oligomerization of VPAC1 and VPAC2 receptors occurred in living cells, the pharmacological consequences would not be significant, at least for the variables tested here.

Methods

Cell culture and transfection

Details of the cell lines expressing VPAC1 or VPAC2 receptors have been given in a previous publication (Langer *et al*., 2001). Briefly, to obtain a cell line expressing both receptors, 15 μg of the receptor-coding region for human VPAC1 and for human VPAC2 receptors were transfected by electroporation in a CHO cell line expressing aequorin (kindly provided by Vincent Dupriez, Euroscreen SA, Belgium) as described (Langer *et al*., 2001). Selection was carried out in culture medium (50% HamF12; 50% DMEM; 10% fetal calf serum; 1% penicillin (10 mU ml^{-1}); 1% streptomycin (10 μg ml^{-1}); 1% L-glutamine (200 mM); PAA, Pashing, Austria), supplemented with 600 μg geneticin (G418) ml^{-1} culture medium. After 10–15 days of selection, isolated colonies were transferred to 24-well plates and grown until confluence, trypsinized and further expanded in six-well plates. Selection of cell clones expressing both receptors was performed by fluorescence-activated cell sorting (FACS) analysis using monoclonal antibodies against the N-terminal domains of VPAC1 or VPAC2 receptors (see below and Langlet *et al*., 2004; 2005).

Immunohistochemistry

Cells were cultured on 22 mm glass slides for 72 h, washed in phosphate-buffered saline (PBS) and fixed with absolute methanol (−20°C) for 10 min. Nonspecific protein binding was prevented by 15 min incubation with 5% normal sheep serum. The cells were then incubated overnight at 4°C with a mixture of monoclonal anti-VPAC1 antibody (2 μg ml^{-1}) and polyclonal anti-VPAC2 antibody (1 μg ml^{-1}) or a mixture of monoclonal anti-VPAC2 antibody (2 μg ml^{-1}) and polyclonal anti-VPAC1 antibody (1 μg ml^{-1}). The primary antibodies were diluted in PBS – 1% normal sheep serum – 1% sodium azide. After three washes, and a second 15 min incubation at room temperature with normal sheep serum, the cells were incubated for 30 min at room temperature with a mixture of FITC-conjugated γ-chain-specific goat anti-mouse IgG (1 : 100) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1 : 100) and diluted in the same solution as the primary antibody. Omission of the primary or secondary antibody resulted in the absence of labeling. After three rinses in PBS, coverslips were mounted with Calbiochem mounting medium before viewing under a fluorescent microscope (Axioplan, Zeiss).

Immunoprecipitation and Western blots

Cells were lysed following 20 min incubation on ice in buffer consisting of 50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM orthovanadate, 1.5% CHAPS and a mixture of protease inhibitors (Complete®); pH 7.5. The remaining insoluble material was eliminated by centrifugation at 15,000 × *g* for 20 min at 4°C. Protein concentration was evaluated by using a BCA assay kit. For each sample, the same amount of protein was added to 50 μl of a 10% protein A Sepharose suspension coated during 2 h with 2 μg of monoclonal anti-VPAC1 or anti-VPAC2 antibody. After 150 min incubation under rotating agitation at 4°C, the Sepharose beads were separated by centrifugation and washed three times with buffer consisting of HEPES 30 mM, NaCl 30 mM and Triton X-100 0.1%. The final pellet of beads was resuspended in a buffer consisting of 125 mM Tris, 10% β-mercaptoethanol, 4% SDS, 20% glycerol, 0.02% bromophenol blue, pH 6.8. After heating at 60°C for 10 min, the samples were resolved by SDS–PAGE using a 10% gel and transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated in blocking buffer (TBS with 3% milk and 0.1% Tween 20) for 1 h at room temperature then probed with polyclonal anti-VPAC1 or anti-VPAC2 antibody diluted 1 : 2000 in TBS + 1% milk + 0.1% Tween 20 overnight at 4°C. Membranes were washed three times in TBS + 0.1% Tween 20 + 0.3% BSA, then probed with HRP goat anti-rabbit antibody diluted 1 : 50.000 in TBS + 0.1% Tween 20 0.3% BSA for 1 h at room temperature. Proteins were visualized using SuperSignal® West Pico Chemiluminescent Substrate.

Membrane preparation

Membranes were prepared from scraped cells lysed in 1 mM $NaHCO_3$ by immediate freezing in liquid nitrogen. After thawing, the lysate was first centrifuged at 4°C for 10 min at 400 × *g* and the supernatant was further centrifuged at 20,000 × *g* for 10 min. The resulting pellet, re-suspended in 1 mM $NaHCO_3$ was used immediately as a crude membrane fraction.

Adenylate cyclase activation assay

Adenylate cyclase activity was assayed, as described previously (Salomon *et al*., 1974). Membrane proteins (3–15 μg) were incubated in a total volume of 60 μl containing 0.5 mM [α^{32}P]-

ATP, 10 μM GTP, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM cAMP, 1 mM theophylline, 10 mM phospho-enol pyruvate, 30 μg ml^{-1} pyruvate kinase and 30 mM Tris-HCl at a final pH of 7.8. The reaction was initiated by addition of membrane protein and was terminated after 15 min incubation at 37°C by adding 0.5 ml of a 0.5% sodium dodecylsulphate solution containing 0.5 mM ATP, 0.5 mM cAMP and 20 000 c.p.m. [^{3}H]-cAMP. cAMP was separated from ATP by chromatography on Dowex 50 W × 8 and then on neutral alumina.

Binding studies

Binding studies were performed by using as tracers [^{125}I]VIP (showing a mild preference for the VPAC1 receptor) and [^{125}I]-(Ro 25-1553), a highly selective VPAC2 receptor agonist. The nonspecific binding was defined as the residual binding in the presence of 1 μM unlabeled agonist. Binding was performed for 30 min at 23°C in a total volume of 120 μl containing 20 mM Tris-maleate, 2 mM MgCl2, 0.1 mg ml^{-1} bacitracin, 1% bovine serum albumin (pH 7.4) buffer. Three to thirty micrograms of protein were used per assay. Bound and free radioactivity were separated by filtration through glass-fiber GF/C filters presoaked for 24 h in 0.01% polyethyleneimine and rinsed three times with a 20 mM (pH 7.4) ice-cold sodium phosphate buffer containing 0.8% bovine serum albumin.

Receptor internalization and trafficking

Receptor internalization was defined as the percentage of cell surface receptors that were no longer accessible to the monoclonal antibody, after agonist exposure. Cells expressing VPAC1, VPAC2 or both receptors were incubated with agonist at 37°C. After three washings with ice-cold PBS, cells were detached from the plates using a 5 mM EDTA/EGTA PBS solution, harvested by centrifugation (500 × *g*, 4°C, 4 min), washed once with PBS solution and re-suspended at density of 3×10^5 cells assay^{-1} in 100 μl PBS-BSA 0.1%, containing 0.1 μg purified anti-VPAC1 or anti-VPAC2 receptor monoclonal antibody. After 30 min incubation at 4°C, the cells were washed with PBS–BSA 0.1% and then incubated with FITC-conjugated γ-chain-specific goat anti-mouse IgG for 30 min, on ice, in the dark. The labelled cells were washed and re-suspended in 250 μl PBS–BSA 0.1%. The fluorescence level was analysed using a FACScalibur (BD Biosciences, Belgium) and the data processed using the Cell Quest software (Becton Dickinson). Basal fluorescence was determined from samples of untransfected CHO cells. Propidium iodide (10 μg ml^{-1}) was used to exclude debris and dead cells from the analysis. Details on specific protocols for evaluation of receptor recovery are given in the legends of the figures.

Peptide synthesis

The peptides used were synthesized in our laboratory as described (Gourlet *et al*., 1997a, b). Peptide purity was assessed by capillary electrophoresis, and conformity by electrospray MS. The sequence of the selective VPAC1 agonist was [His^1,K^{15},R^{16},L^{27}]-VIP (1–7)/GRF (8–27) and that of the selective VPAC2 agonist (Ro 25-1553) was AcHis^1[E^8,K^{12},N-le^{17},A^{19}]-VIP (1–24)/[DLKKGGT]$^{(28–31)}$ with a lactam ring between K^{21} and K^{28}, respectively.

Materials

The FITC-conjugated γ-chain-specific goat anti-mouse IgG and the 10% protein A Sepharose suspension were from Sigma Chemicals Co. (MO, U.S.A.), Alexa Fluor 488-conjugated goat anti-rabbit IgG from Molecular Probes (CA, U.S.A.), and the polyclonal anti-VPAC antibodies were a gift from Dr Stephan Schulz (Schulz *et al*., 2004). The mixture of protease inhibitors (Complete®) was from Roche Diagnostics, Belgium and Pierce, Perbio Science, Belgium supplied the HRP goat anti-rabbit antibody, the BCA assay kit and the SuperSignal® West Pico Chemiluminescent Substrate.

Data analysis

All competition curves, dose–response curves, IC_{50} and EC_{50} values were calculated using nonlinear regression (GraphPad Prism software). Statistical analyses were performed with the same software.

Results

Immunohistochemistry

Selection of CHO cell clones expressing both VIP receptors was first performed by determination of adenylate cyclase activity, stimulated by VIP. Among the 48 clones tested, 30 displayed cAMP increase in response to 1 μM VIP. These 30 clones were further analysed by dot-blot using polyclonal antibodies raised against the C-terminus of each receptor. Eight clones expressed both receptors whereas 11 clones expressed VPAC1 receptors only and the last 11 clones expressed VPAC2 receptors only. The clone used in this study was chosen on the basis of a similar magnitude of FACS signal, using the two monoclonal antibodies raised against the N-terminus of each receptor. Immunohistochemistry was finally used to verify that cells of the clone selected by FACS analysis did express VPAC1 and VPAC2 receptors simultaneously, by using four specific anti-VPAC receptors antibodies: two monoclonal antibodies raised against the N-terminus and two polyclonal antibodies raised against the C-terminus of each receptor. Whatever the combination used, all cells expressed both VPAC1 and VPAC2 as shown in Figure 1.

Immunoprecipitation and Western blots

Receptor density was evaluated using immunoprecipitation followed by Western blots for extracts of CHO cells expressing VPAC1 and VPAC2 receptors, either alone or in combination. As shown in Figure 2, receptor density in cells expressing both receptors (VPAC1/2) was about two-fold lower than for cells expressing each receptor alone. We then used co-immunoprecipitation to evaluate VPAC1 and VPAC2 receptor association in VPAC1/2 cells. As shown in Figure 3, following immunoprecipitation of CHO cells extracts expressing both receptors (VPAC1/2) with either monoclonal anti-VPAC1 or anti-VPAC2 antibody, the polyclonal anti-VPAC1 antibody recognized specifically a protein band of 75 kDa compatible with the molecular weight of glycosylated VPAC1 receptor, whereas for the mixture of protein extracts prepared from cells expressing each receptor individually (VPAC1 + VPAC2), the polyclonal anti-VPAC1 antibody detected a specific protein

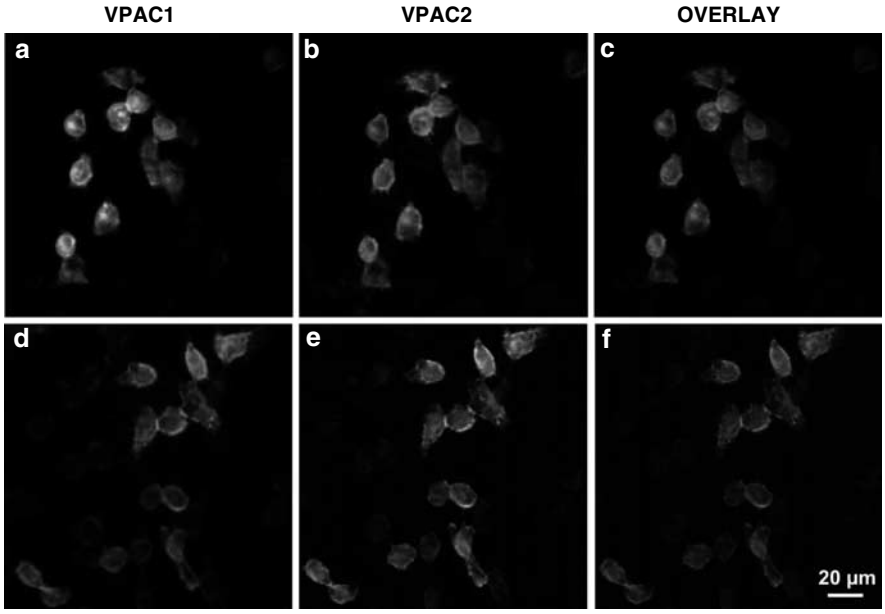

Figure 1 Visualization by double-colour immuofluorescence of VPAC1 and VPAC2 receptors. Cells cotransfected with VPAC1 and VPAC2 receptors were labelled using a mixture of mouse monoclonal anti-VPAC1 and rabbit polyclonal anti-VPAC2 (a–c) or a mixture of rabbit polyclonal anti-VPAC1 and mouse monoclonal anti-VPAC2 antibodies (d–f). Receptors were visualized using FITC-conjugated goat anti-mouse IgG (green) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (red). (For colour figure see online.)

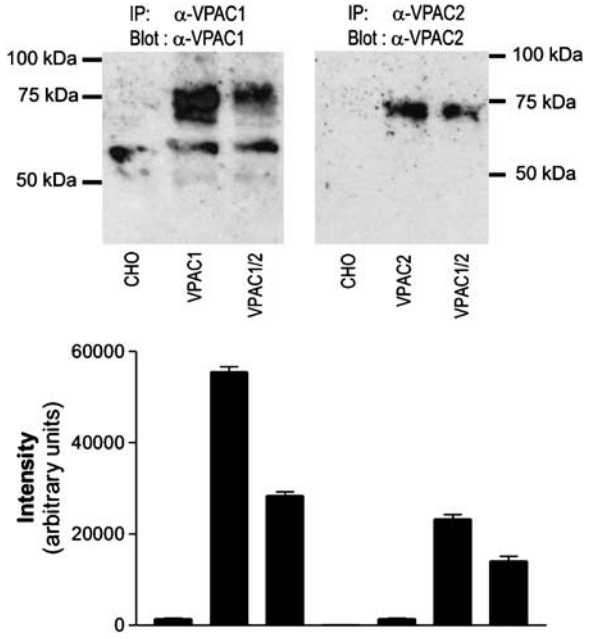

Figure 2 Relative quantification of VPAC1 and VPAC2 receptors by Western blots. The same amount of protein, extracted from wild-type CHO cells (lanes 1 and 4) or from CHO cells stably transfected with VPAC1 receptor (lane 2), VPAC2 receptor (lane 5) or both receptors (VPAC1/2, lanes 3 and 6), was incubated with monoclonal anti-VPAC1 (left panel) or monoclonal anti-VPAC2 (right panel) antibody. The immunoprecipitates were then immunoblotted using polyclonal anti-VPAC1 (left panel) or polyclonal anti-VPAC2 (right panel) antibody. Polyclonal anti-VPAC1 recognized a 75 kDa protein band compatible with glycosylated VPAC1 receptor molecular weight (left panel) and polyclonal anti-VPAC2 a 70 kDa protein band compatible with glycosylated VPAC2 receptor molecular weight (right panel). The blot shown is representative of three separate experiments. The lower graph represents the integration of the bands in arbitrary units (means of three independent experiments).

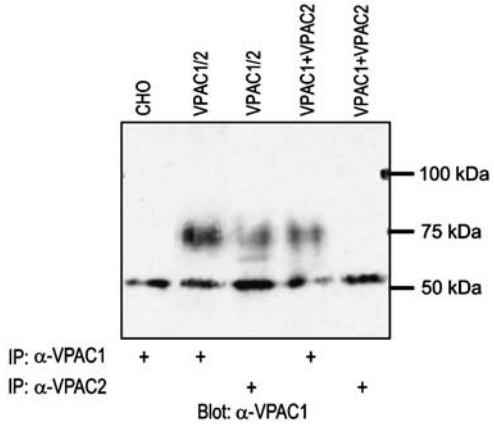

Figure 3 Evaluation by co-immunoprecipitation of VPAC1 and VPAC2 receptor association in VPAC1/2 cells. Protein extracts of wild-type CHO cells (lane 1), of CHO cells stably cotransfected with VPAC1 and VPAC2 receptor (VPAC1/2, lanes 2 and 3), or a mixture of protein extracts of CHO expressing each receptor individually (VPAC1 + VPAC2, lanes 4 and 5) were incubated with monoclonal anti-VPAC1 (lanes 1, 2 and 4) or monoclonal anti-VPAC2 (lanes 3 and 5) antibody (the same total amount of protein was used in each condition). The immunoprecipitates were then immunoblotted using polyclonal anti-VPAC1. Coimmunoprecipitation of VPAC1 receptor with VPAC2 receptor can be seen for VPAC1/2 cells (lane 3) but not for the mixture of cells expressing each receptor individually, indicating that the two receptors interact together when coexpressed in the same cell. The figure is representative of three experiments.

band following immunoprecipitation with the monoclonal anti-VPAC1 antibody only.

Binding studies

Binding experiments were performed on membranes prepared from cells expressing each receptor (VPAC1 or VPAC2), both

receptors (VPAC1/2) or a mixture of membranes prepared from cells expressing each receptor individually (VPAC1 + VPAC2). [^{125}I]VIP competition curves analysis in presence of agonists indicated that, for both VPAC1/2-expressing cells and the VPAC1 + VPAC2 mixture, the selective VPAC1 and the VPAC2 agonists bound to one affinity site and inhibited partially [^{125}I]VIP binding (Figure 4 and Table 1). Competition curves in presence of VIP were better fitted to a two affinity site model, $P=0.0009$ for VPAC1/2 ($F=9.843$, $n=5$) and $P=0.0037$ for VPAC1 + VPAC2 ($F=8.675$, $n=5$) than to a one site model (Table 1). K_i values of VIP, VPAC1 and VPAC2 agonists for VPAC1 or VPAC2 expressing cells are given in Table 1. When using the selective VPAC2 agonist as tracer ([^{125}I]VPAC2 agonist), all the agonists tested bound to one affinity site in membrane preparations from VPAC1/2-expressing cells and VPAC1 + VPAC2 cell mixture (Table 2). The VPAC1 agonist inhibited partially [^{125}I]VPAC2 agonist binding (19±4% for VPAC1/2 cells and 17±3% for VPAC1 + VPAC2 mixture, $n=3$).

Adenylate cyclase activation studies

We measured cAMP production with a range of concentrations of VIP, VPAC1 agonist and VPAC2 agonist. For membranes prepared from cells expressing both VPAC1 and VPAC2 receptors (VPAC1/2), VIP, VPAC1 agonist and VPAC2 agonist potencies (as EC_{50} values) to stimulate cAMP production were 3.0±0.5, 4.0±0.8 and 2.4±0.5 nM, respectively. Maximal cAMP production was 224±7, 154±5 and 210±6 cAMP pmol (mg protein)$^{-1}$ min^{-1} for VIP, VPAC1 agonist and VPAC2 agonist, respectively. Similar results were obtained for the mixture of membranes prepared from cells expressing each receptor individually (VPAC1 + VPAC2); the EC_{50}s of cAMP production being, for VIP 1.8±0.3 nM, for the VPAC1 agonist 1.2±0.4 nM and for the VPAC2 agonist 1.4±0.3 nM. Maximal cAMP production was 193±5, 113±4 or 126±4 cAMP pmole (mg protein)$^{-1}$ min^{-1} for VIP, VPAC1 agonist or VPAC2 agonist, respectively (Figure 5). The EC_{50}s for VIP at the VPAC1 and VPAC2 receptors were 1.5±0.3 and 2.1±0.5 nM, respectively. For the VPAC1 agonist, the EC_{50} was 1.9±0.4 nM at the VPAC1 receptor and, for the VPAC2 agonist, the EC_{50} was 1.0±0.2 nM at the VPAC2 receptor.

Receptor internalization and trafficking

We evaluated receptor internalization and trafficking following VIP, VPAC1 agonist and VPAC2 agonist exposure of cells coexpressing VPAC1 and VPAC2 receptor. As shown in Figure 6, 30 (min exposure to 1 μM VIP or VPAC1 agonist reduced by 57±2 and 50±2% the VPAC1 receptor population expressed at the cell surface, respectively, whereas the VPAC2 agonist did not modify VPAC1 receptor expression. Once internalized, the VPAC1 receptors were not re-expressed at the cell surface within 2 h after agonist washing. These results were not significantly different from those obtained for cells expressing VPAC1 receptors alone. VPAC2 receptor population expressed at the cell surface was reduced by 58±6 and 65±6% following 30 min exposure to 1 μM VIP or VPAC2 agonist, respectively. The VPAC1 agonist did not modify VPAC2 receptor expression. Following washing after exposure to VIP, VPAC2 receptors were re-expressed at the

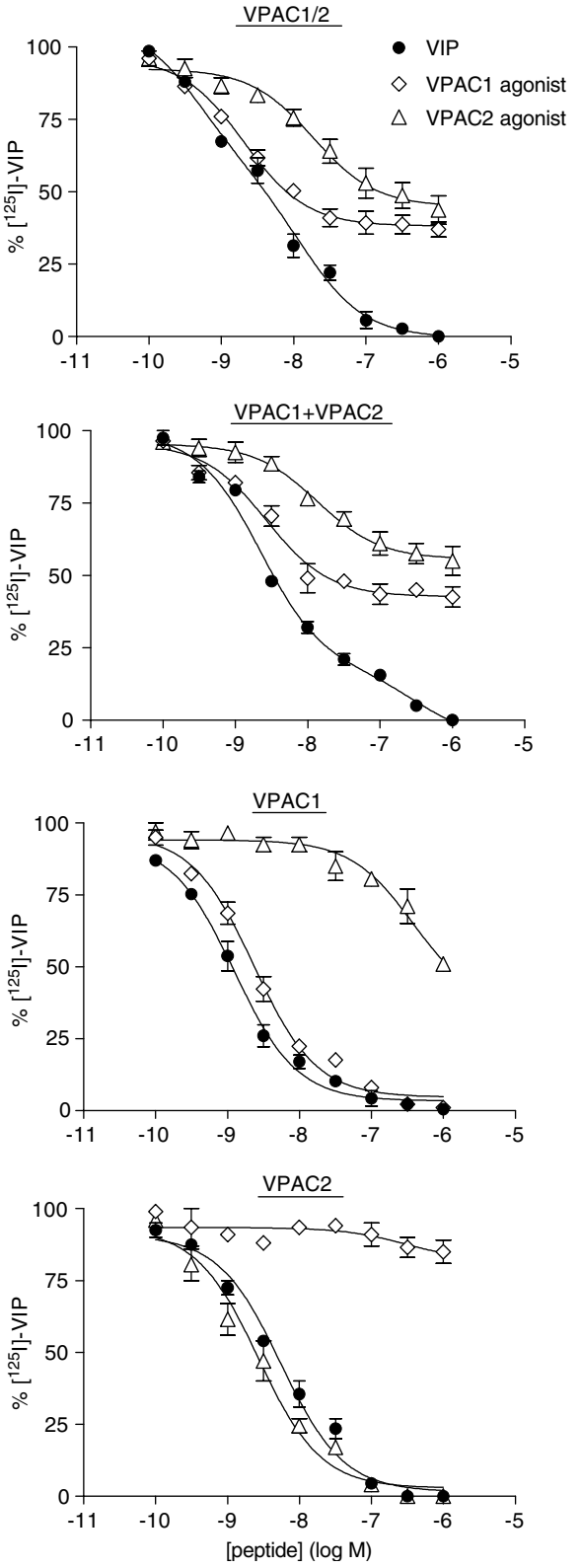

Figure 4 [^{125}I]VIP binding competition curves in presence of VIP (circles), VPAC1 agonist (lozenges) or VPAC2 agonist (triangles) of membranes prepared from CHO cells expressing both VPAC1 and VPAC2 receptors (VPAC1/2), a mixture of membranes prepared from CHO cells expressing each receptor individually (VPAC1 + VPAC2) and membranes prepared from cells expressing VPAC1 or VPAC2 receptors alone. Results represent the means±s.e.m. of five independent experiments performed in duplicate.

Table 1 Summary of binding studies using [^{125}I] VIP

	VPAC1 K_i (nM)	*VPAC2* K_i (nM)	*VPAC1/2* K_{i1} (nM)	K_{i2} (nM)	*VPAC1+VPAC2* K_{i1} (nM)	K_{i2} (nM)
VIP	1.3±0.2	5.5±0.4	0.6±0.3	14±3	1.1±0.1	20±4
VPAC1 agonist	2.3±0.3	—	2.1±0.2	NA	2.4±0.2	NA
VPAC2 agonist	1000±120	2.7±0.2	18±3	NA	14±3	NA

Results represent the means±s.e.m. of five independent experiments in duplicate.
NA: not applicable.

Table 2 Summary of binding studies using [^{125}I]-VPAC2 agonist

	VPAC2 K_i (nM)	*VPAC1/2* K_i (nM)	*VPAC1+VPAC2* K_i (nM)
VIP	5.3±0.3	6.8±0.5	5.7±0.4
VPAC1 agonist	95±10	101±18	150±20
VPAC2 agonist	3.1±0.3	3.3±0.4	3.8±0.4

Results represent the means±s.e.m. of three independent experiments in duplicate.

cell surface within 2 h (this re-expression was blocked by monensine, data not shown), whereas following VPAC2 agonist exposure and washing, the receptors were not re-expressed at the cell surface. When compared to results obtained for cells expressing VPAC2 receptors alone, the only difference found was a slight increase (15%) of the rate of internalization for VPAC2 receptors following VIP or VPAC2 agonist exposure (Figure 6).

Discussion

The classical concept of GPCRs functioning as individual entities has been challenged by recent findings that many GPCRs may exist as homo- and hetero-dimers in the cell membrane (Milligan, 2004; Terrillon & Bouvier, 2004; Prinster *et al*., 2005). If the concept of GPCR oligomerization is accepted, the new task is to evaluate the specificity and the physiological relevance of the partners, and the functional consequences of the oligomerization.

The VIP receptors, namely the VPAC1 and the VPAC2 receptors, belong to the B family of GPCR that share with the A family the heptahelical structure but lack the typical signature of the A-family: the E/DRY sequence located at the junction between the third transmembrane domain and the second intracellular loop and the NPXXY sequence located in the distal part of the seventh transmembrane domain. A few GPCR-B family members are known to form oligomers: the secretin receptor (Ding *et al*., 2002), the calcitonin receptor (Seck *et al*., 2003) and the VIP receptors (Harikumar *et al*., 2006). In ductal pancreatic adenocarcinoma, heterodimerization of the secretin receptor with a naturally occurring, inactive, splice variant leads to inactivation of the wild-type receptor (Ding *et al*., 2002). Heterodimerization of the C1a isoform and the Δe13 splice variant of the rabbit calcitonin receptor leads to a significant reduction in cell surface expression and consequently to reduced activity of the C1a

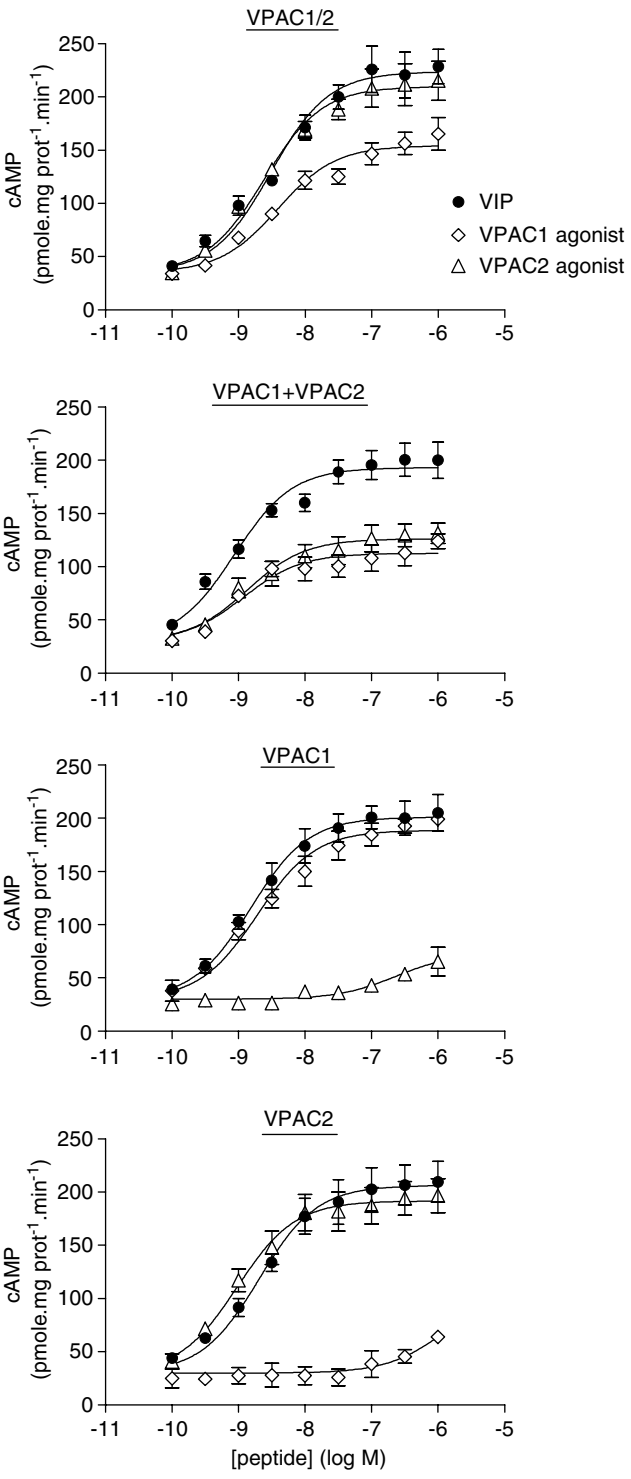

Figure 5 Dose–effect curves of VIP (circles), VPAC1 agonist (lozenges) or VPAC2 agonist (triangles) stimulating cAMP production in membranes prepared from CHO cells expressing both VPAC1 and VPAC2 receptors (VPAC1/2), a mixture of membranes prepared from CHO cells expressing each receptor individually (VPAC1 + VPAC2) and membranes prepared from cells expressing VPAC1 or VPAC2 receptors alone. Results represent the means±s.e.m. of three independent experiments performed in duplicate.

calcitonin receptor (Seck *et al*., 2003). Recently, Harikumar *et al*. (2006) demonstrated that VPAC1 and VPAC2 receptors were able to form homo- and hetero-dimers with themselves

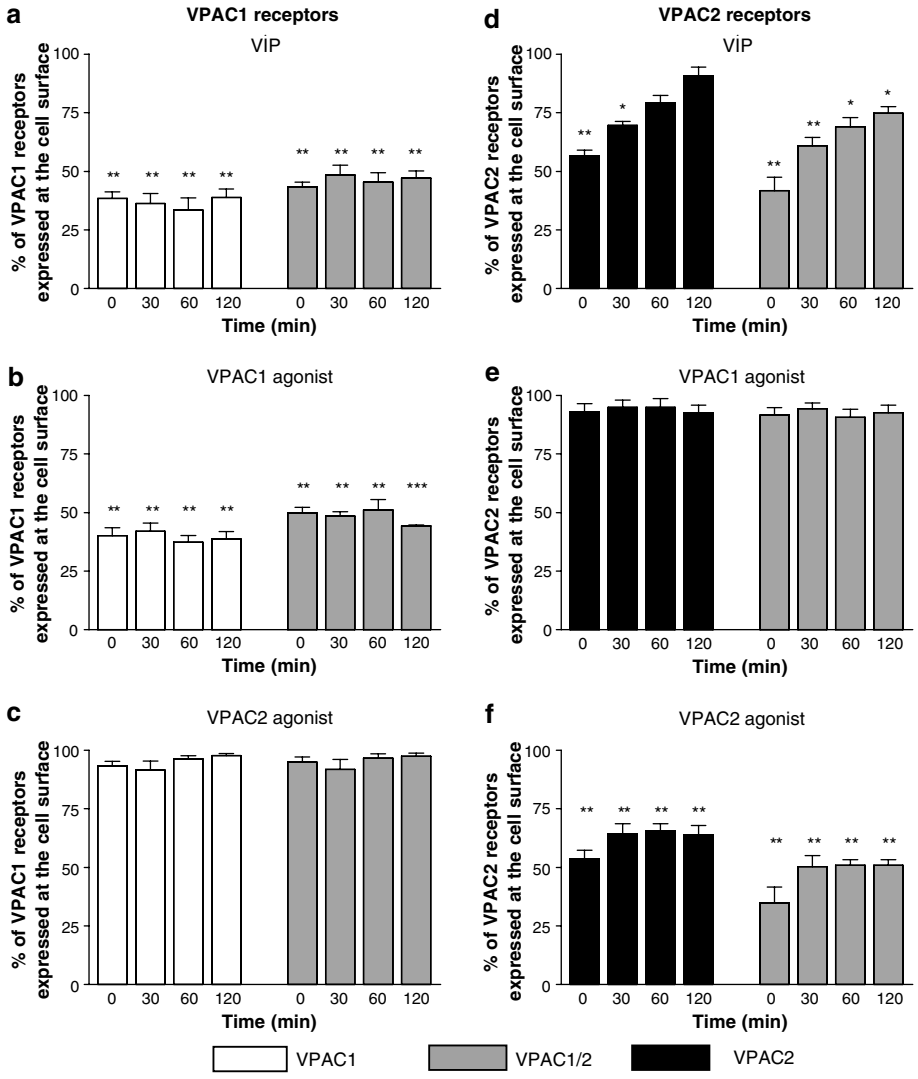

Figure 6 Evolution of the expression of VPAC1 (a–c) and VPAC2 (d–f) receptors at the surface of CHO cells expressing VPAC1 (white boxes), VPAC2 (black boxes) or both receptors (grey boxes) after 30 min exposure (time 0) to 1 μM VIP (a, d), VPAC1 agonist (b, e) or VPAC2 agonist (c, f) followed by three washings and incubation for 30, 60 and 120 min in absence of agonists. Results represent the means $\pm$ s.e.m. of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ evaluated by Student's *t*-test, as compared to receptor expression level before agonist exposure (100%).

but also with the secretin receptor (Harikumar *et al*., 2006). However the functional properties of VPAC1–VPAC2 heterodimers were not investigated.

In the present work, we have studied the pharmacological properties of VPAC1 and VPAC2 receptors coexpressed in CHO cells and confirmed, by co-immunoprecipitation, that the two receptors did interact. We found that receptor coexpression did not modify VIP or selective agonist affinities (IC_{50} values), relative to those in a mixture of cells expressing VPAC1 and VPAC2 alone. Similarly, the potency of agonists to stimulate adenylate cyclase activity was unaffected (EC_{50} values). However, the efficacy of the agonists was modified. The highly selective VPAC1 agonist stimulated adenylate cyclase maximally in cells coexpressing VPAC1 and VPAC2 receptors, a result different from that obtained with a membrane mixture. This difference could be explained as a change in the coupling efficacy of the dimerized receptors, but also by the fact that $G\alpha_s$ proteins are limiting in that system. Indeed, the maximal effect of VIP in those cell lines was identical to the maximal stimulatory effect in presence of Gpp(NH)p, a stable analogue of GTP, acting independently of receptor-mediated stimulation. We also investigated the internalization and the trafficking pattern of the two receptors following VIP, VPAC1 agonist and VPAC2 agonist exposure by FACS analysis using two monoclonal antibodies raised against the N-terminus of each receptor. Again, no qualitative changes were observed, relative to the behaviour of VPAC1 and VPAC2 receptors expressed alone in CHO cells. The only difference was a slight increase, by 15%, of the VPAC2 receptor internalization rate following VIP or VPAC2 agonist exposure.

These results thus strongly suggest that coexpression of VPAC1 and VPAC2 receptors does not modify ligand recognition, agonist signalling or capacity for internalization of receptors. This apparent lack of functional consequences of the constitutive heterodimerization of VPAC1–VPAC2 recep-

tors may be explained by the observation of Harikumar *et al*. (2006) that VIP receptor oligomers dissociate upon agonist binding (Harikumar *et al*., 2006). Our present results suggest also that the pharmacological profile of the selective VPAC1 and VPAC2 receptors that was established in cells expressing one receptor subtype was also applicable to cells that could express both receptors endogenously, such as activated lymphocytes and macrophages (Delgado *et al*., 2004) and cells from hippocampus (Harmar *et al*., 1998), prostate (Garcia-Fernandez *et al*., 2003) or lung (Busto *et al*., 2000). This would make possible the activation, antagonism or downregulation of one receptor subtype without affecting the other. This observation is of special interest in lymphocytes, where the VPAC1 receptors mediated inhibition of mobility and of Fas ligand expression (Delgado *et al*., 1995; 2001), whereas the VPAC2 receptor-mediated cell differentiation (Pankhaniya *et al*., 1998).

The GPCR-A/Rhodopsin family has been the most extensively studied group of receptors and data collected indicate that the consequences of oligomerization may be detected at the level of the recognition of both physiological and pharmacological ligands, of pathways of intracellular signalling, and of desensitization and internalization processes. For example, coactivation of D_1 D_2 dopamine receptors on cells coexpressing D1 and D2 receptors resulted in PLC-mediated increase of intracellular calcium, a signalling pathway not activated when either one of the receptors was singly activated (Lee *et al*., 2004). When coexpressed with angiotensin AT1A receptors, the AT2 receptor interacted directly with the AT1A receptor and antagonized its function (AbdAlla *et al*., 2001). Adenylate cyclase activity stimulated by activated β-adrenoceptors was found to be identical in cells expressing β_1-, β_2-, or both, adrenoceptors at similar levels. However for ERK1/2 MAPK activity, a significant agonist-promoted activation was detected in β_2- but not β_1-adrenoceptor-expressing cells and no ERK1/2 phosphorylation was observed in cells coexpressing the two receptors. A similar inhibition of agonist-promoted internalization of the β_2-adrenoceptor was observed upon coexpression of the β_1 receptor, which by itself internalized to a lesser extent (Lavoie *et al*., 2002). Coexpression of somatostatin SST2a and SST3 receptors led to a pharmacological and functional profile resembling that of the SST2a receptor in terms of agonist specificity, adenylate cyclase inhibition and ERK activation but with a greater resistance to agonist-induced desensitization (Pfeiffer *et al*., 2001). When μ- and δ-opioid receptors were coexpressed, the highly selective synthetic agonists for each receptor had a reduced potency and altered rank order. Moreover, in contrast to the individually expressed μ- and δ-receptors, the coexpressed receptors showed insensitivity to *Pertussis* toxin, most probably due to interaction with a different G protein subtype (George *et al*., 2000). Vasopressin-1 (V1) and 2 (V2) receptor coexpression led to altered receptor internalization and trafficking. Thus, activation with a nonselective agonist promoted internalization of both receptors and inhibition of V1 recycling. Activation with a V1 selective agonist however, induced internalization of both receptors that recycled back to the cell surface (Terrillon *et al*., 2004).

Besides pharmacological and functional regulation, GPCR oligomerisation also plays a role in receptor maturation and in expression at the cell surface. Thus, a functional metabotropic $GABA_B$ receptor required formation of a dimeric complex made of two subunits, $GABA_BR1$ and $GABA_BR2$ (Jones *et al*., 1998). Consistent with this idea, measurements of fluorescence or luminescence resonance energy transfer (FRET and BRET) revealed that several GPCRs dimerize in the endoplasmic reticulum (Terrillon *et al*., 2003; Salahpour *et al*., 2004). Interestingly, Harikumar *et al*. (2006) observed that VPAC1 and VPAC2 receptors formed heterodimers with secretin receptors that remained trapped in the cells. This result and ours (no evidence of modification of agonist potency and efficacy nor internalization when VPAC1 and VPAC2 receptor were coexpressed) suggest that VIP receptor oligomerization modulates receptor expression rather than receptor activity.

This work was supported by F.R.S.M. Grants no. 3.4507.98 and by an 'Interuniversity Poles of Attraction Program (PAI5/20) – Belgian State, Prime Minister's Office, Federal Office for Scientific, Technical and Cultural Affairs'. I. Langer is 'chargée de recherche' of the 'Fonds National de la Recherche Scientifique'.

References

ABDALLA, S., LOTHER, H., ABDEL-TAWAB, A.M. & QUITTERER, U. (2001). The angiotensin II AT2 receptor is an AT1 receptor antagonist. *J. Biol. Chem.,* **276,** 39721–39726.

AKESSON, L., AHREN, B., EDGREN, G. & DEGERMAN, E. (2005). VPAC2-R mediates the lipolytic effects of pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide in primary rat adipocytes. *Endocrinology,* **146,** 744–750.

BALLESTEROS, J.A., SHI, L. & JAVITCH, J.A. (2001). Structural mimicry in G protein-coupled receptors: implications of the high-resolution structure of rhodopsin for structure–function analysis of rhodopsin-like receptors. *Mol. Pharmacol.,* **60,** 1–19.

BUSTO, R., PRIETO, J.C., BODEGA, G., ZAPATERO, J. & CARRERO, I. (2000). Immunohistochemical localization and distribution of VIP/PACAP receptors in human lung. *Peptides,* **21,** 265–269.

DELGADO, M., DE LA, F.M., MARTINEZ, C. & GOMARIZ, R.P. (1995). Pituitary adenylate cyclase-activating polypeptides (PACAP27 and PACAP38) inhibit the mobility of murine thymocytes and splenic lymphocytes: comparison with VIP and implication of cAMP. *J. Neuroimmunol.,* **62,** 137–146.

DELGADO, M. & GANEA, D. (2001). Vasoactive intestinal peptide and pituitary adenylate cyclase- activating polypeptide inhibit expression of Fas ligand in activated T lymphocytes by regulating c-Myc, NF-kappa B, NF-AT, and early growth factors 2/3. *J. Immunol.,* **166,** 1028–1040.

DELGADO, M., POZO, D. & GANEA, D. (2004). The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacol. Rev.,* **56,** 249–290.

DING, W.Q., CHENG, Z.J., MCELHINEY, J., KUNTZ, S.M. & MILLER, L.J. (2002). Silencing of secretin receptor function by dimerization with a misspliced variant secretin receptor in ductal pancreatic adenocarcinoma. *Cancer Res.,* **62,** 5223–5229.

GARCIA-FERNANDEZ, M.O., SOLANO, R.M., CARMENA, M.J., BUSTO, R., BODEGA, G., RUIZ-VILLAESPESA, A., PRIETO, J.C. & SANCHEZ-CHAPADO, M. (2003). Expression of functional PACAP/VIP receptors in human prostate cancer and healthy tissue. *Peptides,* **24,** 893–902.

GEORGE, S.R., FAN, T., XIE, Z., TSE, R., TAM, V., VARGHESE, G. & O'DOWD, B.F. (2000). Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. *J. Biol. Chem.,* **275,** 26128–26135.

GETHER, U. & KOBILKA, B.K. (1998). G protein-coupled receptors. II. Mechanism of agonist activation. *J. Biol. Chem.,* **273,** 17979–17982.

GOURLET, P., VANDERMEERS, A., VERTONGEN, P., RATHE, J., DE NEEF, P., CNUDDE, J., WAELBROECK, M. & ROBBERECHT, P. (1997a). Development of high affinity selective VIP1 receptor agonists. *Peptides,* **18,** 1539–1545.

GOURLET, P., VERTONGEN, P., VANDERMEERS, A., VANDERMEERS-PIRET, M.C., RATHE, J., DE NEEF, P., WAELBROECK, M. & ROBBERECHT, P. (1997b). The long-acting vasoactive intestinal polypeptide agonist RO 25-1553 is highly selective of the VIP_2 receptor subclass. *Peptides,* **18,** 403–408.

HARIKUMAR, K.G., MORFIS, M.M., LISENBEE, C.S., SEXTON, P.M. & MILLER, L.J. (2006). Constitutive formation of oligomeric complexes between family B G protein-coupled vasoactive intestinal polypeptide and secretin receptors. *Mol. Pharmacol.,* **69,** 363–373.

HARMAR, A.J., ARIMURA, A., GOZES, I., JOURNOT, L., LABURTHE, M., PISEGNA, J.R., RAWLINGS, S.R., ROBBERECHT, P., SAID, S.I., SREEDHARAN, S.P., WANK, S.A. & WASCHEK, J.A. (1998). International union of pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase- activating polypeptide. *Pharmacol. Rev.,* **50,** 265–270.

JONES, K.A., BOROWSKY, B., TAMM, J.A., CRAIG, D.A., DURKIN, M.M., DAI, M., YAO, W.J., JOHNSON, M., GUNWALDSEN, C., HUANG, L.Y., TANG, C., SHEN, Q., SALON, J.A., MORSE, K., LAZ, T., SMITH, K.E., NAGARATHNAM, D., NOBLE, S.A., BRANCHEK, T.A. & GERALD, C. (1998). GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. *Nature,* **396,** 674–679.

JUARRANZ, M.G., BOLANOS, O., GUTIERREZ-CANAS, I., LERNER, E.A., ROBBERECHT, P., CARMENA, M.J., PRIETO, J.C. & RODRIGUEZ-HENCHE, N. (2001). Neuroendocrine differentiation of the LNCaP prostate cancer cell line maintains the expression and function of VIP and PACAP receptors. *Cell Signal.,* **13,** 887–894.

LANGER, I., PERRET, J., VERTONGEN, P., WAELBROECK, M. & ROBBERECHT, P. (2001). Vasoactive intestinal peptide (VIP) stimulates [Ca(2+)](i)and cyclic AMPin CHO cells expressing Galpha16. *Cell Calcium,* **30,** 229–234.

LANGLET, C., GASPARD, N., NACHTERGAEL, I., ROBBERECHT, P. & LANGER, I. (2004). Comparative efficacy of VIP and analogs on activation and internalization of the recombinant VPAC2 receptor expressed in CHO cells. *Peptides,* **25,** 2079–2086.

LANGLET, C., LANGER, I., VERTONGEN, P., GASPARD, N., VANDERWINDEN, J.M. & ROBBERECHT, P. (2005). Contribution of the carboxyl terminus of the VPAC1 receptor to agonist-induced receptor phosphorylation, internalization, and recycling. *J. Biol. Chem.,* **280,** 28034–28043.

LAVOIE, C., MERCIER, J.F., SALAHPOUR, A., UMAPATHY, D., BREIT, A., VILLENEUVE, L.R., ZHU, W.Z., XIAO, R.P., LAKATTA, E.G., BOUVIER, M. & HEBERT, T.E. (2002). Beta 1/beta 2-adrenergic receptor heterodimerization regulates beta 2-adrenergic receptor internalization and ERK signaling efficacy. *J. Biol. Chem.,* **277,** 35402–35410.

LEE, S.P., SO, C.H., RASHID, A.J., VARGHESE, G., CHENG, R., LANCA, A.J., O'DOWD, B.F. & GEORGE, S.R. (2004). Dopamine D1 and D2 receptor Co-activation generates a novel phospholipase C-mediated calcium signal. *J. Biol. Chem.,* **279,** 35671–35678.

MILLIGAN, G. (2004). G protein-coupled receptor dimerization: function and ligand pharmacology. *Mol. Pharmacol.,* **66,** 1–7.

PANKHANIYA, R., JABRANE-FERRAT, N., GAUFO, G.O., SREEDHARAN, S.P., DAZIN, P., KAYE, J. & GOETZL, E.J. (1998). Vasoactive intestinal peptide enhancement of antigen-induced differentiation of a cultured line of mouse thymocytes. *FASEB J.,* **12,** 119–127.

PFEIFFER, M., KOCH, T., SCHRODER, H., KLUTZNY, M., KIRSCHT, S., KREIENKAMP, H.J., HOLLT, V. & SCHULZ, S. (2001). Homo- and heterodimerization of somatostatin receptor subtypes. Inactivation of sst(3) receptor function by heterodimerization with sst(2A). *J. Biol. Chem.,* **276,** 14027–14036.

PIERCE, K.L., PREMONT, R.T. & LEFKOWITZ, R.J. (2002). Seven-transmembrane receptors. *Nat. Rev. Mol. Cell Biol.,* **3,** 639–650.

PRINSTER, S.C., HAGUE, C. & HALL, R.A. (2005). Heterodimerization of g protein-coupled receptors: specificity and functional significance. *Pharmacol. Rev.,* **57,** 289–298.

SALAHPOUR, A., ANGERS, S., MERCIER, J.F., LAGACE, M., MARULLO, S. & BOUVIER, M. (2004). Homodimerization of the beta2-adrenergic receptor as a prerequisite for cell surface targeting. *J. Biol. Chem.,* **279,** 33390–33397.

SALOMON, Y., LONDOS, C. & RODBELL, M. (1974). A highly sensitive adenylate cyclase assay. *Anal. Biochem.,* **58,** 541–548.

SCHULZ, S., ROCKEN, C., MAWRIN, C., WEISE, W., HOLLT, V. & SCHULZ, S. (2004). Immunocytochemical identification of VPAC1, VPAC2, and PAC1 receptors in normal and neoplastic human tissues with subtype-specific antibodies. *Clin. Cancer Res.,* **10,** 8235–8242.

SECK, T., BARON, R. & HORNE, W.C. (2003). The alternatively spliced deltae13 transcript of the rabbit calcitonin receptor dimerizes with the C1a isoform and inhibits its surface expression. *J. Biol. Chem.,* **278,** 23085–23093.

TERRILLON, S., BARBERIS, C. & BOUVIER, M. (2004). Heterodimerization of V1a and V2 vasopressin receptors determines the interaction with beta-arrestin and their trafficking patterns. *Proc. Natl. Acad. Sci. U.S.A,* **101,** 1548–1553.

TERRILLON, S. & BOUVIER, M. (2004). Roles of G-protein-coupled receptor dimerization. *EMBO Rep.,* **5,** 30–34.

TERRILLON, S., DURROUX, T., MOUILLAC, B., BREIT, A., AYOUB, M.A., TAULAN, M., JOCKERS, R., BARBERIS, C. & BOUVIER, M. (2003). Oxytocin and vasopressin V1a and V2 receptors form constitutive homo- and heterodimers during biosynthesis. *Mol. Endocrinol.,* **17,** 677–691.

(Received April 18, 2006
Accepted May 11, 2006
Published online 19 June 2006)