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Stable expression of the recombinant human VIP_1 receptor in clonal Chinese hamster ovary cells: pharmacological, functional and molecular properties

Pascale Gaudin, Alain Couvineau, Jean-José Maoret, Christiane Rouyer-Fessard, Marc Laburthe *

Institut National de la Santé et de la Recherche Médicale, INSERM U 410, Neuroendocrinologie et Biologie Cellulaire Digestives, Faculté de Médecine Xavier Bichat, 75018 Paris, France

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Abstract

We stably transfected Chinese hamster ovary (CHO) cells with the recombinant human vasoactive intestinal peptide $(VIP)_1$ receptor. A clone referred to as Clone 15 was isolated and studied for receptor properties. The following data were obtained: (1) one class of binding site was identified by Scatchard analysis of [^{125}I]VIP binding to cell membranes with a K_d of 0.41 nM and a B_{max} of 1.62 pmol/mg protein; (2) the constant K_i for the inhibition of [^{125}I]VIP binding by VIP and related peptides was: VIP (0.9 nM) = pituitary adenylate cyclase-activating peptide (PACAP)-27 (1.3 nM) < PACAP-38 (6.8 nM) < helodermin (46.0 nM) < human growth hormone-releasing factor (GRF) (0.6 μM) < peptide histidine methionineamide (2.0 μM) < secretin (> 10 μM); (3) cross-linking experiments using [^{125}I]VIP identified a single M_r 67 000 recombinant receptor; (4) VIP stimulated cAMP production in Clone 15 cells with an EC_{50} of 0.20 nM; (5) some previously described VIP receptor antagonists including [4-Cl-D-Phe^6,Leu^{17}]VIP, [Ac-Tyr^1,D-Phe^2]GRF-(1–29) amide and VIP-(10–28) inhibited [^{125}I]VIP binding with a K_i of 0.7, 1.6 and 2.5 μM, respectively. They failed to stimulate cAMP production in Clone 15 cells and inhibited, at least partially, the VIP (0.3 nM)-evoked cAMP production; (6) exposure of Clone 15 cells to 10 nM VIP for 24 h resulted in a sharp decrease in B_{max} in Clone 15 cells (0.43 vs. 1.62 pmol/mg protein in control cells) and in the potency and efficacy of VIP in stimulating cAMP. Moreover, immunofluorescence studies using confocal microscopy indicated that the receptor was internalized and sequestered in vesicular structures within the cells. It is concluded that Clone 15 cells provide a valuable tool to further characterize various functional and pharmacological aspects of the human VIP_1 receptor.

Keywords: VIP receptor, human; VIP receptor antagonist; Desensitization; Internalization; Recombinant protein; Transfection

1. Introduction

The functional and biochemical properties of vasoactive intestinal peptide (VIP) receptors have been described for many years (see Laburthe et al., 1993 for a review) but only recent advances permitted isolation of VIP receptor subtypes. Following the purification of a VIP receptor from porcine liver (Couvineau et al., 1990), VIP receptors were cloned from rat lung (Ishihara et al., 1992) and then from human intestine (Couvineau et al., 1994a). These receptors display 81% sequence homology and can be classified as VIP_1 receptors. Indeed a VIP_2 receptor has been cloned from rat hypophysis (Lutz et al., 1993) and later isolated from a human lymphoblast cell line (Svoboda et al., 1994). The VIP_1 and VIP_2 receptors have ~50% homology and, together with receptors for secretin, pituitary adenylate cyclase-activating peptide (PACAP), growth hormone-releasing factor (GRF), gastric inhibitory polypeptide, glucagon, glucagon-like peptide 1, parathyroid hormone and calcitonin, belong to an emerging subfamily within the superfamily of G protein-coupled heptahelical receptor (see for reviews Segré and Goldring, 1993; Couvineau et al., 1994b).

The present availability of VIP receptor cDNAs makes it possible to investigate the individual properties of recombinant VIP_1 and VIP_2 receptors, as VIP receptors co-exist in some organs of mammals (Usdin et al., 1994). In this context and in view of the poor development of VIP

* Corresponding author. INSERM U 410, Faculté de Médecine Xavier-Bichat, B.P. 416, 75870 Paris Cedex 18, France. Tel.: +33 1 44 85 61 35; fax: +33 1 44 85 61 24.

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receptor pharmacology, it would be very useful to establish cell lines stably transfected with the cDNAs encoding VIP receptor subtypes. We describe here the development of a cell clone, Clone 15, isolated from Chinese hamster ovary (CHO) cells stably transfected with the human VIP_1 receptor cDNA (Couvineau et al., 1994a) and analyze the pharmacological, functional and molecular properties of the recombinant receptor.

2. Materials and methods

2.1. Chemicals and reagents

VIP (human), PACAP-27 (ovine), PACAP-38 (ovine), helodermin, peptide histidine methionineamide (PHM), hGRF, secretin (porcine), VIP-(10–28), [4-Cl-D-Phe^6,Leu^{17}]VIP, [Ac-Tyr^1,D-Phe^2]GRF-(1–29) amide were purchased from Peninsula Laboratories, Belmont, CA. [^{125}I]VIP was prepared by the chloramine-T method and purified as reported (Laburthe et al., 1978). The culture media Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 and other culture compounds were purchased from Gibco BRL (Eragny, France). The monoclonal anti-Tag antibodies were obtained from the hybridoma MYC I-9E 10.2 (Evan et al., 1985), which was available through ATCC (no. CRL-1729). Other compounds used in this study were of the highest quality commercially available.

2.2. Transfection, selection and culture of cell lines

The entire coding region of the human VIP_1 receptor from Clone hIVR8 (Couvineau et al., 1994a) was cloned in the vector pcDNA3 (Invitrogen, San Diego, CA) that contains the selectable neomycin gene. For immunofluorescence experiments using confocal laser scanning microscopy (see below), the clone was tagged at the C-terminus with the following marker dodecapeptide (Tag) MEQKLISEEDLN as described (Couvineau et al., 1994a). The recombinant plasmid was transfected into the CHO cell line (ECACC 85050302) by electroporation. Briefly, 4×10^6 cells were preincubated on ice for 5 min with 20 μg of plasmid DNA and 20 μg of salmon sperm DNA carrier in cold Ham's F-12. Electroporation was performed at 330 V and 1000 μF and infinite resistance. After electroporation, cells were kept on ice for 5 min and then transferred into Petri dishes containing 10 ml of culture medium (Ham's F-12, 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin). After 48 h, cells were selected by addition of geneticin (G418) at a final concentration of 400 μg/ml for 2 weeks. Resistant cells were cloned by limiting dilution. Clone 15 was selected from 23 other clones on the basis of its high binding capacity for [^{125}I]VIP (see below), and then maintained in the above described culture medium containing 10% fetal calf serum and 100 μg/ml G418 in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were passaged every 7 days in 25 cm^2 plastic culture flasks and used between passages 4 and 20.

2.3. Membrane preparation

Cells were grown to confluency for 3–4 days in plastic flasks, washed three times using phosphate-buffered saline (pH 7), then harvested with a rubber policeman and centrifuged at $2000 \times g$ for 5 min at 4°C. The cell pellet was then exposed for 30 min to hypoosmotic 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (pH 7.4) as described (Salomon et al., 1993). The resulting broken cell suspension was centrifuged at 20000 $\times g$ for 10 min, washed with 20 mM Hepes buffer (pH 7.4), repelleted, and stored at −80°C until used. This particulate fraction is referred to as the membrane preparation.

2.4. Ligand binding assay

Membranes (~ 80 μg protein/ml) were incubated at equilibrium for 60 min at 30°C with 0.05 nM [^{125}I]VIP with or without unlabeled VIP or other competing peptides. The incubation medium was 20 mM Hepes, pH 7.4, containing 2% (w/v) bovine serum albumin and 0.1% (w/v) bacitracin. The reaction was stopped by transferring the incubation medium to an excess of ice-cold buffer. Bound and free peptides were separated by centrifugation at $20000 \times g$ for 10 min, and membrane pellets were washed twice with 10% (w/v) sucrose in 20 mM Hepes. The radioactivity was then counted with a γ counter. Specific binding was calculated as the difference between the amount of [^{125}I]VIP bound in the absence (total binding) and presence (nonspecific binding) of 1 μM unlabeled peptide. The nonspecific binding represented about 10% of the total radioactivity bound to membranes. All binding data were analyzed using the LIGAND computer program (Munson and Rodbard, 1980). The constants K_i for the inhibition of [^{125}I]VIP binding by unlabeled peptides were calculated as described (Laburthe et al., 1986).

2.5. Cross-linking experiments and PNGase F treatment

Cells were grown to confluency in culture plastic flasks and then washed 3 times with 0.1% (w/v) bovine serum albumin in phosphate-buffered saline. Cells were then incubated for 4 h at 4°C with 0.6 nM [^{125}I]VIP in DMEM containing 15 mM Hepes (pH 7.4), 150 μM phenylmethylsulfonyl fluoride, 1% (w/v) bovine serum albumin and 0.1% (w/v) bacitracin. After incubation, cells were washed three times with ice-cold phosphate-buffered saline and subsequently incubated for 30 min at 37°C in 60 mM Hepes buffer (pH 8.0) containing 150 mM NaCl and 2 mM dissucinimidyl suberate (DSS) as a cross-linker. The reaction was stopped and quenched by the addition of 20 mM

Hepes buffer (pH 8.0) containing 150 mM NaCl and 60 mM ammonium acetate. The cells were harvested, then centrifuged at 20000 × *g* for 10 min, and the pellets were suspended in 60 mM (hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 6.8, containing 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 3% (w/v) sodium dodecylsulfate (SDS). The solubilization was carried out overnight at room temperature. The solubilization medium was centrifuged at 20000 × *g* for 10 min and the supernatant was applied to a 10% polyacrylamide gel with a 5% stacking gel. In some experiments cross-linked [^{125}I]VIP-receptor complexes were submitted to PNGase F treatment prior to electrophoresis as described (Couvineau et al., 1996). The gel was run and fixed as previously described (Couvineau and Laburthe, 1985). It was dried and exposed for 1 week at −80°C to a Trimax type XM film (3M) with a Trimax intensifying screen.

2.6. Cyclic AMP measurement

Cells were seeded in 12-well trays and grown for 3–4 days. They were then incubated with VIP and other compounds, when necessary, under continuous agitation for 30 min at room temperature in phosphate-buffered saline (pH 7) containing 2% (w/v) bovine serum albumin, 0.1% (w/v) bacitracin, 0.01 mg/ml aprotinin and 1 mM 3-isobutyl-1-methylxanthine as described (Servin et al., 1989). The reaction was stopped by 1 M perchloric acid. After centrifugation for 15 min at 4000 × *g*, the cAMP present in the supernatant was succinylated and its concentration was measured by radioimmunoassay as described (Laburthe et al., 1982).

2.7. Isolation of RNA and Northern blot analysis

Total RNAs were isolated from cells by using guanidium isothiocyanate and centrifugation through a CsCl gradient (Darmoul et al., 1991). An aliquot (20 μg) of total RNA sample was separated by electrophoresis on 1% agarose gels after denaturation in 1 mM glyoxal. RNAs separated by electrophoresis were transferred onto Hybond N (Amersham International), prehybridized for 12–18 h at 65°C in hybridization buffer (Darmoul et al., 1991) and then hybridized for 24 h at 60°C in the same buffer containing the hIVR8 cDNA probe (Couvineau et al., 1994a) labeled with [α-^{32} P]dCTP (using a multiprime DNA labeling system from Amersham International). Blots were washed three times for 15 min at 60°C in 2 × standard saline citrate (SCC) (1 × SCC = 150 mM NaCl, 15 mM sodium citrate), 1 × SCC, 0.5 × SCC and finally 0.1 × SCC before autoradiography.

2.8. Immunofluorescence experiments using confocal microscopy

Cells were grown on glass slides in 24-well trays. They were washed with phosphate-buffered saline, fixed for 15 min with 2% (w/v) paraformaldehyde and permeabilized with 0.075% (w/v) saponin in phosphate-buffered saline. Cells were incubated with anti-Tag antibodies (1/250) for 30 min, washed twice with phosphate-buffered saline saponin, then incubated for 30 min with anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate. After washing, slides were mounted in Glycergel and selected fields were scanned using a true confocal scanner Leica TCS 4D comprising a Leica Diaplan inverted microscope equipped with an argon-krypton ion laser (488 nm) with an output power of 2–50 mW and a VME bus MC 68020/6881 computer system coupled to an optical disc for image storage (Leica Laserchnik). The emitted light was collected through a long-pass filter on the target of the photo multiplier. Each sample was treated with a kalman filter to increase the ratio signal vs. background. All image generating and processing operations were carried out using the Leica CLSM software package. Screen images were taken either on Kodak TMAX using a 35 mm camera.

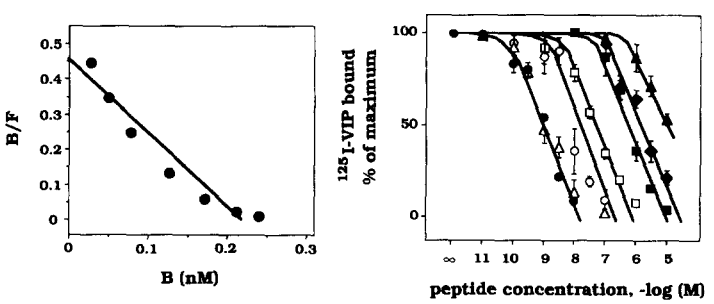

Fig. 1. Binding of [^{125}I]VIP to recombinant human VIP_1 receptors expressed in Clone 15 cells. Left: Scatchard plot of the inhibition of [^{125}I]VIP binding by unlabeled VIP in membranes prepared from Clone 15 cells. Membranes were incubated at 30°C for 1 h with a fixed concentration of [^{125}I]VIP (0.05 nM) and increasing concentrations of unlabeled VIP. The LIGAND computer program (Munson and Rodbard, 1980) was used to determine nonspecific binding and to analyze binding parameters. One representative experiment of four others is shown. Right: competitive inhibition of specific [^{125}I]VIP (0.05 nM) binding to Clone 15 cell membranes by unlabeled VIP (●), PACAP 27 (△), PACAP 38 (○), helodermin (□), PHM (◆), human GRF (■) and secretin (▲). Results are expressed as percent of maximal specific binding in the absence of competing unlabeled peptides. Data are means ± S.E. of six experiments.

2.9. Protein determination

Protein concentration was measured by the procedure of Bradford (1979) with bovine serum albumin as standard.

3. Results

Of several clones obtained after selection in G418-containing culture medium, Clone 15 was selected on the basis of a high level of specific [^{125}I]VIP binding. This clone was grown in culture medium (see Materials and methods) containing 100 μg/ml G418 and studied between passages 4 and 20 under conditions at which the level of recombinant human VIP_1 receptor appeared to be stable. Scatchard analysis of VIP binding to membranes prepared from Clone 15 cells gave a linear plot (Fig. 1) indicating the presence of one class of high affinity receptors with a B_{max} of 1.62 ± 0.19 pmol/mg protein and a dissociation constant of 0.41 ± 0.07 nM (four experiments). Further experiments were carried out to determine the pharmacological profile of the recombinant receptor using several naturally occurring peptides of the VIP family. The competitive inhibition of [^{125}I]VIP binding by these peptides is shown in Fig. 1. The constant K_i was as follows: VIP (0.9 ± 0.2 nM) = PACAP-27 (1.3 ± 0.3 nM) < PACAP-38 (6.8 ± 1.6 nM) < helodermin (46.0 ± 18.5 nM) < hGRF (0.6 ± 0.3 μM) < PHM (2.0 ± 0.8 μM) < secretin (> 10 μM). This order of affinity is identical to that previously observed for the endogenous VIP_1 receptor in human intestine (Laburthe et al., 1986; Salomon et al., 1993). In order to estimate the molecular mass of the human recombinant VIP_1 receptor expressed in Clone 15 cells, cross-linking experiments were carried out using [^{125}I]VIP (Fig. 2). SDS-PAGE analysis of cross-linked material revealed a single M_r 70000 band whose labeling was completely inhibited in the presence of 1 μM unlabeled VIP. Assuming one molecule of tracer (~ 3000) was bound per molecule of receptor, a M_r 67000 protein was identified as the recombinant human VIP_1 receptor in Clone 15 cells. As a control, cross-linking experiments with untransfected CHO cells gave no labeled band (Fig. 2). This supports that CHO cells did not express a VIP receptor in keeping with the absence of specific [^{125}I]VIP binding (not shown). This was further supported by Northern blot analysis of total RNAs isolated from Clone 15 cells and CHO cells which showed a 2.6 kb mRNA species in Clone 15 cells vs. no hybridized band in CHO cells (not shown). After cross-linking the recombinant human VIP_1 receptor was incubated with PNGase F. SDS-PAGE analysis of cross-linked material revealed a single M_r 45000 band (not shown) instead of the M_r 70000 band observed in the absence of PNGase F treatment (Fig. 2). These data show that the apparent M_r 67000 for the recombinant receptor expressed in CHO cells was due to N-linked glycosylation.

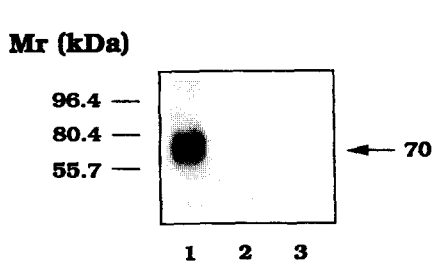

Fig. 2. Covalent labeling of the recombinant human VIP_1 receptor. Clone 15 cells (lanes 1 and 2) or control CHO cells (lane 3) were incubated with [^{125}I]VIP in absence (lanes 1 and 3) or presence (lane 2) of 1 μM unlabeled VIP, then treated with 2 mM disuccinimidyl suberate and submitted to SDS-PAGE analysis as described in Materials and methods. The labeled band (lane 1) corresponded to a molecular weight of 70000. The gel was calibrated with molecular weight marker proteins whose molecular mass is indicated to the left of the gel.

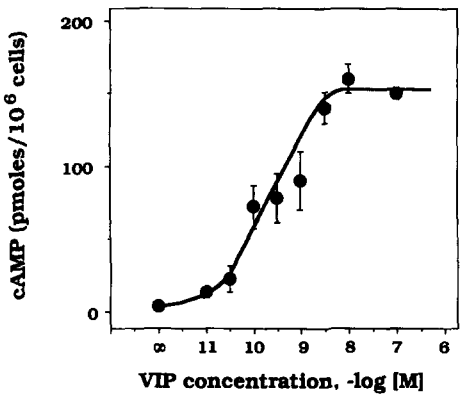

Fig. 3. Stimulation by VIP of cAMP production in Clone 15 cells expressing the recombinant human VIP_1 receptor. Cells were incubated for 30 min at room temperature with various concentrations of VIP. The intracellular amount of cAMP was measured as described in Materials and methods. Data are means ± S.E. of six experiments.

As shown in Fig. 3, VIP stimulated cAMP accumulation in intact Clone 15 cells with half-maximal stimulation obtained at 0.20 ± 0.09 nM peptide (six experiments). At a maximally active dose (0.1 μM), VIP elicited a 38 ± 10-fold increase of cAMP above basal cAMP level. These data support an efficient coupling of the recombinant human VIP_1 receptor to adenylyl cyclase in Clone 15 cells due to the endogenous population of Gs proteins. When naturally occurring peptides of the VIP family were tested for their ability to stimulate cAMP accumulation in Clone 15 cells, they stimulated cAMP (not shown) with an order of potency which was similar to their order of affinity for the VIP_1 receptor (see Fig. 1). Some poorly potent peptide antagonists of VIP receptors were described several years ago (Waelbroeck et al., 1985; Laburthe et al., 1986; Pandol et al., 1986; Turner et al., 1986), including [4-Cl-D-Phe^6,Leu^{17}]VIP, [Ac-Tyr^1,D-Phe^2]GRF-(1–29) amide and VIP-(10–28). In the context of the existence of VIP receptor subtypes (Ishihara et al., 1992; Lutz et al., 1993; Couvineau et al., 1994a; Svoboda et al., 1994), it was of interest to look at the effect of these peptide antagonists on

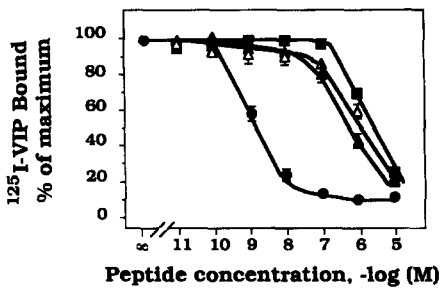

Fig. 4. Competitive inhibition of specific [^{125}I]VIP binding to Clone 15 cell membranes by increasing concentrations of unlabeled VIP (●), [4-Cl-D-Phe^6, Leu^{17}]VIP (▲), [Ac-Tyr^1,D-Phe^2]-GRF-(1–29) amide (△) and VIP-(10–28) (■). Results are expressed as percent maximal specific binding in the absence of competing unlabeled peptides. Data are means ± S.E. of three experiments.

recombinant human VIP_1 receptors expressed in Clone 15 cells. Fig. 4 shows that the three peptides inhibited [^{125}I]VIP binding to Clone 15 cell membranes with the following K_i: 0.7 ± 0.1 μM for [4-Cl-D-Phe^6,Leu^{17}]VIP; 1.6 ± 0.2 μM for [Ac-Tyr^1,D-Phe^2]GRF-(1–29) amide and 2.5 ± 0.2 μM for VIP-(10–28). In sharp contrast these peptides, when tested at 5 μM, were unable to stimulate cAMP accumulation in Clone 15 cells (Fig. 5). When added together with VIP (0.3 nM), the three peptides inhibited partially the VIP-stimulated cAMP level (Fig. 5), supporting the notion that they behave as human VIP_1 receptor antagonists in Clone 15 cells. Finally we investigated the possible down-regulation of recombinant human VIP_1 receptors expressed in Clone 15 cells upon exposure to 10 nM VIP for 24 h. It appeared that such exposure resulted in a sharp decrease in the binding capacity of Clone 15 cells (0.43 ± 0.03 vs. 1.62 ± 0.19 pmol/mg protein) without a significant change in the dissociation constant of receptors (0.26 ± 0.09 nM and 0.41 ± 0.07 nM in treated cells and control cells, respectively). This was associated with an important decrease in the efficacy and potency (half-maximal stimulations were observed for 0.09 ± 0.03 nM and 1.23 ± 0.54 nM peptide in treated cells and control cells, respectively) of VIP in stimulating cAMP accumulation in Clone 15 cells (Fig. 6). Owing to the presence of a Tag at the C-terminus of the recombinant human VIP_1 receptor, it was possible to localize the receptor in Clone 15 cells by confocal laser scanning microscopy using anti-Tag antibodies. As shown in Fig. 7, receptors were diffusely distributed over the plasma membrane of Clone 15 cells. Upon exposure of cells to 10 nM VIP for 24 h, receptors were internalized and sequestered in vesicular structures.

4. Discussion

The present study represents the first development and extensive characterization of a cell clone expressing a stably transfected human VIP_1 receptor. This receptor has been cloned from a cDNA library prepared from normal epithelial cells isolated from human small intestine (Couvineau et al., 1994a). Another human VIP receptor has been cloned from the cancer cell line HT29 (Sreedharan et al., 1993) and expressed in CHO cells (Sreedharan et al., 1994). Other receptors of this family have been also stably expressed in CHO cells such as the rat VIP_1 receptor (Ciccarelli et al., 1994) and the rat $PACAP_1$ receptor (Ciccarelli et al., 1995). The basic properties of the recombinant human VIP_1 receptor stably expressed in Clone 15 cells compare well with those described for VIP receptors on epithelial cells isolated from human small intestine (Salomon et al., 1993). This includes dissociation constant and EC_{50} for cAMP production, both in the subnanomolar range, and peptide specificity towards all natural peptides of mammalian origin belonging to the structural family of VIP e.g. PACAP-27, PACAP-38, PHM, GRF and secretin (Salomon et al., 1993 and this paper). It is also worth pointing out that cross-linking experiments showed that the molecular weight of the recombinant human VIP_1 receptor expressed in Clone 15 cells is 67,000 (see Fig. 2). This is identical to the molecular weight of VIP receptors on epithelial cells isolated from human small intestine (Salomon et al., 1993). Since VIP receptors are glycoproteins with ~ 20 kDa N-linked carbohydrate in intestinal cells (Laburthe and Couvineau, 1988), these data strongly support that the recombinant human VIP_1 receptor undergoes normal glycosylation, at least quantitatively, when expressed in Clone 15 cells. This was confirmed by PNGase treatment of the recombinant human VIP_1 receptor expressed in clone 15 cells.

The recombinant human VIP_1 receptor expressed in Clone 15 cells displayed pharmacological properties which are typical of the human species. In particular, the order of affinity of VIP-related peptides e.g. VIP = PACAP-27 < PACAP-38 < helodermin < hGRF < PHM < secretin is

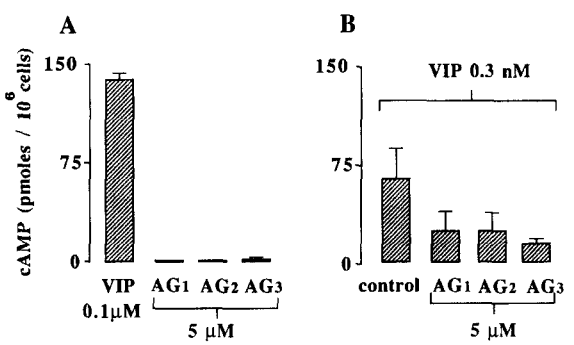

Fig. 5. Effect of the three putative VIP receptor antagonists, VIP-(10–28) [AG1], [Ac-Tyr^1,D-Phe^2]GRF-(1–29) amide [AG2] and [4-Cl-D-Phe^6, Leu^{17}]VIP [AG3] on intracellular cAMP level in Clone 15 cells expressing the recombinant human VIP_1 receptor. Cells were incubated for 30 min with 5 μM AG1, AG2 or AG3 in the absence (A) or the presence (B) of 0.3 nM VIP. To the left (A), the effect of 5 μM AG1, AG2 or AG3 is compared to the effect of 0.1 μM VIP. The intracellular amount of cAMP was measured as described in Materials and methods. Data are means ± S.E. of three experiments.

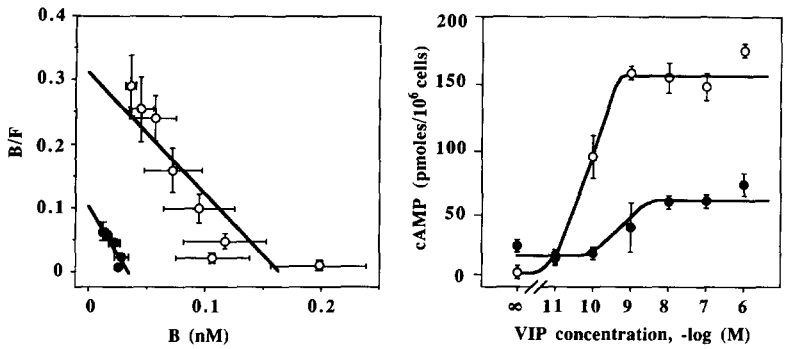

Fig. 6. Desensitization of the recombinant human VIP_1 receptor expressed in Clone 15 cells. Effect of the pretreatment of cells with 10 nM VIP for 24 h on binding parameters (left) and cAMP production (right). All experiments were performed after pretreatment of cultured cell monolayers with 10 nM VIP for 24 h at 37°C. The culture medium was then discarded and cell monolayers were washed three times with PBS before the cells were used for further studies. Left: membranes from Clone 15 cells pretreated (●) or not (○) with VIP were prepared as described in Materials and methods and incubated for 1 h at 30°C with a fixed concentration of [^{125}I]VIP (0.05 nM) and increasing concentrations of unlabeled VIP. The binding data were analyzed by the LIGAND computer program (Munson and Rodbard, 1980) and Scatchard plots are shown. Data are means ± S.E. of three experiments. Right: Clone 15 cells pretreated (●) or not (○) with VIP were washed as described above and then incubated with various concentrations of VIP (see Materials and methods and the legend to Fig. 3). Data are means ± S.E. of three experiments.

observed in human tissues but not in rat tissues (Laburthe et al., 1982, 1993). Our results also evidence that the previously described VIP receptor antagonists (Waelbroeck et al., 1985; Laburthe et al., 1986; Pandol et al., 1986; Turner et al., 1986) compete with VIP for binding to the recombinant human VIP_1 receptor and behave as antagonists of this receptor (see Figs. 5 and 6). Since these antagonists have a very low potency in the micromolar range and in the case of GRF analogs may behave as GRF agonists (see Laburthe et al., 1986), they cannot be used in practice for biological studies in vivo (Laburthe et al., 1993). Therefore, it was not in the aim of this work to fully characterize their behaviour in Clone 15 cells. This is nevertheless an interesting observation in view of the fact that VIP receptors in different species are pharmacologically distinct (Laburthe et al., 1986, 1993). In this respect, the recombinant human VIP_1 receptor stably expressed in Clone 15 cells represents a new and pertinent tool for screening VIP receptor agonists or antagonists acting in human tissues.

Clone 15 cells preincubated with 10 nM for 24 h showed a marked decrease in the potency and efficacy of VIP in stimulating cAMP production, indicating homologous VIP receptor desensitization. This was associated with a marked decrease in the binding capacity of VIP receptors in Clone 15 cells without there being a significant change in the dissociation constant after VIP treatment. Similar data were previously reported for secretin receptors stably transfected in CHO cells (Vilardaga et al., 1994) supporting that recombinant receptors expressed in this cell line undergo typical desensitization. This phenomenon had been previously described for native VIP

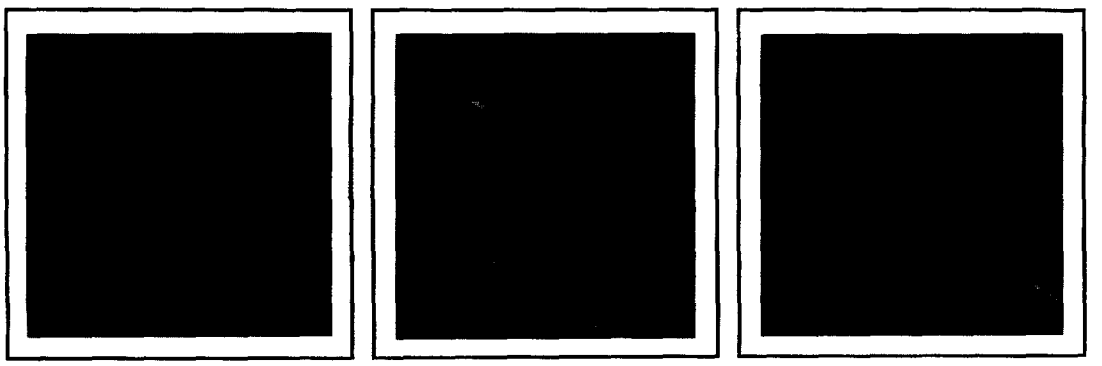

Fig. 7. Desensitization of the recombinant human VIP_1 receptor expressed in Clone 15 cells. Effect of the pretreatment of cells with 10 nM VIP for 24 h on the subcellular localization of receptors. Experiments were performed after pretreatment of cultured cell monolayers with 10 nM VIP for 24 h at 37°C. The culture medium was then discarded and cell monolayers were washed three times with phosphate-buffered saline. After permeabilization with saponin, cell monolayers were incubated with the mouse monoclonal anti-Tag antibody, washed and incubated with anti-mouse immunoglobulin G conjugated to fluorescein isothiocyanate as described in Materials and methods. The cell monolayers were then analyzed by confocal laser scanning microscopy. A: untransfected CHO cells incubated with the monoclonal anti-Tag antibody gave no staining; B: Clone 15 cells which were not pretreated with VIP; C: Clone 15 cells pretreated for 24 h with 10 nM VIP.

receptors and was shown to be associated with the internalization of VIP (see Rosselin et al., 1988). However, due to the lack of VIP receptor antibodies, the reality of VIP receptor internalization upon incubation of intact cells with VIP and during receptor desensitization was only supported by indirect evidence (Rosselin et al., 1988; Turner et al., 1988). In the present work, owing to the presence of a Tag at the C-terminus of the recombinant VIP receptor, we were able to demonstrate by confocal microscopy that upon incubation of Clone 15 cells with VIP, VIP receptors were internalized and sequestered in vesicular structures. This represents the first direct evidence for VIP receptor sequestration in vesicular structures during desensitization. The nature of the endocytic pathway(s) and vesicular compartment(s) is unknown but is most probably complex, as recently shown for cholecystokinin receptors (Roettger et al., 1995). Clone 15 cells stably expressing the tagged human VIP_1 receptor provide for the first time a valuable cellular model for further analysis of the internalization of VIP receptors. In addition, our work indicates that the receptor domain(s) responsible for VIP receptor desensitization and internalization can now be investigated by transfecting mutant VIP receptors in CHO cells.

In conclusion, this study shows that cells engineered by molecular biology may provide a powerful model to study the pharmacological properties of the human VIP_1 receptor. This should be valuable for further characterization of this receptor and will be instrumental in developing more selective and specific human VIP_1 receptor agonists and antagonists.

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