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Comparative efficacy of VIP and analogs on activation and internalization of the recombinant $VPAC_2$ receptor expressed in CHO cells

Christelle Langlet, Nathalie Gaspard, Ingrid Nachtergael, Patrick Robberecht*, Ingrid Langer

Department of Biochemistry and Nutrition, Faculty of Medicine, Université Libre de Bruxelles, Bât G/E, CP 611, 808 route de Lennik, B-1070 Bruxelles, Belgium

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Abstract

Using a monoclonal antibody interacting with the extracellular amino-terminus of the human $VPAC_2$ receptor but that did not interfere with ligand binding, we measured by flow cytometry receptor internalization and trafficking induced by full agonists, partial agonists and an antagonist in Chinese hamster ovary cells expressing the recombinant receptor. The agonists, but not the antagonist, induced a rapid, dose-dependent receptor internalization blocked by hypertonic sucrose that was more pronounced for the VIP analog *N*-hexanoyl-VIP (80%) than for VIP and Ro 25-1553 (50%) and the [A^{11}]-VIP (20%). Re-expression of the receptors at the membrane was achieved within two hours after exposure to VIP and Ro 25-1553 was blocked by 25 μM monensin but not by 10 μg/ml cycloheximide. Re-expression was much slower after exposure to the acylated peptide and was blocked by preincubation with 25 μM monensin and 10 μg/ml cycloheximide.

Keywords: $VPAC_2$ receptor; Receptor internalization; Receptor trafficking; VIP analogs

1. Introduction

Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) are neuropeptides that modulate exocrine and endocrine secretions, smooth cell contractility and the immune system [5,7]. The effects of VIP are mediated through interactions with two receptor subclasses named as $VPAC_1$ and $VPAC_2$ receptors [10]. The effects of PACAP are also mediated not only through interactions with the same receptors but also through a selective receptor named as PAC_1 receptor [31]. All these receptors are members of a large family of G-protein-coupled receptors named the GPCR-B family that includes the receptors for the peptide hormones secretin, glucagon, glucagon-like peptides, calcitonin, parathormone, and GRF. These receptors are preferentially coupled to $G_{\alpha s}$ protein, which stimulates adenylate cyclase activity and increases cyclic AMP levels. Like most of the GPCR receptors, the VIP receptors undergo, after agonist exposure, a rapid desensitization followed by internalization [24,25]. The pathway generally considered involves receptor phosphorylation, the binding of arrestin that in turn promotes receptor desensitization and subsequent internalization via clathrin-coated pits that drives the receptors to acidic early endosomes leading to receptor recycling or degradation [6]. However, recent studies suggest that GPCR trafficking may also occur for some receptors (or in some cell lines) through a clathrin-independent pathway, like the lipid-raft endocytosis [4,32]. Although the existence of desensitization/internalization of both $VPAC_1$ and $VPAC_2$ receptors is established [18,24,25], the mechanisms involved are still a matter of debate: considering the $VPAC_1$ receptor, Shetzline et al. [28] concluded that receptor regulation involves agonist-stimulated, GRK-mediated phosphorylation,

Abbreviations: CHO, Chinese hamster ovary cells; EC_{50}, concentration of agonist required for half maximal response; GRF, growth hormone releasing factor; IC_{50}, concentration of ligand required for 50% inhibition of tracer binding; PACAP, pituitary adenylate cyclase activating polypeptide; PG99-465, N^{α}-Myristoyl-H^1, K^{12}, K^{27}, K^{28}, G^{29}, G^{30}, T^{31} VIP(1-26); Ro 25-1553, Acetyl-His^1[E^8, K^{12}, Nle^{17}, A^{19}, D^{25}, L^{26}, K^{27}, K^{28}, G^{29}, G^{30}, T^{31}] cyclo 21-25 VIP (2-24); VIP, vasoactive intestinal polypeptide

* Corresponding author. Tel.: +32 2 5556228; fax: +32 2 5556230.
E-mail address: probbe@ulb.ac.be (P. Robberecht).

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β-arrestin translocation, and dynamin receptor internalization. However, neither overexpression of β-arrestin nor expression of a dominant negative mutant altered internalization. Claing et al. [2] studied the possible involvement of the GIT1 (a GTPase-activating protein of small GTP-binding protein) and concluded that $VPAC_1$ receptor internalization occurred through a dynamin-sensitive pathway insensitive to GIT1 or β-arrestin.

The following questions are considered in the present work using the model of recombinant human $VPAC_2$ receptor expressed in Chinese hamster ovary (CHO) cells: is receptor internalization a clathrin dependent phenomenon? is the internalized receptor recycled to the membrane? is there a correlation between agonist efficacy to stimulate adenylate cyclase and to induce receptor internalization?

We developed for that study a monoclonal antibody that binds to the amino-terminal extracellular domain of the receptor that does not interfere with ligand binding and therefore allows the quantification of the receptors expressed at the cell surface by fluorescent assisted cell sorting (FACS) technique.

2. Materials and methods

2.1. Cell cultures

All the experiments were conducted on Chinese hamster ovary cells expressing the recombinant wild-type human $VPAC_2$ receptor obtained as previously described [1,14]. Cells were grown in culture medium consisting in 50% HamF12; 50% DMEM; 10% fetal calf serum; 100 μg/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Sigma–Aldrich, St.-Louis, MO, U.S.A.).

2.2. Membrane preparations

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 5 min at 400 × *g* and the supernatant was further centrifuged at 20,000 × *g* for 15 min. The pellet was resuspended in 1 mM $NaHCO_3$ and used immediately.

2.3. Binding studies

Binding studies, using [^{125}I]-Ro 25-1553, [^{125}I]-VIP or [^{125}I]-*N*-Hexanoyl-VIP, were performed for 30 min at 23 °C in a total volume of 120 μl containing 20 mM Tris–maleate, 2 mM $MgCl_2$, 0.1 mg/ml bacitracin and 1% bovine serum albumin (pH 7.4). Protein (3–30 μg) was used per assay. The assays were performed in such conditions that specific binding was strictly proportional to the amount of protein. Bound and free radioactivity were separated by filtration through glass-fiber GF/C filters presoaked for 24 h in 0.01% polyethyleneimine. The filters were rinsed three times with 20 mM (pH 7.4) sodium phosphate buffer containing 0.5% bovine serum albumin.

2.4. Adenylate cyclase activity

Adenylate cyclase activity was determined by the procedure of Salomon et al. as described previously [26]. 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 pyruvate kinase and 30 mM Tris–HCl at a final pH of 7.8. The reaction was initiated by membrane addition and was terminated after 15 min incubation at 37 °C by adding 0.5 ml of a 0.5% sodium dodecyl sulfate solution containing 0.5 mM ATP, 0.5 mM cAMP and 20,000 cpm [^{3}H]-cAMP. cAMP was separated from ATP by two successive chromatographies on Dowex 50W × 8 and neutral alumina.

2.5. Peptide synthesis and purification

All peptides were synthesized by solid-phase methodology using the Fmoc (9-fluorenyl-methoxycarbonyl) strategy with an automated Symphony apparatus. The peptides were cleaved, precipitated with 10 volumes of cold ether and purified on reverse phase and ion exchange chromatographies. The purity (>95%) was assessed by capillary electrophoresis and the conformity by electrospray mass spectrometry.

2.6. Preparation of a monoclonal antibody for $VPAC_2$ receptor identification

2.6.1. Genetic immunization and generation of monoclonal antibody

The protocol described by Costagliola et al. was followed [3]: 6-week-old Balb/c female mice were anaesthetized by injections of 6–10 mg/kg ketamine HCl and 0.1 ml/kg Rompum. The anterior tibialis muscle of each leg was injected at day 0 with 100 μl of 10 mM cardiotoxin (Latoxan, Rosans, France) and 5 days later with 50 μg of the plasmid construct consisting in the human $VPAC_2$ receptor coding region inserted into the mammalian expression vector pcDNA 3.1 (Invitrogen, Carlsbad, CA, U.S.A.), in a final volume of 100 μl of 0.09% NaCl. Injections were repeated 3 and 6 weeks thereafter. Blood samples were collected from retro-ocular puncture 7 weeks after the initial immunization and serum tested by FACS and Western Blotting for the presence of antibodies against $VPAC_2$ receptor. The mouse selected for mAb production was boosted by an i.v. injection of 10^6 CHO cells expressing the human $VPAC_2$ receptor resuspended in 100 μl saline solution. Three days later, splenocytes were fused with SP2O, a non-secreting myeloma cells line, at a 3:1 ratio in presence of polyethylene glycol. Fused cells were then delivered into 96-well plates and selected by 100 μM hypoxantine, 400 μM aminopterine and 16 μM thymidine. Irradiated macrophages from mouse peritoneum were also

added to supply cytokines and growth factors. After 10 days, culture supernatants were screened by FACS (see below) and the cells producing antibodies cloned by dilution. The monoclonal antibody selected (mAb $VPAC_2$) was purified using ImmunoPure IgG Purification kit (Pierce). Using the mouse mAb isotyping kit (Isotrip, Boehringer Mannheim Corp.) we established that it was of the IgG 2a subtype.

2.6.2. Evaluation of the presence of an antibody by flow cytometry and testing its selectivity

Chinese hamster ovary cells expressing the recombinant human $VPAC_2$ receptor were detached from the plates using a 5 mM EDTA/5 mM EGTA PBS solution, harvested by centrifugation (500 × *g*, 4 °C, 4 min), washed once with PBS solution and resuspended to 3×10^5 cells/tube in 100 μl of PBS–BSA 0.1%, containing purified mAb at a final concentration of 1 μg/ml. 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 (Sigma Chemicals) for 30 min on ice in the dark. The cells were washed and resuspended in 250 μl PBS–BSA 0.1%. The level of fluorescence was analyzed using a FACScalibur (Becton Dickinson) and the data processed using the CellQuest software (Becton Dickinson). Basal fluorescence was determined from sample of untransfected CHO cells. Propidium iodide (10 μg/ml) was used to exclude dead cells from the analysis.

The following controls were performed to evaluate (a) the specificity (b) the location of the recognized epitope and (c) the eventual interference with the ligand-binding site and therefore receptor activation.

(a) Using the above-described FACS technique, the fluorescence level was not different when the antibody was applied on untransfected CHO cells or expressing the human $VPAC_1$, the rat $VPAC_1$ and the rat $VPAC_2$ receptor. A significant fluorescence was detected on $SUPT_1$ cells expressing human $VPAC_2$ receptor [29] but not on LoVo cells expressing $VPAC_1$ receptor only [9].
(b) A significant fluorescence signal was observed in CHO cells transfected with a chimeric $VPAC_2/VPAC_1$ receptor consisting in the 1–127 $VPAC_2$ receptor sequence grafted on the TM_1, IC_1, TM_2, EC_1, TM_3, IC_2, TM_4, EC_2, TM_5, IC_3, TM_6, EC_3, TM_7 and the carboxy-terminus of $VPAC_1$ receptor (codon 144–457) [15]; no signal was detected in cells transfected with a chimeric $VPAC_1/VPAC_2$ receptor consisting in the 1–143 $VPAC_2$ receptor sequence followed by the $VPAC_2$ receptor sequence 128–438. The expression of these two chimeric receptors on the studied clones was confirmed by binding and functional data.
(c) The antibody at concentrations ranking from 0.5 to 50 μg/ml did not inhibit ^{125}I-Ro 25-1553 binding on $VPAC_2$ expressing membranes. Membranes expressing $VPAC_2$ receptors were preincubated 30 min at 4 °C in presence or not of the antibody (final concentration 20 μg/ml). VIP, Ro 25-1553 and *N*-hexanoyl-VIP dose–effect curves of adenylate cyclase activation were performed thereafter in the standard conditions (in the assay, the final concentration of the antibody was 6 μg/ml). Preincubation did not modify either the agonist EC_{50} values, or the maximal stimulatory effect.

2.7. Receptor internalization

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 the $VPAC_2$ receptor were incubated with agonist at 37 °C. After washing three times with ice-cold phosphate buffer saline, cells were detached from the plates using a 5 mM EDTA/5 mM EGTA PBS solution. They were processed for FACS analysis as described in Section 2.6. The mean channel fluorescence (MCF) was used to compare the levels of receptor expression at the cell surface. Results were normalized for the MCF obtained for wt $hVPAC_2$ receptor with the specific antibody (100%) after subtraction of the non-specific fluorescence obtained with non-transfected cells (0%). Details on specific protocols for evaluation of receptor recovery are given in the legends of the figures.

3. Results

3.1. Ability of the different peptides used to occupy the human recombinant $VPAC_2$ receptor expressed in CHO cells and to stimulate adenylate cyclase activity

The detailed characteristics of the peptides tested were already published [8,9,16,20,21], and the data presented in Fig. 1 and Table 1 represent the values obtained on the cell line used in the whole study. The choice of the studied peptides was based on the following considerations: the selective agonists Ro 25-1553 and *N*-hexanoyl-VIP had a lower IC_{50} value than VIP but behave differently: the cyclic peptide Ro 25-1553 was as efficient as VIP (same maximal effect) whereas the acyl-VIP was more efficient than VIP; this

Table 1
pIC_{50} of binding and pEC_{50} of adenylate cyclase activation for VIP and analogs on membranes from CHO cells expressing the $hVPAC_2$ receptors

	pIC_{50} (M)	pEC_{50} (M)
VIP	8.10 ± 0.04	9.10 ± 0.02
N-hexanoyl-VIP	8.60 ± 0.02	9.70 ± 0.01
$[R^{16}]$-VIP	7.20 ± 0.01	8.30 ± 0.07
Ro 25-1553	8.50 ± 0.09	9.90 ± 0.02
$[A^{11}]$-VIP	6.30 ± 0.03	7.00 ± 0.03
$[V^{11}]$-VIP	6.50 ± 0.06	7.00 ± 0.05
PG99-465	7.90 ± 0.02	–

The values derived from experiments performed on cell membranes from the clones used during the present study. Values are given with the standard error of the mean (three determinations).

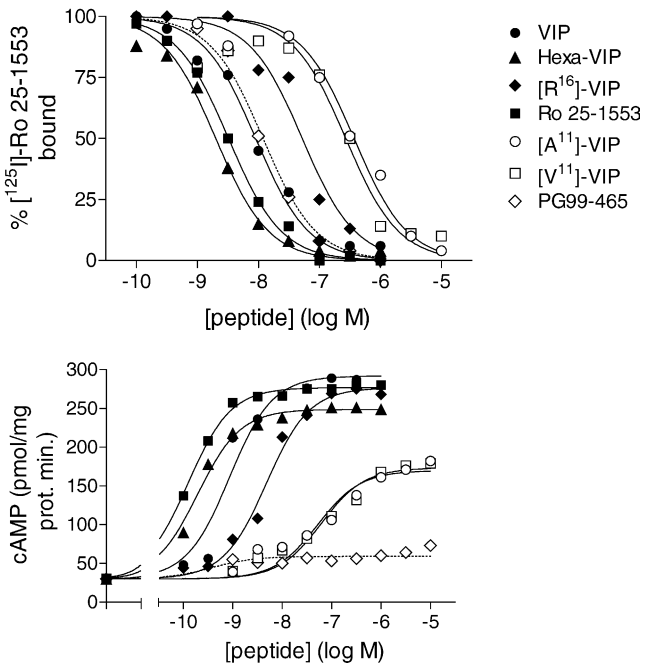

Fig. 1. Upper panel: Inhibition of [^{125}I]-Ro 25-1553 binding to membranes from CHO cells expressing the recombinant hVPAC$_2$ receptor by increasing concentrations of VIP analogs. Lower panel: Adenylate cyclase stimulation by the VIP analogs of the same membranes used in the upper panel. Means of three experiments, the IC_{50} and EC_{50} values are given with the standard error in Table 1.

was already observed in preparations expressing a rather low density of wild-type receptors and obvious on mutated receptors with a poor G-protein coupling. PG99-465 was the most potent and selective VPAC$_2$ receptor antagonist described. The [A^{11}]-VIP, [V^{11}]-VIP were synthesized on the basis of Nicole et al. [22] study of the VIP residues permitting a discrimination between VIP interaction with the VPAC$_1$ and VPAC$_2$ receptors; they had a lower affinity than VIP for the VPAC$_2$ receptor and were partial agonists. [R^{16}]-VIP was initially developed as a VPAC$_1$ selective agonist with a higher affinity than VIP on that receptor but a higher IC_{50} value than VIP on the VPAC$_2$ receptor. Its efficacy was comparable to that of VIP on the VPAC$_2$ receptor. In general, the EC_{50} values for adenylate cyclase activation correlated with the IC_{50} values of binding but, due to the existence of spare receptors, the EC_{50} values were lower than the IC_{50} values. The efficacies of the peptides are presented in Fig. 1.

3.2. Effect of a 30 min incubation with increasing peptide concentrations on VPAC$_2$ receptors expressed at CHO cell surface and identified by binding of the monoclonal antibody

The results are presented in histograms in Fig. 2 and are expressed as % of receptors remaining accessible to the antibody. VIP, [R^{16}]-VIP, and Ro 25-1553 reduced similarly to 40–50% of initial expression the number of receptors; *N*-hexanoyl-VIP was more efficient, eliciting a 75% receptor inaccessibility, whereas the ligands mutated in position 11 reduced this parameter of 25% only and PG99-465 was inactive.

3.3. Effect of forskolin, phorbol esters and various inhibitors

The effect of a 30 min preincubation in presence of 0.5 M hypertonic sucrose, 4 μg/ml Filipin, 10 mM methyl-β-cyclodextrine, 10 μM forskolin, 10 μM H89 and 3 μM phorbol ester was evaluated on VIP and *N*-hexanoyl-VIP stimulated internalization. The pre-treatments in absence of agonist had no effect on the expression of the receptors. 0.5 M sucrose completely inhibited the response to the two agonists whereas the other agents were inactive (Fig. 3). It must be noticed that 10 mM methyl-β-cyclodextrine reduced the number of living cells (as appreciated by the propidium iodide FACS signal) but that only the cells not labeled with that marker were analyzed.

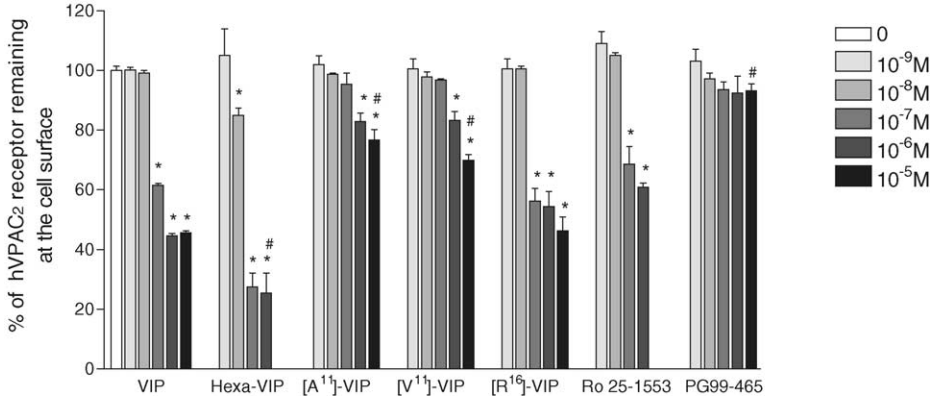

Fig. 2. Dose–effect curves (presented as histograms) of increasing concentrations of VIP and analogs on receptor internalization after a 30 min exposure to the peptide. The results correspond to the number of receptors that remained accessible to the antibody and were expressed in % of the receptors expressed in untreated cells. The results were the means of at least three determinations made in duplicate. * Represents the values different from the control and # the maximal peptides effect different from that obtained in presence of 1 μM VIP ($p < 0.05$ evaluated by Mann–Whitney test).

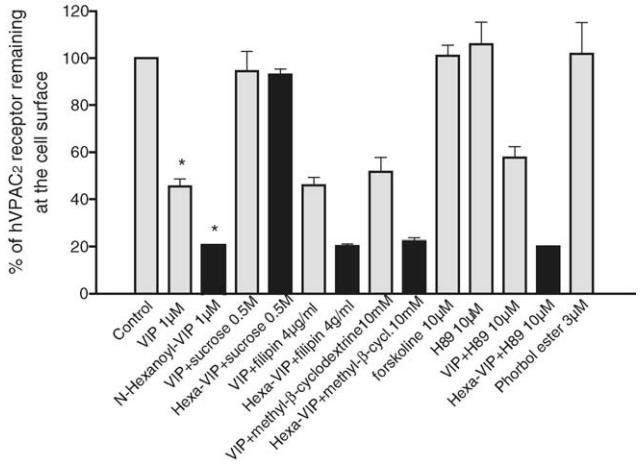

Fig. 3. Effect of hypertonic sucrose, methyl-β-cyclodextrine, filipin and H89 on 10 μM VIP and *N*-hexanoyl-VIP stimulated internalization. A 30 min preincubation was performed before a 30 min exposure to agonists. The results were presented in % of the receptors expressed in absence of added agonist. Forskoline and phorbol ester were also tested during a 30 min period. * indicated the values statistically different from their respective controls ($p < 0.05$ evaluated by Mann–Whitney test).

3.4. Time course of internalization

Internalization was followed from 5 to 120 min in presence of VIP, *N*-hexanoyl-VIP and Ro 25-1553. The number of accessible receptors was already decreased after 5 min incubation and still progressed during the first 60 min (Fig. 4).

3.5. Reappearance of the receptors

After a 30 min exposure to VIP or *N*-hexanoyl-VIP, cells were extensively washed and the receptors expressed at the cell surface were followed during 120 min in absence and presence of 25 μM monensin or 10 μg/ml cycloheximide. Fig. 5A shows that the recovery after exposure to VIP was complete within 2 h and that the phenomenon was inhibited

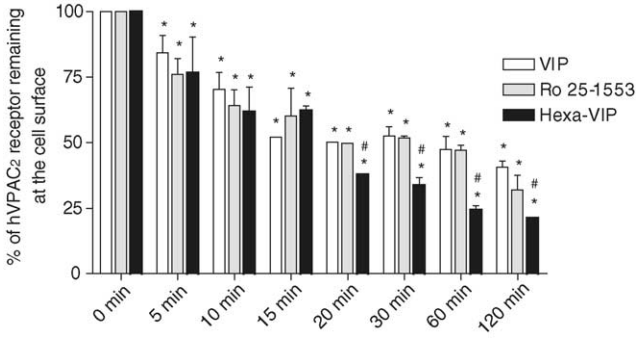

Fig. 4. Time course of receptor internalization in presence of 1 μM VIP, Ro 25-1553 or *N*-hexanoyl-VIP. The results were the mean of at least three determinations in duplicate. * Indicated the values significantly different from the control and # the values obtained in the presence of Ro 25-1553 and *N*-hexanoyl-VIP different from the value observed in the presence of 1 μM VIP ($p < 0.05$ evaluated by Mann–Whitney test).

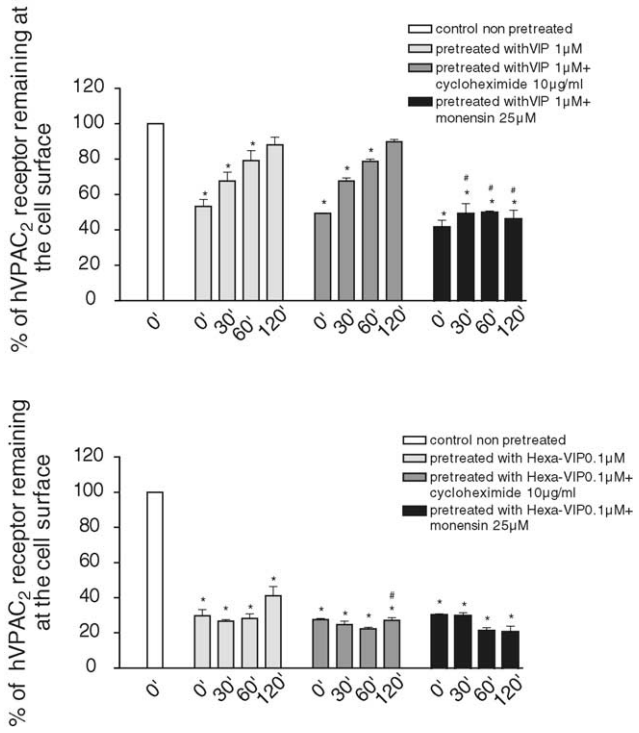

Fig. 5. Evolution of the number of receptors after a 30 min exposure of the cells to 1 μM VIP (upper panel) or 0.1 μM *N*-hexanoyl-VIP (lower panel), followed by three washings and incubation for 30, 60 or 120 min in absence of agonist. Three conditions were compared: first, incubation with the agonists and recovery in absence of added agent; second, addition of 10 μg/ml cycloheximide 2 h before incubation with agonists and during the recovery period; third, addition of 25 μM monensin 20 min before incubation with agonists and during the recovery period. Values were the mean of three experiments made in duplicate. * Indicated the values different from the control and # the values different from the values obtained after washings (time 0 of recovery, $p < 0.05$ evaluated by Mann–Whitney test). The values obtained in presence of monensin or cycloheximide alone were omitted as they were not different from the control (95 ± 5 for monensin, 93 ± 6 for cycloheximide, means ± S.E.M. of at least five experiments).

by monensin that had no effect per se on receptor expression and receptor internalization. Cycloheximide did not influenced receptor reappearance. The reappearance of the internalized receptors after exposure to *N*-hexanoyl-VIP was much slower and was blocked by both monensin and cycloheximide.

3.6. Kinetic properties of the agonist

Considering the significantly higher efficacy of *N*-hexanoyl-VIP as compared to VIP and Ro 25-1553, we evaluated the kinetics properties of the three compounds: the three agonists were iodinated, tracer binding was performed and the dissociation rate was measured by addition at steady state of a large excess of unlabelled ligand. The results presented in Fig. 6 indicated that the half-life of iodinated *N*-hexanoyl-VIP was significantly longer than that of iodinated VIP and Ro 25-1553 that were comparable. In another set of experiments (Fig. 7), we appreciated the inhibitory kinetics of the $VPAC_2$

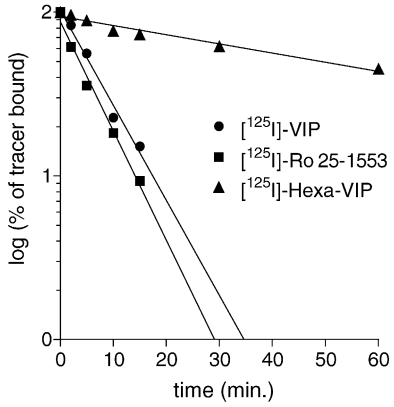

Fig. 6. Dissociation rate of the three labeled ligands: after a 30 min binding at 25 °C with the appropriate tracer, 10 μM VIP was added and the remaining bound radioactivity was followed for 15–60 min. The results were expressed in % of the tracer bound before addition of unlabeled peptide and were presented in a semi log scale. These results were one representative experiment of three others.

antagonist PG99-465 on VIP, Ro 25-1553 and *N*-hexanoyl-VIP stimulated adenylate cyclase. Fig. 7 indicated that the antagonist exerted its effect more rapidly on VIP and Ro 25-1553 stimulated adenylate cyclase than on *N*-hexanoyl-VIP effect (the values obtained with and without added antagonist were significantly different [$p < 0.05$, Mann–Whitney test, $n = 3$] 4, 5 and 18 min after antagonist addition on VIP, Ro 25-1553 and *N*-hexanoyl-VIP stimulation, respectively) suggesting a slower rate of exchange for that agonist.

4. Discussion

The present study on the dynamics of the human recombinant $VPAC_2$ receptor expressed in CHO cells is essentially based on quantification of the receptors expressed at the cell surface of intact cells by using a monoclonal antibody obtained by genetic immunization [3], that binds to the amino-terminal extracellular part of the receptor on an undetermined epitope but that does not interfere with the ligand binding site. This is essential for data interpretation: (a) in membranes, preincubation with antibody does not interfere with tracer binding, competition curves and agonist-stimulated adenylate cyclase activity (see details in Section 2); (b) incubation of intact cells with antagonist concentrations that saturate the receptors does not modify the binding of the antibody (Fig. 2).

The decrease in antibody binding corresponds thus to the inaccessibility of the amino-terminus of the receptor and can be interpreted as receptor sequestration near the membrane, receptor internalization in endocytotic vesicles or receptor degradation. The term “internalization” is used in the present study to describe the observed phenomenon without interpretation of the mechanisms supporting it and without identification of the cellular compartment in which the receptor is send.

Exposure to rather high agonist concentrations induces a rapid internalization of the $VPAC_2$ receptor blocked by hypertonic sucrose that is reported to reduce the clustering of receptors into endosomes [11] but not modified by preincubation with Filipin and methyl-β-cyclodextrine that blocked the lipid raft dependent internalization [19,30]. Thus the $VPAC_2$ receptor is likely driven to an endocytic compartment but if this occurs through the successive formation of clathrin-coated vesicles, arrestin interaction, dynamin interaction [6] remains a not yet proved chain of events. As already mentioned in the Section 1, the precise chain of events that promotes $VPAC_1$ receptor internalization is still under discussion [28] and there is not a unique scheme for all the GPCR class II and differences were obvious also between GPCRI and II receptors: for instance, the secretin receptor is internalized in

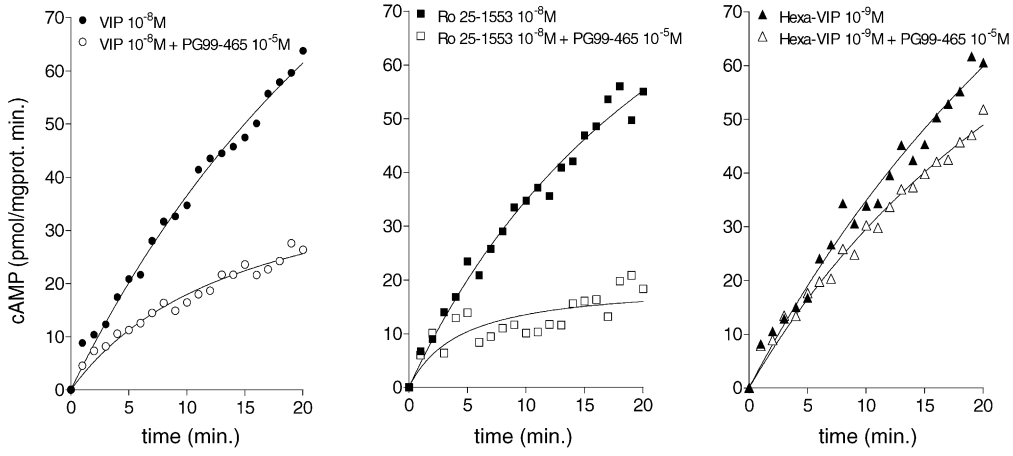

Fig. 7. Kinetics studies of adenylate cyclase activation of CHO cell membranes expressing the recombinant $VPAC_2$ receptor in presence of VIP (l-panel), Ro 25-1553 (middle panel) and *N*-hexanoyl-VIP (right panel) in absence (dark symbols) or presence (open symbols) of the receptor antagonist PG99-465. Agonist was added 15 min before starting the experiment in a medium including all the reagents necessary for adenylate cyclase activation but without labeled [^{32}P]-ATP that was added at time 0. At that moment also the antagonist was added in the appropriate controls. The results were one representative experiment of three others. Control experiments (not shown) were performed by adding antagonist at the same time than agonist: the agonist effect was completely inhibited whatever the agonist tested.

endocytic vesicles after a second messenger-dependent phosphorylation [27] and is conducted in a compartment different from that of the adrenergic receptor, but common with the angiotensin AT1 receptor [13].

After endocytosis, the receptors can be recycled to the membranes, submitted to a lysosomal proteolysis or degraded in the proteasomes [12]. Considering the rather rapid reappearance of the receptor to the membrane after exposure to VIP and also to Ro 25-1553 (but not *N*-hexanoyl-VIP see below), a phenomenon not affected by protein synthesis inhibitors but completely blocked by monensin, an ionophore that prevents receptor recycling from early endosomes by neutralizing intraluminal pH, it is likely that most of the internalized receptors are indeed recycled. As monensin had no effect per se in absence of agonist, a quantitatively important agonist-independent constitutive trafficking is unlikely.

The present work compares the capability of different VIP receptors ligands to occupy the receptors, to stimulate adenylate cyclase activity and to induce receptor internalization. VIP, Ro 25-1553 and [R^{16}]-VIP were equally efficient to stimulate adenylate cyclase and had a comparable affinity for the receptor. They induced the same maximal 50% receptor internalization within 30 min, a value that remained stable for a further 90 min in the continuous presence of the agonist. [A^{11}] and [V^{11}]-VIP were partial agonists on adenylate cyclase stimulation of a lower affinity for the receptor than VIP. They induced at a maximal concentration of 10 μM only 20% of receptor internalization and were thus also partial agonists on this effect. The selective antagonist PG99-465 did not induce receptor internalization. The correlation between the efficacy of the stimulation of the signaling pathway and the internalization, and the observation that both forskolin and phorbol esters were unable to induce $VPAC_2$ receptor internalization suggest that cyclic AMP increase and PKC activation are not the sole determinant of receptor internalization in line with other observations [17]. *N*-hexanoyl-VIP induced a 75% internalization of the receptors, a value significantly higher than that observed with the other agonists. As compared to VIP, this agonist had lower EC_{50} and IC_{50} values on $VPAC_2$ receptor and higher values on the $VPAC_1$ receptor and was therefore a $VPAC_2$ preferring agonist. Its selectivity was however limited. On the $VPAC_2$ wild-type receptor, *N*-hexanoyl-VIP exerted a robust stimulation of adenylate cyclase activity, equal or slightly superior to that of VIP [16]. However, on mutated $VPAC_2$ receptors or on chimeric $VPAC_1$/$VPAC_2$ receptors with a reduced efficacy of VIP, the *N*-acylated peptide behaved as a "super agonist" with an increased coupling efficacy to the second messengers [14]. It is tempting to link the higher efficacy of *N*-hexanoyl-VIP on agonist mediated internalization to its capacity to favor the active state of the receptor. Furthermore, this higher internalization efficacy was also associated with a poor reappearance of the receptors to the membrane that appears qualitatively different from the receptor recycling as it is blocked by cycloheximide, suggesting that it corresponds to the synthesis of new receptors rather than to the re-expression of internalized ones. It is actually suggested that the routing of the internalized receptors back to the membrane or to proteolysis compartments depends on the capability of the internalized receptor to remain associated with arrestin [23]. This could be modulated by the rate of receptor dephosphorylation or by receptor configuration. It could be possible that *N*-hexanoyl-VIP induced a receptor configuration different from that induced by the other agonists. The observation, by the direct measurement of the dissociation rate of the labeled derivative, and indirectly by the rate of exchange with an antagonist in a functional assay, that the *N*-acylated peptide dissociated much slowly from the receptor may indicate a stabilization of the active conformation of the receptor or that the receptor adopts an original conformation.

Whatever the explanation, if VIP analogs are to be developed for therapeutic purposes, it must be kept in mind that agonists with a comparable affinity for the receptor and an apparent comparable efficacy may differ in their capacity to induce a sustained receptor internalization.

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