CONTRIBUTION OF THE CARBOXY-TERMINUS OF THE VPAC₁ RECEPTOR TO AGONIST INDUCED RECEPTOR PHOSPHORYLATION, INTERNALIZATION, AND RECYCLING

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When exposed to Vasoactive Intestinal Peptide (VIP), the human wild type VPAC₁ receptor expressed in Chinese Hamster Ovary (CHO) cells is rapidly phosphorylated, desensitized and internalized in the endosomal compartment and is not re-expressed at the cell membrane within two hours after agonist removal. The aims of the present work were first to correlate receptor phosphorylation level, measured after metabolic labeling and receptor immunoprecipitation, to internalization and recycling, measured by flow cytometry and in some cases by confocal microscopy using a monoclonal antibody that did not interfere with ligand binding, and second to identify the phosphorylated Ser/Thr residues, as there was no evidence for a Tyr residue phosphorylation. Combining receptor mutations and truncations allowed identification of S250 (in the second intracellular loop), T429, S435, S448 or S449 and S455 (all in the distal part of the C-terminus) as candidates for VIP stimulated phosphorylation. The effects of single mutations were not additive, suggesting alternative phosphorylation sites in mutated receptors. Replacement of all the Ser/Thr residues in the carboxy-terminal tail and truncation of the domain containing these residues completely inhibited VIP stimulated phosphorylation and receptor internalization. There was, however, no direct correlation between receptor phosphorylation and internalization: in some truncated and mutated receptors, a 70% reduction in phosphorylation had little effect on internalization. In contrast to results obtained on the wild type and all the mutated or truncated receptors that still underwent phosphorylation, internalization of the severely truncated receptor was reversed within two hours incubation in the absence of the agonist. Receptor recovery was blocked by monensin, an endosome inhibitor.

The neuropeptide Vasoactive Intestinal Polypeptide (VIP) exerts its multiple regulatory functions through interaction with two high affinity receptors named VPAC₁ and VPAC₂. These are members of a family of G protein coupled receptors (GPCRs), designated as Class II or B. This class includes also, among others, receptors for peptides of at least 20 amino acid residues like secretin, glucagon, glucagon-like peptides, growth hormone-releasing peptide, parathormone and pituitary adenylate cyclase activating peptide (1). VPAC₁ and VPAC₂ receptors are preferentially coupled to the Gαₛ protein (1) responsible for increasing cyclic AMP concentrations but may also, with a lower efficiency, couple to Gαᵢ and Gαᵣ proteins (2) responsible for a [Ca²⁺]ᵢ and IP₃ increase. As with most, if not all the GPCRs, both VIP receptors are desensitized, sequestered and down-regulated after exposure to agonist (3-5). This was observed in cells expressing native receptors as well as in transfected Chinese Hamster Ovary cells (CHO) cells and HEK 293 cells. It was recently demonstrated that VPAC₁ receptor phosphorylation and desensitization was enhanced by co-transfection with the G protein-receptor-kinases, GRK 2, 3, 5 and 6 (5). Although the overexpression of arrestin or of a dominant negative mutant did not modify receptor internalization, the inhibitory effect of a dominant negative mutant of dynamin suggested the following sequence of events for
receptor regulation: agonist stimulation, G protein kinase mediated phosphorylation, β-arrestin translocation and dynamin-dependant receptor internalization (5).

In the present work we detailed the contribution of the carboxy-terminal intracellular tail to receptor internalization by studying truncated and mutated human VPAC1 receptors expressed in CHO cells. We developed a monoclonal antibody against the amino-terminal extracellular part of that receptor that permitted the quantification, by flow cytometry, of the receptors expressed at the cell membrane. Immunoprecipitation of the receptor after metabolic labeling with $^{32}$P orthophosphate, followed by SDS-PAGE and autoradiography, allowed for phosphorylation quantification. We therefore evaluated the link between receptor phosphorylation and receptor internalization. We also evaluated the recycling of the receptors to the membrane.

We found that VIP induced in the wild type (wt) receptor a rapid phosphorylation and internalization which was not reversible within 2 h. Mutation into Ala or truncation of all the Ser/Thr residues in the C-terminal tail and mutation of one Ser in the second intracellular loop, abolished receptor phosphorylation and internalization. A larger truncation of a domain located between the seven transmembrane helix and the Ser/Thr containing region led to a receptor that was no longer phosphorylated but remained internalized. However, receptors were re-expressed at the membrane within 120 min. Single and combined mutations of the Ser/Thr residues indicated the possibility of alternative phosphorylation sites in mutant receptors and also that phosphorylation of all the identified sites was not necessary for receptor internalization.

MATERIALS AND METHODS

Construction of truncated and mutated receptors - The cell line expressing VPAC1 receptor has been detailed in previous publication (6). Generation of the truncated receptors was achieved by introduction of a stop codon using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla CA, USA) essentially according to the manufacturer’s instructions as described (2). The expected mutation was confirmed by DNA sequencing on an ABI automated sequencing apparatus, using the BigDye Terminator Sequencing Prism Kit from ABI (PerkinElmer, CA, USA). The complete nucleotide sequence of each construction was verified by DNA sequencing. Twenty µg of receptors coding region were transfected by electroporation in CHO cell line expressing aequorin and Gα16 (kindly provided by Vincent Dupriez, Euroscreen SA, Belgium) as described (2). Selection was carried out in culture medium [50% HamF12; 50% DMEM; 10% Fetal Calf Serum; 1% Penicillin (10 mU/ml); 1% Streptomycin (10 µg/ml); 1% L-Glutamine (200 mM)], supplemented with 600 µg Geneticin (G418)/ml culture medium. After 10 to 15 days of selection, isolated colonies were transferred to 24 well plates and grown until confluence, trypsinized and further expanded in 6 well plates, from which cells were scraped and membranes prepared for identification of receptor expressing clones by an adenylate cyclase activity assay in presence of 1 µM VIP. The selected clones were expanded in the same medium than that used for the selection but in absence of Geneticin.

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 400g and the supernatant was further centrifuged at 20,000g for 15 min. The pellet was resuspended in 1 mM NaHCO$_3$ and used immediately.

Binding studies - Binding studies, using $^{125}$I-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, 1% bovine serum albumin (pH 7.4) and 3 to 30 µg protein per assay. The assays were performed in such conditions that specific binding was strictly proportional to the amount of protein. Bound and free radioactivities were separated by filtration through glass-fiber GF/C filters pre-soaked for 24 h in 0.01% polyethyleneimin. The filters were rinsed three times with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5% bovine serum albumin. Binding sites density was evaluated by: density = bound/free x IC$_{50}$ per mg protein.

Adenylate cyclase activity - Adenylate cyclase activity was determined by the Salomon et al. procedure (7) as previously described (8). Membrane proteins (3-15 µg) were incubated in a total volume of 60 µl containing 0.5 mM [$\alpha^{32}$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 [3H]-cAMP. cAMP was separated from ATP by two successive chromatographies on Dowex 50Wx8 and neutral alumina.

**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 vol of cold ether and purified on reverse phase and ion exchange chromatographies. The peptide purity (>95%) was assessed by capillary electrophoresis and their conformity by electrospray mass spectrometry. The peptide mentioned as the VPAC1 antagonist was AcHis1 [D-Phe2,K15,R16,L27]VIP (3-7)/GRF (8-27).

**Preparation of a monoclonal antibody for VPAC1** - a) Genetic immunization and generation of monoclonal antibodies were performed according to Costagliola et al (9). The protocol was approved by the local Ethical Committee for Animal Experimentation. Six weeks old Balb/c female mice were anaesthetized by injections of 6-10 mg/kg Ketamin HCl® associated to 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). Five days later, 50 µg of the plasmid construct was injected in the same region in a final volume of 100 µl 0.09% NaCl. Injections were repeated 3 and 6 weeks thereafter. Blood samples were obtained from retro-ocular puncture 7 weeks after the initial immunization and serum tested for the presence of antibodies against the VPAC1 receptor. The mouse selected (by FACS and Western Blotting) for monoclonal antibody (mAb) production, was boosted by an IV injection of 100 µl of a saline solution containing 10⁶ CHO cells expressing the human VPAC1 receptor. Three days later, splenocytes were fused with SP2O, a non secreting myeloma cell line at a 3:1 ratio in presence of polyethylene glycol. Fused cells were then delivered into ten 96-well plates and selected by 100 µM hypoxanthine, 400 µM aminopterine and 16 µM thymidine. Irradiated macrophages from mouse peritoneum were added to the well for supply of cytokines and growth factors. After 10 days, culture supernatants were screened by FACS (see below) and the cells producing antibodies were cloned by dilution. The monoclonal antibody selected (mAb-VPAC1) was purified using ImmunoPure IgG Purification kit (Pierce) and was of IgG 2a subtype, based on the mouse mAb isotyping kit (Isotrip, Boehringer Mannheim Corp).

b) Properties of the selected antibody. CHO cells expressing the recombinant human VPAC1 receptor were detached from the plates using a 5 mM EDTA/ 5 mM EGTA PBS solution, harvested by centrifugation (500g, 4°C, 4 min), washed once with PBS solution and resuspended to 3.10⁵ cells/tube in 100 µl PBS-0.1% BSA, containing 0.1 µg purified mAb VPAC1. After 30 min incubation at 4°C, the cells were washed in the same buffer and centrifuged in the same conditions. They were then incubated for 30 min, on ice in the dark, with secondary antibody, a FITC-conjugated-γ-chain-specific goat anti-mouse IgG (Sigma Chemicals Co., St. Louis, MO). The cells were again washed and resuspended in 250 µl PBS-0.1% BSA. The level of fluorescence was analyzed using a FACScalibur (Becton Dickinson, Eerenbodegem, Belgium) and the data processed using the cell quest software. Basal fluorescence was determined from sample of non transfected CHO cells. The use of propidium iodide (10 µg/ml) allowed exclusion of debris and dead cells from the analysis. The same procedure was used to evaluate the selectivity of the antibody: the level of fluorescence observed with CHO cells expressing the human VPAC2, the rat VPAC1 and VPAC2 receptors was not different from that of cells that did not express the human VPAC1 receptor. Chimeric receptors made of different parts of the human VPAC1 and VPAC2 receptors and expressed in CHO cells (8) were only detected when the amino-terminal domain of the VPAC1 receptor was conserved (Fig. 1). Furthermore, preincubation of membranes prepared from cells expressing the human VPAC1 receptor with the monoclonal antibody at increasing concentrations did not modify the binding of [125I]-VIP nor the VIP stimulated adenylyl cyclase activity.

**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 the VPAC₁ receptor were incubated with agonist at 37°C and after washing 3 times with ice-cold phosphate buffer saline were processed for FACS analysis as described above. Details on specific protocols for evaluation of receptor recovery are given in the legends of the figures.

Confocal microscopy was also used to confirm receptor sequestration: cells were cultured on 22 mm glass slides for 72 h. After 30 min VIP treatment, cells were washed in Phosphate Buffer Saline (PBS) and fixed with -20°C absolute methanol for 10 min. Unspecific protein binding was prevented by 15 min incubation with 5% normal sheep serum. The cells were then incubated overnight at 4°C with the monoclonal anti-VPAC₁ antibody (1:250). The primary antibody was diluted in PBS- 1% normal sheep serum- 1% azide. The optimal working dilution had been previously determined empirically by serial dilutions for the antibody used. 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 FITC-conjugated-γ-chain-specific goat anti-mouse IgG (Sigma Chemicals Co., St. Louis, MO) (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. Cells were finally incubated for 5 min at room temperature with Hoechst 33258 (Molecular Probes, Eugène, OR). After 3 rinses in PBS, coverslips were mounted with « Slow Fade Light » anti-fade mounting medium (Molecular Probes, Eugène, OR) in 50% glycerol (or the Calbiochem mounting medium) before viewing under a LSM510 NLO confocal microscope fitted on an Axiovert M200 inverted microscope equipped with a C-Apochromat 63x/1.2 N.A. water immersion objective (Zeiss, Iena, Germany). A 2x electronic zoom was used across regions of interest. The 488 nm excitation wavelength of the Argon/2 laser, a main dichroic HFT KP650 and a band-pass emission filter (BP435-485 nm). Optical sections, 2.5 µm thick, were collected for each fluorochrome sequentially. The images generated (512 x 512 pixels, pixel size: 0.14 µm) were merged and displayed with the Zeiss LSM510 software and exported in .jpg image format. All figures show a single optical section across the regions of interest. Scale bars: 10 µm.

Immunoprecipitation, determination of receptor phosphorylation and of receptor density – Cells were first cultured in phosphate-free DMEM for 16 h and then incubated for 2 h at 37°C in presence of 0.1 mCi/ml acid-free [³²P]orthophosphate. At the end of this labeling period, agonist was added for 5 min. Phosphorylation inhibitors were added 30 min prior agonist addition. Cells were then washed three times with ice-cold buffer consisting in 10 mM HEPES, 4.2 mM NaHCO₃, 11.7 mM glucose, 1.2 mM MgSO₄, 4.7 mM KCl, 118 mM NaCl and 1.3 mM CaCl₂ pH 7.4 and then lysed in 1.2 ml of a buffer consisting in 20 mM Tris, 100 mM (NH₄)₂SO₄ and 10% glycerol pH 7.5. The cell lysate was centrifuged at 600g for 4°C followed by the supernatant centrifuged at 19,000g for 30 min. The resulting pellet was resuspended in the same buffer containing 1% dodecylmaltoside (Roche Diagnostics Belgium) and solubilized for 45 min at 4°C. The remaining insoluble material was eliminated by a further centrifugation. The supernatant (200 µl) was added to 50 µl of a 10% protein A Sepharose suspension coated during 2 h with 2 µg of purified mAb VPAC₁. After 150 min incubation under rotating agitation at 4°C, the Sepharose beads were separated by centrifugation and washed successively with the concentrated lysis buffer, then with a two-fold diluted buffer and finally with water. The final beads pellet was resuspended in a buffer consisting in 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. The gel was fixed, dried and the phosphorylated bands detected and quantified by phosphoimaging (Vilber Lourmat, Kaiser).

The contribution of GRK to agonist-dependent phosphorylation was assessed in in vitro assays. CHO cells expressing the VPAC₁ or the CCR5 receptor (kindly provided by Dr C. Blanpain, IRIBHN, Brussels) were grown using a main dichroic HFT KP650 and a band-pass emission filter (BP435-485 nm). Optical sections, 2.5 µm thick, were collected for each fluorochrome sequentially. The images generated (512 x 512 pixels, pixel size: 0.14 µm) were merged and displayed with the Zeiss LSM510 software and exported in .jpg image format. All figures show a single optical section across the regions of interest. Scale bars: 10 µm.
as previously described and resuspended in a buffer consisting in 20 mM Tris HCl, 10 mM MgCl₂, 10 mM NaCl, 1 mM EGTA added of a protease inhibitor cocktail (Complete, Roche Diagnostic, Belgium). Fifty µl of membrane (2 µg/µl of protein) were incubated with 50 µM \([\gamma^{32}P]\)-ATP for 5 min at 37°C in the presence of the tested agents. The final volume was 100 µl. Reactions were started by the addition of ATP, and stopped by a 30 sec centrifugation at 13,000g, removal of the supernatant and solubilization of the membranes with 250 µl of a buffer consisting in 20 mM Tris, 100 mM (NH₄)₂SO₄, 10% glycerol, a protease inhibitor cocktail (Complete, Roche Diagnostics, Belgium), and 1% dodecylmaltoside, pH 7.5.

Human VPAC₁ solubilized receptor was then immunoprecipitated as previously described and protein resolved on 10% SDS-PAGE. CCR5 solubilized receptor was then immunoprecipitated in 80 µl of a 50% protein G Sepharose suspension and 3 µg of purified monoclonal anti-human CCR5 antibody (2D7, PharMingen Diego, CA). After 5 successive washes, the sample was resuspended in the buffer, heated at 50°C for 20 min and processed as for the VPAC₁ receptor.

Receptor density was evaluated in all cases by binding studies using \(^{125}\text{I}\)-VIP as ligand as previously described (10) and confirmed in some cases by Western Blotting. As Western Blotting could not be performed with the monoclonal antibody used for FACS and immunoprecipitation studies, we used a polyclonal antibody generated by Prof Schulz (Otto-von-Guericke-University, Magdeburg, Germany) directed against the 438-457 sequence of the carboxy-terminus of the receptor (11). This antibody does not recognize any of the truncated receptors under studies (data not shown) but Western Blots performed on wt and selected mutated receptors validated binding data (see results). Membranes were prepared as described above. The protein were resolved by 10% SDS-PAGE, transferred on a nitrocellulose membrane, and incubated with 1µg/ml primary antibody overnight at 4°C. We used as secondary antibody anti-rabbit peroxydase-conjugated antibody. Proteins were visualized using SuperSignal® West Pico reagent Chemiluminescent Substrate (Pierce, Perbio-Science).

**RESULTS**

Agonist induced VPAC₁ receptor phosphorylation and internalization. VIP induced a rapid, dose-dependent stimulation of \(^{32}\text{P}\) incorporation into a protein of an apparent molecular size of 75 kDa that immunoprecipitated with the mAb-VPAC₁ (Fig. 2). No signal was observed in non transfected cells.

Receptor phosphorylation was agonist dependent: forskolin, phorbol esters and the selective VPAC₁ antagonist were inactive per se; forskolin and phorbol esters did not modify VIP induced phosphorylation. VIP stimulated phosphorylation was not affected by 100 nM H-89, 6 µM Staurosporine, 300 nM K252a, 100 µM Genistein, but inhibited by 30 µM H-89, 100 µM A3, and 200-500 µM CKI-7 (Fig. 3). The possible contribution of the GRKinases to receptor phosphorylation was evaluated on membranes incubated in presence of radioactive ATP, 1 µM VIP and 0.1 mM Zn ++ and 1 µg/ml Heparin as inhibitors (12-14). A positive control consisted in CHO cell membranes expressing the chimikine receptor CCR5 stimulated by Rantes (15) (Fig. 3). VPAC₁ receptor internalization was estimated by flow cytometry by the decrease in fluorescence associated to the binding of the mAb-VPAC₁. A typical experiment is shown in Fig. 4. Exposure to VIP induced a rapid and sustained decrease in the receptor number expressed at the cell surface that was completely blocked by preincubation in presence of 0.5 M sucrose, suggesting that the receptor was internalized in endosomes. The selective VPAC₁ receptor antagonist was inefficient. Results observed at 5 and 30 min were presented in Fig. 4. Seventy five % of the receptors disappeared within 30 min.

Receptor internalization was further visualized by confocal microscopy: as shown in Fig. 5 left panels, fluorescence signal was detected in unstimulated cells exclusively at CHO cells membranes whereas the fluorescence was scattered in the cytosol after 30 min stimulation with 1 µM VIP. As negative control (right panel), we used the poly A mutant that was considered as non internalized by FACS technique (see below) and remained indeed at the cell membrane by confocal inspection (Fig. 5).

The reversibility was tested after 30 min exposure to agonist: after three washes, cells were incubated 20 to 120 min and the receptors accessible to the antibody again
evaluated. There was no reappearance of the receptors (Fig. 6). We used as positive control the VPAC₂ receptor expressed in the same CHO cell line, that was similarly internalized but was re-expressed to the membrane within 120 min (Fig. 6); the results of the VPAC₂ receptor were detailed in reference (16).

Properties of carboxy-terminally truncated receptors. The truncated receptors studied were schematized in Fig. 7 and listed in Table I. They were all stably expressed in CHO cells. For each construction at least 4 clones were generated and studied. To compare the different constructions we detailed clones that expressed (when possible) a similar receptor density. The binding receptor properties and the capability of VIP to stimulate adenylate cyclase activity were detailed elsewhere for some of the truncated receptors (17) and are summarized in Table I. The IC₅₀ values of binding, the EC₅₀ values and the maximal stimulatory effect of VIP on adenylate cyclase were comparable for all the truncated receptors. We already reported that two receptors had an elevated basal adenylate cyclase activity that was decreased by the selective VPAC₁ receptor antagonist (17) suggesting a constitutive activity, but that did not modify VIP stimulation.

As compared to the 1-457 wild type receptor, the 1-444, 1-441, and 1-436 truncated receptors had a comparable 30% reduction of phosphorylation measured by densitometry on gels loaded with the same amount of receptors. The 1-433 and 1-429 truncated receptors had a 70% reduction in receptor phosphorylation (Fig. 8). The 1-421 truncated receptor retained only 10% of the VIP stimulated phosphorylation. The shortest receptor tested 1-398 exhibited a still detectable VIP stimulated phosphorylation but to low to be valuably quantified.

Receptor internalization was rapid (65 to 80% of the receptors were no more accessible to the antibody after 5 min incubation with 1 µM VIP and this value remained stable for the next 25 min). Internalization was slow down for the 1-429 VPAC₁ receptor and for the shorter fragments 1-421, 1-417, 1-402. For this last construction, 10 and 20% only of the receptors were not accessible after 5 and 30 min incubation respectively. Surprisingly, the shortest fragments 1-401, 1-400, 1-399 and 1-398 were internalized as rapidly and as efficiently as the wild type receptor (Table I).

As mentioned above, VIP stimulated wild type receptor internalization was not reversible after repeated washings of the treated cells and further incubation for 120 min in absence of agonist. The same behavior was observed for the 1-444 to 1-429 truncated receptors. For the 1-421 to 1-402 truncated receptors, the results were difficult to analyze due to the low level of internalization, but no reappearance of the receptors could be suspected. Surprisingly, reappearance of the internalized 1-401, 1-400, 1-399 and 1-398 truncated receptors was obvious (Fig. 6). Receptor reappearance was in all cases inhibited by 25 µM monensine but not by 10 µg/ml cycloheximide (data not shown).

Properties of carboxy-terminally mutated receptors. Serine and threonine residues were mutated in alanine separately or collectively to precise the phosphorylatable residues and the functional consequences on ligand recognition, adenylate cyclase activation, coupling to G protein, internalization and reappearance of the receptors at the cell membrane. To validate the receptor quantification by binding assay, we performed a Western Blot of the wild type and some point mutated VPAC₁ receptors using the polyclonal anti-VPAC₁ antibody (Fig. 9). A same amount of receptor, evaluated by binding assay, was introduced in each lane. The results obtained after revelation by chemiluminescence showed a labeling of each construction and no labeling of the membrane extracted from untransfected CHO (last lane). Integration of each band revealed that the amount of receptor detected was not different except for the poly A mutant. This last observation could be due to a poor recognition of the mutant by the antibody (6 mutations in the peptide used for antibody generation).

The results are summarized in Tables II and III and location of the mutations can be found in Fig. 7. The S455A mutant was not different from the wild type receptor except for a 40% reduced phosphorylation. The triple mutant S447-448-449A (that eliminates a PKA consensus sequence), had also properties undistinguishable from those of the wild type receptor except for a 30% reduction in receptor phosphorylation. Internalization was comparable to that of the wild type receptor and re-expression of the receptor at the cell surface was not observed within 120 min. The
S447A, S441A, T438A, T432A, S431A, S425A and S422A mutants were on all the parameters tested, not different from the wild type receptor. The S435A and the T429A had as sole difference with the wild type receptor a 66% reduction in VIP stimulated receptor phosphorylation. Combining the three mutations that decreased from at least 50% receptor phosphorylation led to the S455,S435,T429A triple mutant: surprisingly the individual effects on receptor phosphorylation were not additive, the phosphorylation level reaching as for the single mutant 40% of that of the wild type receptor. However, at variance with the single mutants, internalization of the receptor was significantly slow down (Tables II and III).

Mutations of Ser residues in IC2. As mentioned in the above paragraph, the deletion of all the Ser and Thr residues of the carboxy-terminus markedly reduced, but did not abolish the VIP stimulated receptor phosphorylation. We hypothesized therefore the possibility of a phosphorylable Ser/Thr residue in the intracellular loops connecting the transmembrane domains. As we previously shown (18) the importance of the distal part of the IC3 for receptor coupling to the G proteins and as a poorly coupled receptor could not be helpful, we first mutated the S247 and S250 residues in IC2. The single mutated S247A receptor was undistinguishable from the wild type receptor, but the S250A had a 50% reduction of VIP induced phosphorylation (Fig. 10) without change in ligand recognition, basal and VIP stimulated adenylate cyclase values, receptor internalization and trafficking (Table II).

Combining truncation of the receptor C-terminus containing all the Ser/Thr residues and S250A mutation (S250A 1-421) completely abolished VIP stimulated receptor phosphorylation and markedly slowed down receptor internalization (Fig. 10).

Surprisingly, combination of the Ser/Thr mutations in the C-terminus that reduced receptor phosphorylation with the S250A mutation did not further reduce phosphorylation or did not further slow down receptor internalization: the S250,435,455,T429A receptor was not different from the S435,455,T429A receptor. The activity, including receptor phosphorylation, internalization and trafficking, of S250,435,455A, S250,455,T429A and the S250,435,T429A were not different from any corresponding single mutant (Table III).

Mutation in Ala of all the Ser and Thr residues of the C-terminal tail and of S250 led to a receptor with binding properties and adenylate cyclase activity not different from that of the wild type receptor but that was neither phosphorylated nor internalized (by FACS technique and confocal microscopy; see carboxy poly A in Table III and Fig. 5).

DISCUSSION

The aims of the present work were to identify the amino acid residues of the human VPAC1 receptors that are phosphorylated during agonist stimulation and to correlate the phosphorylation level with receptor internalization and eventually re-expression to the membrane.

To identify the phosphorylated residues, we first searched for consensus sequences (19,20) and identified a PKA (S447,S448,S449 in the C-terminus), a PKC (F249,S250,E251,R252 in IC2), and casein kinase (S247,F248,F249,S250 in IC2; S331,D332,S333,S334 and S334,P335,Y336,S337 in IC3; S422,G423,G424,S425 in C-terminus) consensus sites. As the receptor was not phosphorylated by forskolin, nor by phorbol esters, and as the VIP stimulated phosphorylation was not inhibited by low concentrations of H-89, nor by staurosporone or K252a, we hypothesized that VIP induced phosphorylation was not mediated by PKA and PKC. The partial inhibitory effect of CKI-7 (21), a selective inhibitor of casein kinase 1-α, did not exclude involvement of that enzyme. However, phosphorylation by casein kinases implies the presence of an acidic function in the consensus (22), preferentially a phosphorylated Ser/Thr, to anchor the enzyme and trigger a phosphorylation cascade. There was no evidence that these potential initiators (S247, S331 and S334 in the intracellular loops and S422 in the first part of the C-terminus) were indeed phosphorylated. In the distal part of the C-terminus tail however, a phosphorylation cascade starting with T429 could involve T432, S435, T438 and S441. However, by single mutation, only T429 and S435 were identified as candidates for phosphorylation. GRK remained by exclusion
the main kinase(s) identified. This was tested on membranes as there is no known selective cell permeable inhibitor: Zn\(^{++}\) and heparin are reported to antagonize GRK activity (12-14). In our experimental conditions, Zn\(^{++}\) was only partially efficient. However, in the positive control used (15), similar results were obtained. Finally, only Ser and/or Thr residues were phosphorylated: the tyrosine kinase inhibitor genistein was inactive and the non-selective Ser/Thr kinase inhibitor A3 (23) completely blocked VIP stimulated phosphorylation. Recent data (24) suggest that S447 in the PKA consensus in the carboxy-terminal could be phosphorylated but it was not demonstrated that this was performed through kinase A activation: replacement of S447 with Ala increased basal unstimulated phosphorylation and blunted the VIP induced phosphorylation, a finding that was not observed in the present work.

The strategy used to identify the phosphorylated residues consisted of the progressive truncation of the carboxy-terminus, individual mutations in Ala of the suspected residues, followed by combined mutations.

For the interpretation of the results on the truncated receptors, we made the assumption that a decreased phosphorylation was due to the suppression of one phosphorylatable residue and that an unchanged phosphorylation level meant that the residues that were suppressed were not phosphorylated. In other words we did not consider that the truncated receptors may be phosphorylated on other residues than those used in the wild type receptor. We also made the assumption that the phosphorylation level was directly linked to the number of phosphorylated residues and we did not consider possible kinetic changes in the kinase and phosphatase activities. We also considered that the evaluation by binding studies of the number of receptors was appropriate and that phosphorylation was in any case proportional to the receptor density.

Considering these points, the similar 30% reduction in receptor phosphorylation of the three truncated forms 1-444, 1-441 and 1-436 suggested that at least one of the following S447, S448, S449 and S455 was phosphorylated. As 447-448-449 was a PKA consensus sequence and as PKA has been excluded, we first considered the residue S455 as a good candidate. Mutation into Ala reduced receptor phosphorylation by 40%. However, the simultaneous mutation to Ala of the three adjacent Ser residues reduced the phosphorylation by 30%. The single mutation of the S447 residue did not significantly modify VIP induced phosphorylation under our conditions as already discussed. The marked decrease in receptor phosphorylation when comparing the 1-436 and the 1-433 mutants focuses on the S435 residue; indeed, its replacement by Ala decreased by 70% the phosphorylation level. As phosphorylation of the 1-429 truncated receptor was comparable to that of the 1-433, it was unlikely that S431 and T432 were phosphorylated. Mutation of these residues in Ala confirmed this hypothesis. The VIP stimulated phosphorylation of the 1-421 receptor was extremely low but detectable. Removal of either T429, S425 or S422 could be responsible for that decrease. Individual mutations of these Thr and Ser residues into Ala indicated that T429 was the only residue to be phosphorylated. From the results on the truncated and the single mutation receptors, as well as the effect of forskolin, TPA and inhibitors, we considered that the following residues were likely candidates for VIP stimulated VPAC1 receptor phosphorylation: S455, S448 or S449, S435 and T429 in the C-terminus and also S250 in the IC2 loop. A recent study on the 5-HT2A receptor also implicated a serine located in the IC2 and a second in the C-terminus in the agonist-mediated receptor desensitization (25).

However, the results obtained when combining mutations of these identified residues indicated that the effects on phosphorylation were not additive. This contrasts with results published, for instance, for the CCR5 receptor where four phosphorylatable serine residues were identified and each contributed equally to the total phosphorylation level (15).

In our model, combining double (data not shown), triple and quadruple mutations of the target residues identified by point mutation maintained a phosphorylation level of about 30% of that observed in the wild type receptor, a value reached with some single mutations. However, mutation of all the phosphorylatable residues of the carboxy-terminus abolished receptor phosphorylation. This suggests that phosphorylation can operate on other residues when the preferred ones are missing. This
alternative phosphorylation has been described for rhodopsin: rhodopsin kinase can efficiently phosphorylate other serine and threonine residues in the absence of the 3 sites preferentially phosphorylated (26,27).

A second point to be considered is the fact that single mutation of S435 and T429 to Ala induced a more pronounced decrease in receptor phosphorylation than mutation of S250, S455 and of the sequence S447-S448-S449. This suggested a hierarchy in the phosphorylation of the VPAC₁ receptor that could be explained by the fact that some residues are better substrates, constitute a kinase binding site or trigger a phosphorylation cascade. This last point was already discussed. Hierarchical phosphorylation has already been reported for the δ-Opioid receptor (28), the N-formyl peptide receptor (29) and the CCK receptor (30,31). Whatever the explanation, we concluded that there is variability as to which residues are phosphorylated in mutated and probably also in the truncated receptors.

Due to this variability, it is difficult to correlate phosphorylation data and receptor internalization. A quantitative aspect can be discussed: if we consider the mutant and the truncated receptors longer than 1-402 residues, the receptor phosphorylation level must be reduced to at least 30% of the wild type receptor level to decrease receptor internalization. Thus phosphorylation rate is in excess for internalization. Such a low phosphorylation requirement has already been described for other receptors: internalization of the CCR5 receptor only requires the presence of two phosphorylated serines in the C-terminus (32), even if, in vivo, four distinct C-terminal residues are phosphorylated (15). A stoichiometry of 2 mol of phosphate/mol for the β2-adrenergic- (33) and m2 muscarinic receptor (34) is sufficient for their internalization, while additional phosphorylation of up to 10 to 11 mol of phosphate/mol of receptor does not amplify the phenomenon. For these two receptors, the position of the phosphorylated sites was not critical. Receptor internalization was directly correlated to arrestin binding and complete phosphorylation of the receptor was not necessary for arrestin-receptor complex stability.

Several mechanisms are possible for receptor internalization: first, an arrestin-, clathrin-, dynamin-dependent process, second, an arrestin and clathrin-independent but dynamin-dependent process through caveolae, third, an arrestin and clathrin-independent but dynamin-dependent process that does not require caveolae, and fourth, an arrestin-, clathrin-, dynamin-independent process. Concerning the VPAC₁ receptor, the established facts are as follows: a) a dynamin-dependent mechanism. b) a VIP-dependent arrestin recruitment to the membrane without any effect of dominant negative mutant (5). c) internalization in endocytic vesicles which could be blocked by sucrose (present work). d) a relative dependence on receptor phosphorylation (present work). Considering other class 2 GPCRs, it appears that a) the secretin receptor is phosphorylated after agonist exposure, but phosphorylation is not required for internalization (35). Arrestin is recruited to the membrane but there is no effect of dominant negative construction. Dominant negative dynamin was also without effect (36). b) the PTH receptor is internalized by an arrestin-dependent mechanisms but requires the presence of two highly conserved residues located in the core of the receptor: N289 and K382. These residues could regulate a conformational modification necessary to translocation toward the endocytic endosomes (37). The use of a pathway that differs from the classical clathrine coated pit pathway is not limited to class 2 GPCR: internalization of the class 1 GPCR 5HT₂A receptor involves also atypic mechanisms (38).

In the present work, we showed that VPAC₁ receptor internalization occurs by two different mechanisms: a phosphorylation dependent non-reversible pathway and a phosphorylation-independent pathway which allows rapid recycling of the receptor to the plasma membrane. This is the case of the truncated 1-421 to 1-402 receptors. A possible explanation for this is that the multiple positive charges in the 402 to 421 domain (R403, R404, H406, K417, H420) may prevent interactions of negatively charged residues located in the intracellular domains (E394, E398 in the C-terminal tail, but also D327 and D332 in IC3 and E251 in EC2) with an unidentified intracellular partner. It must be noticed that E394 was identified as necessary for coupling of VPAC₁ receptor to Gαs (39).

In conclusion, the present data do not allow an unambiguous identification of the Ser/Thr residues of the VPAC₁ receptor that
are phosphorylated in response to VIP. This is likely due to the possibility of alternative phosphorylation when key residues are mutated or eliminated by truncation. They clearly demonstrate that when all the potential phosphorylation sites located in the C-terminus and one Ser residue in the IC2 loop are mutated into Ala, the VIP stimulated phosphorylation is abolished and the receptor is no longer internalized, although it is still fully active. Truncation of the distal part of the C-terminus containing all the Ser/Thr residues also abolishes receptor phosphorylation and internalization. However, a receptor more proximally truncated, although still fully active and not phosphorylated is internalized rapidly supporting the notion of recruitment of arrestin-insensitive/GRK-insensitive pathways. This internalization differs from that of the wild type receptor by its reversibility within 2 h, suggesting new interactions with the receptor trafficking machinery.

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FIGURE LEGENDS

Fig. 1. Evaluation by flow cytometry of the capacity of the monoclonal antibody used in this study to recognize the human VPAC₁ receptor (black serpentine) expressed at the surface of CHO cells (panel 1) or LOVO cells (panel 2), the human VPAC₂ receptor (grey serpentine) (panel 3), and different VPAC₁/VPAC₂ chimera (panel 4 to 8) expressed in CHO cells. The grey histograms represent untransfected CHO cells. The presence of receptors in the cell lines that did not recognize the antibody was established by binding studies using appropriate ligands (8). The antibody was used at a 2 ng/µl concentration. Representative of 3 independent experiments.

Fig. 2. VIP induced phosphorylation of VPAC₁ receptor. Receptor phosphorylation levels were evaluated after preincubation of the cells with inorganic [³²P] by VIP stimulation and receptor immunoprecipitation with anti-VPAC₁ receptor monoclonal antibody. The arrow indicates a size of 75 kDa corresponding to the labeled receptor detected by autoradiography after SDS page. Upper panel: Dose-effect study after 5 min VIP stimulation. Lower panel: Time course study following 1 µM VIP stimulation. The two last rightward lanes show the phosphorylation of untransfected CHO cells after 2 and 15 min stimulation with 1 µM VIP respectively. Representative of 3 independent experiments.

Fig. 3. Effect of protein kinases inhibitors on receptor phosphorylation. Upper panels: Receptor phosphorylation levels were evaluated after preincubation of the cells with inorganic [³²P] by 5 min stimulation and receptor immunoprecipitation with anti-VPAC₁ receptor monoclonal antibody. The arrow indicates a 75 kDa size corresponding to the labeled VPAC₁ receptor, detected by autoradiography after SDS page. Left: Effect of 30 min incubation with 30 µM H-89, 300 nM K252 or 100 µM genistein on 1 µM VIP, 10 µM forskolin or 30 nM PMA induced VPAC₁ receptor phosphorylation. Right: dose-effect curve of A3 (a general serine-threonine kinase inhibitor) and CKI-7 (a casein kinase 1-α inhibitor) on the phosphorylation of the VPAC₁ receptor. Lower panel: membrane phosphorylation of the VPAC₁ and of the CCR5 receptor. The arrows indicating a 75 kDa or 40 kDa size correspond to the labeled VPAC₁ or CCR5 receptor respectively. The membrane preparation was incubated 5 min in presence of 100 µM [γ³²P]-ATP and in presence of 1 µM VIP or 100 nM RANTES (the agonist ligand of CCR5), in presence or absence of 0.1 mM Zn²⁺ or 1 µg/ml heparin (inhibitors of GRK). Representative of 3 independent experiments.

Fig. 4. Flow cytometry analysis of CHO expressing the human VPAC₁ receptor. Left panel: the internalization is evaluated by the disappearance of the receptor from the cell surface after a treatment of 5 min (grey histogram) or 30 min (grey dark histogram) with 1 µM VIP, as compared to untreated cells (full dark histogram). Middle panel: internalization of VPAC₁ receptor after 30 min treatment with 1 µM VPAC₁ antagonist (grey histogram). Right panel: internalization of VPAC₁ receptor after 30 min pretreatment with sucrose 0.45 M, followed by 5 min treatment with 1 µM VIP. Representative of 3 independent experiments.

Fig. 5. Visualization of wt and poly A mutated VPAC₁ receptor internalization by confocal microscopy. CHO cells expressing the wt VPAC₁ receptor (left panels) or the poly A mutated receptor (right panels) were exposed to 1 µM VIP for 0 (upper panels) and 30 min (lower panels). The receptors were labeled with the anti-VPAC₁ monoclonal antibody and revealed by a secondary FITC conjugated-antibody. After a 30 min treatment to VIP, the VPAC₁ receptor is found within the cytosol (lower left panel), even though the poly A receptor is still localized at the plasma membrane (upper right panel). The nuclei were labeled in blue by the Hoechst reagent. Three slides were examined for each experimental condition. Scale bars: 10 µm.

Fig. 6. Evolution of receptors expression after a 30 min exposure of the cells to 1 µM VIP, followed by three washings and incubation for 30 or 120 min in absence of agonist. Values are the means ± SEM of three experiments made in duplicate. * p<0.05 as compared to control evaluated by Mann-Whitney test. The VPAC₂ receptor expressed also in CHO cells was also presented as control of a wt receptor re-expressed to the membrane after VIP washing (ref 16).
Fig. 7. Schematic representation of the second, the third intracellular loops, and the carboxy-terminus tail of the human VPAC₁ receptor. The serine and threonine are in grey. The black traces indicate the positions for receptor truncation.

Fig. 8. VIP induced phosphorylation of the truncated 1-429, 433, 436, 441 and the wild type VPAC₁ receptor. Receptor phosphorylation levels evaluated after preincubation of the cells with inorganic [³²P] followed by 5 min stimulation with 1 µM VIP and receptor immunoprecipitation with anti-VPAC₁ receptor monoclonal antibody. The arrow indicates a size of 75 kDa corresponding to the labeled receptor detected by autoradiography after SDS page. The same amount of receptor, determined by binding assay, was loaded in each electrophoresis lane. Representative of 3 independent experiments.

Fig. 9. Relative quantification of wild type and mutated VPAC₁ receptor by Western Blot. The assay was performed with an anti-VPAC₁ antibody directed against the 438-457 sequence of the carboxy-terminal domain of the receptor. The membrane was incubated with a secondary antibody coupled to peroxydase and revealed by chemiluminescence (upper panel). On each lane the same amount of receptor, based on binding studies, was charged. The lower panel represented the integration of the bands in arbitrary units and the results were the mean of three separate experiments, the upper panel being one of these experiments.

Fig. 10. VIP induced phosphorylation of VPAC₁ wild type and mutated receptor. Receptor phosphorylation levels evaluated after preincubation of the cells with inorganic [³²P] followed by 5 min stimulation with 1 µM VIP and receptor immunoprecipitation with anti-VPAC₁ receptor monoclonal antibody. The arrow indicates a size of 75 kDa corresponding to the labeled receptor detected by autoradiography after SDS page. Left panel: S250A mutant and VPAC₁ wild type receptor. Right panel: truncated S250A 1-421 mutant and VPAC₁ wild type receptor. The same amount of receptor, determined by binding assay, was loaded in each electrophoresis lane. Representative of 3 independent experiments.
Table 1: Summary of binding, adenylate cyclase activation, phosphorylation and internalization studies performed on wild type and truncated VPAC₁ receptors. Results represent the means ± SEM of at least three independent experiments in duplicate. * p<0.05 evaluated by Mann-Whitney test as compared as wild type receptor value. The phosphorylation levels are expressed in % of wild type receptor phosphorylation level normalized for the amount of receptors as determined by binding. Internalization corresponds to the percentage of receptors that are no more expressed at the cell surface after 5 or 30 min treatment with 1 µM VIP.

| Receptors   | Binding pIC50 | cAMP pEC50 | cAMP Emax (fold stim) | Phosphorylation (in % of wt) | Internalization (%) |
|-------------|---------------|------------|-----------------------|-----------------------------|---------------------|
| VPAC₁ WT    | 8.8 ± 0.02    | 8.6 ± 0.08 | 3.3 ± 0.3             | 100                         | 66 ± 5             |
| VPAC₁ 1-444 | 8.8 ± 0.03    | 8.4 ± 0.03 | 3.9 ± 0.4             | 62 ± 3*                     | 82 ± 2             |
| VPAC₁ 1-441 | 8.5 ± 0.05    | 8.6 ± 0.06 | 3.1 ± 0.3             | 67 ± 7*                     | 85 ± 4             |
| VPAC₁ 1-436 | 8.8 ± 0.04    | 8.6 ± 0.02 | 2.7 ± 0.2             | 70 ± 8*                     | 73 ± 3             |
| VPAC₁ 1-433 | 8.3 ± 0.03    | 8.7 ± 0.04 | 3.1 ± 0.3             | 30 ± 4*                     | 77 ± 9             |
| VPAC₁ 1-429 | 8.6 ± 0.05    | 8.5 ± 0.04 | 2.8 ± 0.2             | 32 ± 5*                     | 37 ± 6*            |
| VPAC₁ 1-421 | 8.8 ± 0.07    | 8.3 ± 0.05 | 3.6 ± 0.4             | 10 ± 2*                     | 14 ± 4*            |
| VPAC₁ 1-417 | 8.5 ± 0.06    | 8.5 ± 0.06 | 3.5 ± 0.3             | <5*                         | 13 ± 1*            |
| VPAC₁ 1-402 | 8.2 ± 0.08    | 8.5 ± 0.07 | 6.0 ± 0.5*            | <5*                         | 9 ± 3*             |
| VPAC₁ 1-401 | 8.6 ± 0.04    | 8.3 ± 0.03 | 4.8 ± 0.4*            | <5*                         | 67 ± 2             |
| VPAC₁ 1-400 | 8.9 ± 0.05    | 8.8 ± 0.04 | 2.4 ± 0.2             | <5*                         | 74 ± 8             |
| VPAC₁ 1-399 | 8.7 ± 0.04    | 8.4 ± 0.05 | 5.1 ± 0.4*            | <5*                         | 71 ± 5             |
| VPAC₁ 1-398 | 8.8 ± 0.08    | 8.4 ± 0.04 | 3.6 ± 0.3             | <5*                         | 65 ± 4             |
Table II: Summary of binding, adenylate cyclase activation, phosphorylation and internalization studies performed on wild type and point mutated VPAC₁ receptors.
Results represent the means ± SEM of at least three independent experiments in duplicate. * p<0.05 evaluated by Mann-Whitney test as compared as wild type receptor value. The phosphorylation levels are expressed in % of wild type receptor phosphorylation level normalized for the amount of receptors as determined by binding. Internalization corresponds to the percentage of receptors that are no more expressed at the cell surface after 5 or 30 min treatment with 1 µM VIP.

| Receptors | Binding  | cAMP  | cAMP  | Phosphorylation | Internalization (%) |
|-----------|---------|-------|-------|-----------------|--------------------|
|           | pIC50   | pEC50 | Emax  | (in % of wt)    | 5 min  | 30 min |
| VPAC₁ wt  | 8.8 ± 0.02 | 8.6 ± 0.03 | 3.3 ± 0.3 | 100 ± 3 | 66 ± 3 | 77 ± 4 |
| S247A     | 8.4 ± 0.03 | 8.3 ± 0.05 | 5.0 ± 0.6 * | 100 ± 2 | 63 ± 2 | 76 ± 5 |
| S250A     | 8.7 ± 0.04 | 8.6 ± 0.02 | 3.2 ± 0.2 | 53 ± 9* | 74 ± 4 | 83 ± 6 |
| S422A     | 8.4 ± 0.01 | 8.8 ± 0.04 | 5.2 ± 0.5 * | 100 ± 4 | 62 ± 5 | 64 ± 3 |
| S425A     | 8.6 ± 0.03 | 8.7 ± 0.01 | 5.1 ± 0.4 * | 100 ± 1 | 54 ± 7 | 64 ± 1 |
| T429A     | 8.6 ± 0.05 | 8.6 ± 0.03 | 3.6 ± 0.3 | 33 ± 6* | 73 ± 1 | 90 ± 6 |
| S431A     | 8.8 ± 0.06 | 8.7 ± 0.02 | 3.4 ± 0.3 | 100 ± 3 | 63 ± 4 | 70 ± 2 |
| T432A     | 8.8 ± 0.02 | 8.5 ± 0.03 | 4.5 ± 0.4 * | 100 ± 2 | 54 ± 3 | 73 ± 3 |
| S435A     | 8.9 ± 0.04 | 8.5 ± 0.01 | 4.1 ± 0.3 | 33 ± 8* | 58 ± 2 | 69 ± 4 |
| T438A     | 8.3 ± 0.04 | 8.6 ± 0.02 | 5.5 ± 0.6 * | 100 ± 4 | 64 ± 4 | 76 ± 2 |
| S441A     | 8.3 ± 0.05 | 8.6 ± 0.05 | 4.9 ± 0.4 * | 100 ± 2 | 78 ± 3 | 83 ± 4 |
| S447A     | 8.6 ± 0.03 | 8.1 ± 0.02 | 6.6 ± 0.5 * | 100 ± 5 | 67 ± 5 | 77 ± 1 |
| S455A     | 8.7 ± 0.02 | 8.7 ± 0.01 | 3.1 ± 0.3 | 59 ± 7* | 69 ± 4 | 85 ± 3 |
Table III: summary of binding, adenylate cyclase activation, phosphorylation and internalization studies performed on wild type and mutated VPAC₁ receptors. Results represent the means ± SEM of at least three independent experiments in duplicate. * p<0.05 evaluated by Mann-Whitney test as compared as wild type receptor value. The phosphorylation levels are expressed in % of wild type receptor phosphorylation level normalized for receptor levels determined by binding. Internalization corresponds to the percentage of receptors that are no more expressed at the cell surface after 5 or 30 min treatment with 1 µM VIP.

| Receptors                         | Binding pIC50 | cAMP Emax (fold stim) | cAMP pEC50 | Phosphorylation (% of wt) | Internalization (%) |
|-----------------------------------|---------------|-----------------------|------------|----------------------------|---------------------|
|                                   |               |                       |            |                            | 5 min     | 30 min     |
| VPAC₁ wt                          | 8.8 ± 0.02    | 3.3 ± 0.3             | 8.6 ± 0.03 | 100                        | 66 ± 3    | 77 ± 2     |
| S447-448-449A                     | 8.9 ± 0.01    | 3.1 ± 0.3             | 8.6 ± 0.02 | 71 ± 9*                    | 63 ± 6    | 74 ± 1     |
| S250-435-455A                     | 8.8 ± 0.02    | 2.8 ± 0.2             | 8.9 ± 0.02 | 74 ± 18*                   | 70 ± 5    | 81 ± 6     |
| S250-455-T429A                    | 8.9 ± 0.03    | 3.2 ± 0.3             | 8.8 ± 0.01 | 31 ± 9*                    | 65 ± 2    | 85 ± 2     |
| S250-435-T429A                    | 8.4 ± 0.04    | 4.1 ± 0.4             | 8.6 ± 0.02 | 54 ± 2*                    | 69 ± 7    | 78 ± 4     |
| S435-455-T429A                    | 8.3 ± 0.03    | 3.2 ± 0.3             | 9.2 ± 0.04 | 39 ± 4*                    | 26 ± 9*   | 59 ± 3*    |
| S250-435-455-T429A                | 8.4 ± 0.01    | 2.7 ± 0.2             | 8.3 ± 0.03 | 35 ± 5*                    | 37 ± 8*   | 69 ± 5     |
| S250-435-447-448-449-455-T429A    | 8.7 ± 0.02    | 5.4 ± 0.5             | 8.7 ± 0.01 | 46 ± 8*                    | 76 ± 4    | 88 ± 7     |
| Carboxy poly A                    | 8.9 ± 0.03    | 3.9 ± 0.4             | 8.1 ± 0.02 | <5*                        | <5*       | <10*       |
Figure 1

Fluorescence intensity
Figure 2

| VIP (nM) | 0 | 0.1 | 1.0 | 10 | 100 | 1000 | VPAC<sub>1</sub> ANTAGO |
|----------|---|-----|-----|----|-----|------|-----------------------|
|          | 0 | 2'  | 5'  | 15'| 30' | 60'  | 2'                    |

**CHO expressing the VPAC<sub>1</sub> receptor**

| VIP | 0' | 2' | 5' | 15' | 30' | 60' | 2' | 15' |
|-----|----|----|----|-----|-----|-----|----|-----|
|     | 75 kDa |    |    |     |     |     |    |     |

**Untransfected CHO**

| VIP | 2' | 15' |
|-----|----|-----|
|     | 75 kDa |    |
Figure 3

Cont   VIP 1µM   Fors 10µM   VIP   VIP PMA 30nM   VIP
H-89 30µM  H-89 30µM  K252 300nM  K252 300nM  Gen
H-89 30µM  K252 300nM  K252 300nM  Gen

Cont   VIP 1µM
A3 [µM]  CKI-7 [µM]
100 200 500 100 200 500

75 kDa

VIP 10^{-4}M
RANTES 10^{-7}M
Zn^{+} 0.1mM  Heparin 1 µg/ml
Zn^{+} 0.1mM  Heparin 1 µg/ml

75 kDa

40 kDa
Figure 4
Figure 5
Figure 6

![Graph showing the percentage of receptor remaining at the cell surface for different constructs over time. The x-axis represents different constructs, and the y-axis shows the percentage remaining. The bars indicate the control, 30 min, and 120 min conditions. Significant differences are marked with asterisks.](http://www.jbc.org/Downloadedfrom)
Figure 7
Figure 8

|    | 1-429 | 1-433 | 1-436 | 1-441 | VPAC1 wt |
|----|-------|-------|-------|-------|---------|
| VIP| VIP   | VIP   | VIP   | VIP   | VIP     |

75 kDa
Figure 9

[Image of a gel blot with bands labeled VPAC1wt, S250A, T429A, S435A, S455A, PolyA, and control. The gel shows a 75 kDa marker. A bar graph below the gel shows arbitrary units with error bars.]
Figure 10

| VPAC<sub>1</sub> S250A | VPAC<sub>1</sub> wt | S250A 1-421 | VPAC<sub>1</sub> wt |
|-----------------------|---------------------|-------------|---------------------|
| VIP 1µM               | VIP 1µM             | VIP 1µM     | VIP 1µM             |

75 kDa

[Image of the figure showing the bands for VPAC<sub>1</sub> S250A, VPAC<sub>1</sub> wt, S250A 1-421, and VPAC<sub>1</sub> wt with arrow indicating 75 kDa]
Contribution of the carboxy-terminus of the VPAC1 receptor to agonist induced receptor phosphorylation, internalization, and recycling

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