0026-895X/03/6406-1565–1574$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 64:1565–1574, 2003

Vol. 64, No. 6
2450/1114435
Printed in U.S.A.

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Serine 447 in the Carboxyl Tail of Human $VPAC_1$ Receptor Is Crucial for Agonist-Induced Desensitization but Not Internalization of the Receptor

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Received February 6, 2003; accepted September 11, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The $VPAC_1$ receptor for vasoactive intestinal peptide (VIP) belongs to the class II family of G protein-coupled receptors and is coupled to Gs protein/adenylyl cyclase. We assessed whether 10 different Ser/Thr residues in human $VPAC_1$ receptor intracellular domains play a role in the process of VIP-induced desensitization/internalization by performing a site-directed mutagenesis study. The Ser/Thr residues mutated to Ala include potential G protein-coupled receptor kinase, protein kinase A and protein kinase C targets that are of particular interest for $VPAC_1$ receptor desensitization. The data show that when Chinese hamster ovary cells expressing wild-type receptors were pretreated for 5 min with VIP (50 nM), receptor desensitization occurred with a 10-fold right shift of the ED_{50} for adenylyl cyclase activation. When the construct with the widest span of mutations was studied, there was no longer any short-term desensitization. By using constructs with fewer and fewer mutations, we identified Ser447 in the C-terminal tail to be crucial for rapid desensitization. We also showed that Ser447 plays an essential role for VIP-induced $VPAC_1$ phosphorylation in Chinese hamster ovary cells. Furthermore, we demonstrated that none of the mutated Ser/Thr residues was involved in down-regulation after a 12-h treatment of cells with 50 nM VIP. Neither were they involved in VIP and VIP-induced receptor internalization as shown using a novel fluorescein-tagged VIP and $VPAC_1$ receptor bearing a Flag epitope in the N-terminal domain and a green fluorescent protein at the C terminus. We conclude that Ser447, a likely G protein-coupled receptor kinase target, is crucial for VIP-induced phosphorylation and rapid desensitization of $VPAC_1$ receptor.

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Receptors ($VPAC_1$ and $VPAC_2$) for vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP) are members of the family of class II G protein-coupled receptors (GPCRs) (Laburthe et al., 2002a). This family is composed of several distinct peptide receptors, such as PACAP, secretin, glucagon, glucagon-like peptide-1 (GLP-1), GLP-2, parathyroid hormone (PTH), gastric inhibitory polypeptide, calcitonin, calcitonin gene-related peptide (CGRP) and corticotropin-releasing factor receptors, that have different physiological functions. The class II receptors for peptides have a low sequence homology with other members of the superfamily of GPCRs (Laburthe et al., 1996).

On the basis of the extensively studied β_2-adrenergic receptor, a type I GPCR, the process of agonist-mediated desensitization involves G protein-coupled kinases (GRKs) that rapidly phosphorylate receptors leading to subsequent binding of β-arrestins to phosphorylated receptors (Pitcher et al., 1998). The role of arrestins in this process is crucial because they alter the affinity of the receptor for the G-protein. They also recruit different clathrin adapter proteins to signal internalization of the activated receptor (Claing et al., 2002; Pierce et al., 2002). Some GPCRs, which include muscarinic, serotoninergic and various other peptidergic receptors including the $VPAC_1$ receptor, can follow a pathway of internalization that is dependent on dynamin rather than arrestins (Claing et al., 2000; Bhatnagar et al., 2001; Delaney et al., 2002).

It has been very recently shown that upon binding to recombinant $VPAC_1$ receptors, VIP induced receptor phosphorylation, β-arrestin translocation to plasma membrane, and a dynamin-dependent receptor internalization (Shetzline et al., 2002). A few studies have been reported on recep-

ABBREVIATIONS: VPAC, vasoactive intestinal peptide/pituitary adenylate cyclase-activating polypeptide type; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylyl cyclase-activating protein; GPCR, G protein-coupled receptor; C-T, C-terminal tail; GLP, glucagon-like peptide; PTH, parathyroid hormone; CGRP, calcitonin gene-related peptide; PKA, protein kinase A; PKC, protein kinase A; CHO, Chinese hamster ovary; BSA, bovine serum albumen; PBS, phosphate-buffered saline; KSCN, potassium thiocyanate; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine B isothiocyanate; GFP, green fluorescent protein; IL, intracellular loop; H-89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; FK, forskolin; HEK, human embryonic kidney.

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tor desensitization or internalization for other class II GPCRs. There are speculations that $VPAC_1$ receptor and the closely related secretin receptor are desensitized by the action of GRK2 and GRK5 (Shetzline et al., 1998, 2002). A further indication that members of type II GPCRs are desensitized by GRKs is provided by CGRP receptors whereby GRK6 is particularly effective (Aiyar et al., 2000). In homologous or agonist-induced processes, desensitization of GPCRs is essentially mediated by GRKs, whereas receptor internalization can involve PKA action as with secretin receptors (Walker et al., 1999). Another example is that of the PTH-1 receptor subtype, which is coupled to cyclic AMP/PKA and phospholipase C/cytosolic calcium and diacylglycerol/PKC pathways. It has been reported that internalization of PTH-1 receptor is dependent on protein kinase PKC (Ferrari et al., 1999). Taken together, these observations highlight that GRKs and PKA/PKC can play a role in the desensitization and internalization, respectively, of type II GPCRs.

Extensive structure and function studies of $VPAC_1$ receptors and other members of this receptor family have been performed to elucidate ligand binding motifs, whereas those implicated in the processes of receptor desensitization and internalization remain poorly explored (Lins et al., 2001; Du et al., 2002; Laburthe and Couvineau, 2002b).

Because members of the GRK family potentially mediate phosphorylation and thereby desensitization of $VPAC_1$ receptors, we searched for potential targets for GRKs and other protein kinases in the intracellular domain of $VPAC_1$ receptors. The consensus motifs for GRK are still ill-defined, as exemplified by the following observation. Even though GRK1, -2, and -3 preferentially phosphorylate serine/threonine residues in an acidic environment, the majority of GRK2 targets in β_2-adrenergic receptors are also phosphorylated by GRK5 (Fredericks et al., 1996). Thus, in search for GRK targets, we performed a site-directed mutagenesis study of the 9 serine residues that were located in a potentially charged environment because of either acidic or basic amino acid residues. This structure and function study included the consensus sites of phosphorylation by PKA and by PKC as well as one highly conserved threonine residue present in the first intracellular loop (IL1). We show that Ser 447 in the C-terminal tail, one of the potential GRK phosphorylation sites, is crucial for short-term VIP-induced desensitization but not for down-regulation and internalization of human $VPAC_1$ receptors.

Materials and Methods

Materials. Enzymes for site-directed mutagenesis and sequencing were from Promega (Charbonnière, France) and synthetic oligonucleotides were from Invitrogen (Cergy Pontoise, France). The human $VPAC_1$ receptor cDNA was cloned in our laboratory (Couvineau et al., 1994). Synthetic VIP was purchased from Neosystem (Strasbourg, France). Culture medium was from Invitrogen. All antibodies used were from Sigma (St-Quentin-Fallavier, France), and VIP was labeled with ^{125}I purchased from Amersham Biosciences (Les Ulis, France) as described previously (Boissard et al., 1986). The 1.4-kilobase EcoRI fragment containing the entire sequence of human $VPAC_1$ receptor with an inserted octapeptide DYKDDDDK (Flag), at the N-terminal domain between A30 and A31, was subcloned into EcoRI sites of the pAlter-1vector as described previously (Gaudin et al., 1998). Thereafter, Flag epitope-tagged human $VPAC_1$ receptor-green fluorescent protein (Flag/$VPAC_1$/GFP) was prepared by inserting Flag/$VPAC_1$ receptor into pEGFP-N2, an N-terminal protein fusion vector (BD Biosciences Clontech, Palo Alto, CA), via EcoRI restriction sites as described previously (Nicole et al., 2000).

Site-Directed Mutagenesis. Single-stranded DNA [(+) strand] was produced in *Escherichia coli* JM109, and full-length $VPAC_1$ receptor mutants were generated by oligonucleotide-directed mutagenesis as described previously (Couvineau et al., 1996). The expected mutations of the different serine and threonine residues (see Fig. 1) were identified by direct double-strand sequencing of the corresponding $VPAC_1$ receptor regions.

Transfection of $VPAC_1$ Receptor in CHO Cells and Membrane Preparation. All the studies were performed using stably transfected CHO cells. The recombinant plasmids encoding human $VPAC_1$ receptor or mutated receptor constructs were transfected in CHO cells using Fugene (Roche Molecular Biochemical), and stable transfectants were selected in the presence of 800 μg/ml of G418 (Geneticin) for 4 days. Then, the cells were grown for 3 to 4 days without G418. After a second round of G418 selection (800 μg/ml) for 4 days, the cells were grown without G418 for use. The cell culture medium was Ham's F-12 medium supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 mg/ml streptomycin at 37°C in 95% air/5% CO_2. Cell membrane homogenates were prepared after harvesting the cells grown to confluence with a rubber policeman, as described previously (Gaudin et al., 1998). Briefly, they were homogenized using a Dounce homogenizer in 5 mM HEPES buffer, pH 7.4, containing 0.5 mM phenylmethylsulfonyl fluoride. After centrifugation of the cell homogenate at 15,000*g* for 30 min, the pellet or cell membrane preparation was recovered and stored at −80°C until use.

Assays of ^{125}I-VIP Receptor Binding and Adenylyl Cyclase Activity. Specific ^{125}I-VIP binding to the transfected CHO cells was performed as described previously (Marie et al., 1986). Briefly, the subconfluent cells grown in 12-well plates were incubated for 45 min at 37°C in 20 mM HEPES buffer, pH 7.4, containing 2% (w/v) bovine serum albumin (BSA), 0.1% (w/v) bacitracin, 124 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.8 mM glucose, and 50 pM ^{125}I-VIP and in the presence of increasing concentrations of VIP. Thereafter, the cells were washed twice with ice-cold PBS with 1% (w/v) BSA, then solubilized with 1 ml of 0.5 N NaOH, and the amount of radioactivity present was estimated by a γ-counter. For each mutant studied, at least three individual experiments were performed in triplicate. Specific binding was calculated as the difference between the amount of bound ^{125}I-VIP in the absence and the presence of 1 μM unlabeled VIP. The nonspecific binding represented about 10 to 15% of total radioactivity bound. All binding data were analyzed using the LIGAND computer program (Munson and Rodbard, 1980). The dissociation constant (K_D) and binding capacity (B_{max}) were determined by Scatchard analysis. All the data were plotted using the program Prism 2.01 (GraphPad Software). The adenylyl cyclase activity in cell membrane preparations was assayed as described previously (Laburthe et al., 1986). The amount of protein in membrane preparations was estimated according to the method of Bradford (1976).

Short- and Long-Term VIP-Induced Desensitization of $hVPAC_1$ Receptor. For short-term desensitization studies, CHO cells grown in T-75 flasks were incubated in the absence or the presence of 50 nM VIP at 37°C for 5 min. Then, the corresponding cell membrane preparations (see above) were incubated in the presence of increasing concentrations of VIP, and adenylyl cyclase activity was measured. To evaluate long-term desensitization, transfected CHO cells expressing either wild or mutated $VPAC_1$ receptor were incubated for 12 h with 50 nM VIP. Then, VIP-induced adenylyl cyclase activity was measured as above. The adenylyl cyclase activity was calculated as picomoles of cAMP per minute per milligram of protein. To normalize data for each construct, we estimated their percentage of maximal stimulation, which was obtained at 1 μM. The basal and maximal stimulation were similar in the different constructs as indicated in Table 1. The adenylyl cyclase data were

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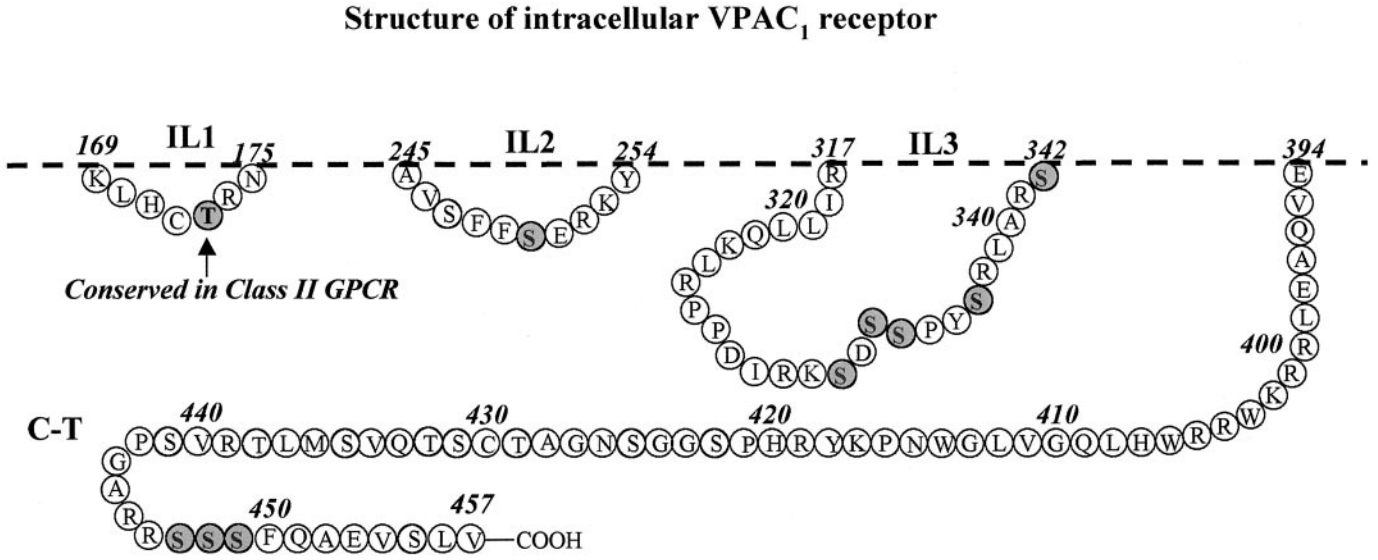
analyzed and plotted using the sigmoidal dose-response regression curve-fitting program Prism 2.01 (GraphPad Software).

Estimation of ^{125}I-VIP Internalization in CHO Cells. Subconfluent CHO cells expressing wild-type or mutated VPAC$_1$ receptors were grown in 12-well plates. They were incubated at 37°C in the binding buffer described above containing 50 pM ^{125}I-VIP in the absence (total binding) and the presence (nonspecific binding) of 1 μM native VIP. At different time intervals (0–60 min), the cells were washed with ice-cold PBS containing 1% (w/v) BSA and thereafter treated differently. On the one hand, the cells were lysed with 500 μl of 0.5 M NaOH at 25°C and the measured radioactivity was used to estimate specific ^{125}I-VIP binding to the cells. On the other hand, bound ^{125}I-VIP at the cell surface was stripped by an incubation with 0.5 M KSCN solution at room temperature during 10 min as described previously (Lyu et al., 2000). The amount of specifically internalized ^{125}I-VIP was then estimated and expressed as a percentage of ^{125}I-VIP specifically bound to CHO cells in function of time.

Synthesis and Characterization of a Fluorescent VIP Derivative. A fluorescent VIP derivative or VIP-FITC was custom-synthesized by Neosystem (Strasbourg, France). A spacer corresponding to the peptide sequence (29–32) of PACAP with a terminal cysteine residue bearing fluorescein was added to the C terminus of VIP. The abilities of VIP-FITC versus VIP to bind to wild-type VPAC$_1$ receptors and stimulate adenylyl cyclase activity in CHO cell membrane preparations was assayed as described previously (Laburthe et al., 1986; Nicole et al., 2000). Briefly, competitive ^{125}I-VIP binding to the cell membranes expressing recombinant VPAC$_1$ receptors was performed in the presence of increasing concentrations of either native VIP or VIP-FITC. Similarly, adenylyl cyclase activity in these membranes was assayed in the presence of increasing concentrations of either compound (see *Results*).

Analysis of VIP-FITC Internalization by Confocal Microscopy. A previously characterized clone of CHO cells (CL15) expressing the wild-type human VPAC$_1$ receptor was grown on 12-mm glass coverslips for 48 h (Gaudin et al., 1998). Cells were then incubated with 50 nM VIP-FITC at 20°C or 37°C during 5 or 60 min in the above described binding buffer. The cells were then fixed at 4°C for 10 min in PBS containing 4% (w/v) paraformaldehyde. The cover slips were mounted with Vectashield medium (Vector Laboratories, Burlingame, CA) and fluorescence of selected fields was observed with a LEICA TCS 4d confocal laser scanning microscope using a



Location of mutated amino-acids

Mutants	IL1	IL2	IL3	C-T
IL1/2/3/C-T	T173A	S247A, S250A	S331A, S333A, S334A, S337A	S447A, S448A, S449A
IL/2/3/C-T		S250A	S331A, S333A, S334A, S337A, S342A	S447A, S448A, S449A
IL2(S250A)		S250A		
IL3			S331A, S333A, S334A, S337A, S342A	
C-T(S447A3)				S447A, S448A, S449A
C-T(S447A)				S447A
C-T(S447D)				S447D

Fig. 1. Schematic representation of intracellular VPAC$_1$ receptor structure with mutated serine/threonine residues. The top shows the intracellular loops (IL1, IL2, and IL3) and the C-terminal tail (C-T) of wild-type VPAC$_1$ receptor bearing the serine/threonine residues that have been mutated in this study (shown in gray). The arrow indicates a threonine residue in IL1 that is highly conserved in class II GPCRs for peptides. The different VPAC$_1$ receptor constructs with their located mutations are indicated at the bottom. They range from a wide span of mutations (IL1/2/3C-T) to a restricted residue in C-T [C-T(S447A)].

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63/1.4 objective. The fluorescent image processing was performed with the on-line "Scan Ware" software, and numeric images were processed with Photoshop 5.5 (Adobe Systems, Mountain View, CA).

Estimation of Cell Surface Expression of hVPAC$_1$ Receptors. Cell surface expression of the Flag/VPAC$_1$/GFP constructs (see *Materials*) in nonpermeabilized CHO cells was assessed using the mouse monoclonal anti-Flag antibodies and a subsequent TRITC-sheep antimouse IgG. This assessment was valid for all VPAC$_1$ receptor constructs because they had a Flag epitope in the N-terminal extracellular domain as well as a GFP molecule at the C terminus. Thus, the epitope-tagged receptors at the surface of nonpermeabilized cells were detected by an intense rhodamine fluorescence caused by the TRITC-labeled sheep antibodies. CHO cells expressing wild or mutated (C-T(S447A) receptors were grown on 12-mm glass cover slips for 48 h and then they were further incubated for 12 h at 37°C in either the absence (control) or the presence of 50 nM VIP. The immunolabeling procedure was performed at 20°C with PBS containing 1% (w/v) BSA (PBS-BSA), starting with a 30-min incubation with the buffer. Then, the cells were subsequently incubated for 30 min with the primary Flag mouse monoclonal antibody (dilution 1/50) and the secondary antibody (TRITC-sheep antimouse IgG at a 1/100 dilution). The cover slips were mounted in glycergel medium (DAKO, High Wycombe, UK). Selected fields were examined on a Leica (Rueil-Malmaison, France) inverted DM IRB microscope using a 40× fluor oil immersion objective and the two filters N2.1 (515–590 nm band pass) and L5 (480–527 nm band pass) for GFP and TRITC fluorescence, respectively.

Phoshorylation of Flag-Tagged VPAC$_1$ receptor. The CHO cells expressing wild-type or the mutated VPAC$_1$ receptor C-T(S447A) were used for receptor phosphorylation experiments as described previously (Shetzline et al., 2002). Briefly, subconfluent CHO cells grown in T-25 flasks were washed in phosphate-free DMEM containing 20 mM HEPES, pH 7.4, and labeled with [^{32}P]orthophosphate (125 μCi/ml) for 1 h in phosphate-free medium at 37°C. The cells were then incubated for 10 min in the absence (basal) or in the presence of 1 μM VIP, the medium was discarded, and cells were lysed for 30 min at 4°C in 50 mM Tris-HCl buffer, pH,8, containing 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 10 mM disodium pyrophosphate, 5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, and 10 μg/ml benzamidine. Lysates were centrifuged at 100,000*g* for 30 min and the supernatant was used for immunoprecipitation. The wild-type and mutated VPAC$_1$ receptors, which are Flag-tagged, were immunoprecipitated with monoclonal anti-M2-Flag-antibody bound to protein G Sepharose beads (Sigma). The proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with monoclonal anti-M2-Flag-antibody and a secondary antibody coupled to alkaline phosphatase. Detection was performed using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate colorimetric detection kit. The detected VPAC$_1$ receptor proteins were scanned and quantitated with the use of NIH Image. Also, the ^{32}P-labeled bands of the autoradiograph corresponding to VPAC$_1$ receptor (90 kDa) were similarly quantitated, and results are expressed as the ratio of ^{32}P-labeled VPAC$_1$ receptor/VPAC$_1$ receptor protein.

Statistical Analysis. All results are expressed as mean ± S.E. Differences among data were tested by analysis of variance by the Student's *t* test for unpaired data. A P value of <0.05 was regarded as statistically significant.

Results

Characterization of Mutated hVPAC$_1$ Receptors Expressed in CHO Cells. Serine/threonine residues in the intracellular region of VPAC$_1$ receptor have been mutated into alanine (Fig. 1). The first construct (IL1/IL2/3/C-T) has widespread mutations starting from the first intracellular loop (IL1) and going through IL2 and -3 to the C-terminal tail. In the other constructs, the extent of mutations is progressively narrowed and ends with a single mutation (S447A) or (S447D) in the C-terminal tail. All mutants, as well as the wild-type receptor, were stably expressed in CHO cells, and VIP binding parameters (K_D and B_{max}) and VIP stimulation of adenylyl cyclase were measured. Table 1 summarizes the experimental results. Scatchard analysis revealed that the B_{max} and K_D were similar for the mutants and wild-type VPAC$_1$ receptors. The EC_{50} values represent the concentrations of VIP that induce half-maximal stimulation of adenylyl cyclase and were similar for wild and mutated VPAC$_1$ receptors. In addition, the efficacies of VIP in stimulating adenylyl cyclase were similar in wild-type and mutated VPAC$_1$ receptor-expressing cells, as inferred from the basal and maximal values shown in Table 1. These data indicate that the performed mutations of VPAC$_1$ receptor do not significantly alter either the stoichiometry of VIP binding to receptors or VIP-induced adenylyl cyclase activity.

VIP-Induced Desensitization of hVPAC$_1$ Receptors. The different VPAC$_1$ receptor mutants made were used to assess whether putative phosphorylation sites for PKA, PKC, or GRKs are directly involved in the desensitization process. To this end, VIP-induced adenylyl cyclase responsiveness in

TABLE 1

^{125}I-VIP Binding and adenylyl cyclase assays in CHO cells expressing wild-type and mutated VPAC$_1$ receptors

Binding parameters (K_D and B_{max}) of CHO cells expressing wild-type and mutated VPAC$_1$ receptors were determined by Scatchard analysis. The data is consistent with one binding site for both wild and mutated receptors. VIP stimulation of adenylyl cyclase activity in membranes prepared from transfected CHO cells was also performed. The EC_{50} values are from VIP stimulation of adenylyl cyclase activity of CHO cell membranes after short-term (5 min) cell exposure to VIP in desensitization studies. The corresponding desensitization curves as well as control curves obtained in the absence of VIP exposure are shown in Fig.2. All data are mean ± S.E. of at least three independent experiments.

	VIP/VPAC$_1$ binding		Adenylyl Cyclase Activity			
					5-Min VIP Treatment	
Constructs	K_D	B_{max}	Basal	Maximal	EC_{50} Without	EC_{50} With
	nM	*fmol/mg of protein*	*pmol/min/mg of protein*		*nM*	*nM*
Wild-type	0.3 ± 0.1	300 ± 22	10.7 ± 0.1	0.27 ± 0.08	0.27 ± 0.08	3.15 ± 0.61**
IL1/2/3/C-T	0.8 ± 0.1	361 ± 37	9.8 ± 2.1	0.23 ± 0.07	0.23 ± 0.07	0.29 ± 0.07
IL2/3/C-T	0.9 ± 0.2	325 ± 29	10.3 ± 0.2	0.31 ± 0.06	0.31 ± 0.06	0.41 ± 0.11
IL2 (S250A)	0.7 ± 0.1	774 ± 65	9.0 ± 0.3	0.11 ± 0.04	0.11 ± 0.04	1.08 ± 0.21**
IL3	0.7 ± 0.2	494 ± 36	9.0 ± 0.1	0.20 ± 0.07	0.20 ± 0.07	1.90 ± 0.40**
C-T (S447A3)	0.5 ± 0.2	500 ± 45	11.8 ± 0.4	0.16 ± 0.05	0.16 ± 0.05	0.16 ± 0.04
C-T (S447A)	0.5 ± 0.2	482 ± 102	10.1 ± 0.2	0.57 ± 0.21	0.57 ± 0.21	0.55 ± 0.16
C-T (S447D)	0.8 ± 0.2	502 ± 39	8.4 ± 1.1	0.37 ± 0.05	0.37 ± 0.05	0.54 ± 0.18

** $p < 0.01$, compared with control EC_{50} values.

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membranes prepared from CHO cells expressing wild-type or mutated VPAC$_1$ receptor was first assayed after a 5-min cell exposure to 50 nM VIP. As shown in Fig. 2, top left, for the wild-type receptor, a 10-fold rightward shift of the EC$_{50}$ for VIP stimulation of adenylyl cyclase is present after a 5-min VIP pretreatment of CHO cells versus control cells (EC$_{50}$, 0.27 ± 0.08 nM versus 3.15 ± 0.61 nM). We then investigated whether mutated VPAC$_1$ receptors were desensitized similarly to the wild-type receptor. When the construct that bears the widest span of mutations (IL1/2/3/C-T) was studied, a 5-min VIP pretreatment of CHO cells did not induce a rapid

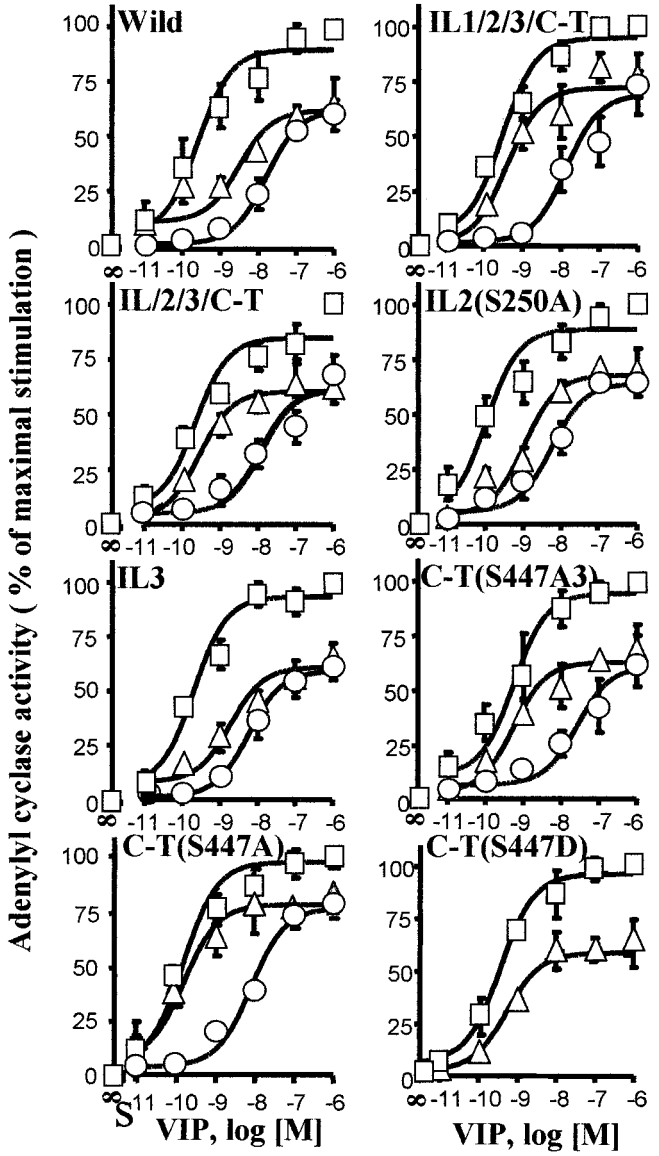

Fig. 2. VIP stimulation of adenylyl cyclase activity of CHO cell membranes after short- or long-term cell exposure to VIP. CHO cells expressing wild-type VPAC$_1$ receptors and the mutants IL1/2/3C-T, IL2/3C-T, IL2(S250A), IL3, C-T(S447A3), and C-T(S447A) were incubated without (□) or with 50 nM VIP for either 5 min (△) or 12 h (○) at 37°C. Then the cells were washed and cell membranes were prepared for VIP stimulation of adenylyl cyclase activity. Data are normalized as percentage of maximal enzyme activity induced by 1 μM native VIP over basal values for each type of transfected cell. The adenylyl cyclase data were analyzed and plotted using the sigmoidal dose-response regression curve-fitting program Prism 2.01 (GraphPad Software). Each value represents the mean ± S.E. of three experiments.

desensitization. Indeed, a similar EC$_{50}$ (0.2 nM VIP) was observed for pretreated versus control cells. To identify the responsible mutated serine or threonine residues that blunted VPAC$_1$ receptor desensitization, we studied other constructs bearing fewer and fewer mutations, as shown in Fig. 1 The threonine residue in the first IL can be excluded because the mutations in construct IL2/3/C-T maintained the loss of VIP-induced desensitization. The serine residues in the two remaining intracellular loops can also be excluded because VIP elicited desensitization of the two constructs IL2(S250A) and IL3 bearing mutations in either IL2 or IL3. In both cases, a 10-fold rightward shift of the EC$_{50}$ for VIP stimulation of adenylyl cyclase was observed after a 5-min VIP pretreatment of CHO cells. The EC$_{50}$ values obtained without and with VIP pretreatment were 0.11 ± 0.04 nM and 1.08 ± 0.21 nM, respectively, for IL2(S250A) and 0.20 ± 0.07 nM and 1.90 ± 0.40 nM, respectively, for IL3. Thus the data indicate that the mutation responsible for blunting short-term desensitization lies within the C-terminal tail of VPAC$_1$ receptor; this was confirmed by studying the last two mutants, C-T(S447A3) and C-T(S447A). There were no changes in the EC$_{50}$ values for VIP stimulation of adenylyl cyclase activity between control and 5-min VIP pretreated CHO cells expressing the two mutants. Taken together, the results indicate that the residue serine 447, which is a GRK putative consensus site is responsible for VPAC$_1$ receptor desensitization. One of the effects of serine phosphorylation is the introduction of a negative charge in a polypeptide sequence that was initially uncharged. We investigated the functional consequence of a pseudophosphorylated VPAC$_1$ receptor mutant C-T(S447D) with a negatively charged aspartate residue. The results in Table1 and Fig. 2 show normal signaling for the construct, and VIP-induced short-term desensitization is blunted as with VPAC$_1$ receptor mutant C-T(S447A). Because serine 448 within the cluster of serine 447–449 is a consensus site for PKA and because serine 447 could be a consensus site for PKA, we further evaluated whether VIP can induce VPAC$_1$ receptor desensitization in the presence of the selective PKA antagonist H-89. CHO cells expressing wild-type VPAC$_1$ receptors were incubated with H-89 before a 5-min incubation in the absence (control) or the presence of 50 nM VIP. The ability for VIP to stimulate adenylyl cyclase activity in membranes prepared from the corresponding cells is shown in Fig. 3. VIP retained its full capacity to desensitize VPAC$_1$ receptors in presence of H-89, thereby suggesting that PKA is not involved in this process and sustaining the above GRK desensitization pathway at serine 447. A similar experiment was performed in which with CHO cells expressing wild-type VPAC$_1$ receptor were incubated with H-89 in the absence (control) or the presence of 1 μM forskolin (FK). The result, shown in Fig. 3, was that VIP-induced responsiveness in membranes prepared from control and FK-treated cells was alike. This ineffectiveness of FK versus VIP in desensitizing VPAC$_1$ receptor is again in line with a GRK- rather than a PKA-mediated process. We also investigated down-regulation of VPAC$_1$ receptors by a 12-h incubation of CHO cells expressing wild-type or mutated VPAC$_1$ receptor in the absence (control) or the presence of 50 nM VIP. In all cases studied, we observed a similar 50-fold rightward shift of the EC$_{50}$ for VIP stimulation of adenylyl cyclase compared with control cells (Fig. 2). This shift is 5-fold greater than that obtained in short-term desensitization, although the

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efficacies were similar. This indicates that unlike what is observed for short-term desensitization, none of the mutated serine residues (Fig. 1) that are consensus phosphorylation sites for PKC, PKA, and GRKs have a crucial role in down-regulation.

Internalization of VIP and Receptors in CHO Cells. We developed a VIP-FITC derivative (see *Materials and Methods*) for visualizing VIP internalization by $VPAC_1$ receptor expressed in CHO cells. This VIP-FITC has the same apparent affinity as native VIP for binding to $VPAC_1$ receptor and stimulating adenylyl cyclase activity through interaction with $VPAC_1$ receptor (Fig. 4, A and B). After a 60-min incubation period at 20°C with VIP-FITC, CHO cells expressing wild-type $VPAC_1$ receptor were labeled throughout their plasmalemmal surface (C1), whereas untransfected CHO cells exhibited no labeling (not shown). When CHO cells expressing wild-type receptor were incubated with VIP-FITC for either 5 or 60 min at 37°C (C2 and C3, respectively), the fluorescence was found to be between the perinuclear region and the inner plasma membrane at 5 min, whereas it was essentially at the perinuclear region of the cells at 60 min. These data indicated that a rapid receptor-mediated internalization of VIP-FITC occurs in CHO cells expressing wild-type $VPAC_1$ receptor. Quantitative aspects of VIP internalization were obtained by studying radiolabeled ^{125}I-VIP. Shown in Fig. 5 are the results of ^{125}I-VIP internalization time course in CHO cells expressing wild-type receptors. The radioligand internalization was rapid, whereby at least 50% of radioligand was internalized within 5 min. The internalization reached a plateau after 15 min, with a maximal radioligand internalization of 70 to 75%. By using this latter quantitative technique, we then studied internalization of ^{125}I-VIP by mutated receptor constructs (Fig. 5). Similar results were obtained for wild-type receptor and all receptor constructs including (IL1/2/3/C-T), (IL3), C-T(S447A3), and C-T(S447A) mutants. These data indicate that the potential GRK, PKC, and PKA phosphorylation sites explored are not involved in the process of internalization of $VPAC_1$ receptors. The rapid internalization of $VPAC_1$ receptor is therefore a distinct process from the short-term desensitization, which is completely blunted by mutation at serine 447.

Next, we explored whether mutation of serine 447 representing GRK phosphorylation site could affect long-term agonist treatment on $VPAC_1$ receptor level in CHO cells. Because VIP derivatives are inconvenient for studying long-

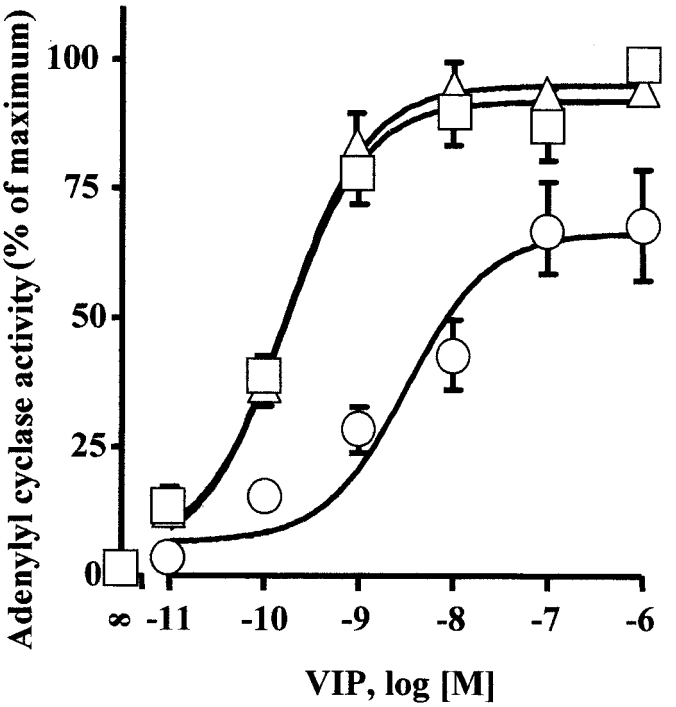

Fig. 3. Wild-type $VPAC_1$ receptors expressed in CHO cells are desensitized by VIP independently of PKA. CHO cells expressing wild-type $VPAC_1$ receptors were incubated with 10 mM H-89 for 30 min at 37°C before a 5-min incubation in the absence (□) and in the presence of either 50 nM VIP (○) or 1 μM forskolin (△). Then the cells were washed and cell membranes were prepared for VIP stimulation of adenylyl cyclase activity. Results are expressed as percentage of maximal enzyme activity induced by 1 μM native VIP over basal value. The data are plotted using Prism, and each value represents the mean ± S.E. of three experiments.

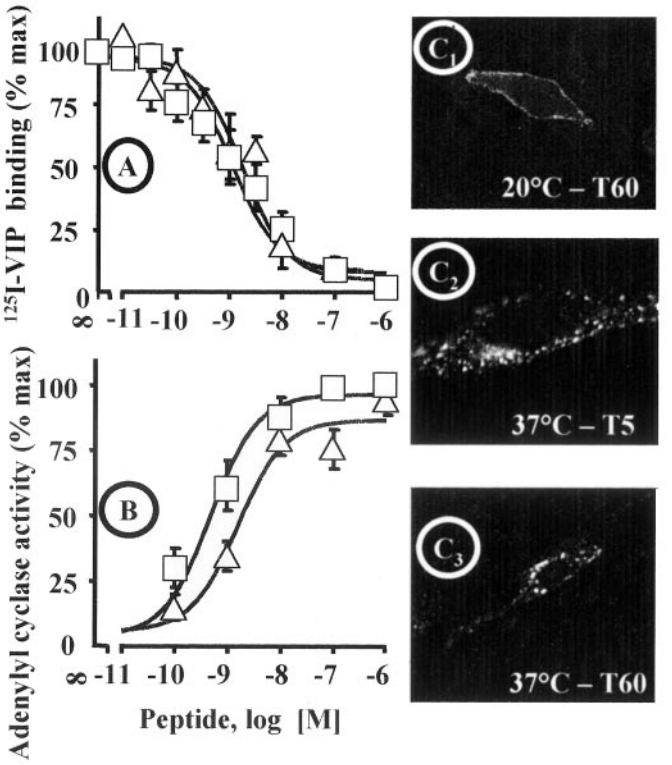

Fig. 4. Characterization of a newly synthesized VIP-FITC and its internalization by wild-type $VPAC_1$ receptor. The ability of the fluorescent VIP derivative referred to as VIP-FITC (△) versus native VIP (□) to compete for ^{125}I-VIP binding to wild-type $VPAC_1$ receptors in cell membranes is shown in A. The results are expressed as a percentage of maximal specific binding in the absence of unlabeled native VIP. The values of nonspecific binding obtained in the presence of 1 μM native VIP represented about 10 to 15% of total membrane bound ^{125}I-VIP associated radioactivity. The competition curves (A) for both native VIP and VIP-FITC were similar, with a half-maximal inhibition (IC_{50}) of 1.5 nM. Furthermore, a dose-dependent stimulation of adenylyl cyclase with VIP and VIP-FITC is shown in B. Results are expressed as a percentage of maximal enzyme activity induced by 1 μM native VIP over basal value. VIP-FITC stimulated adenylyl cyclase with an EC_{50} value of 1.6 ± 0.2 nM versus 0.5 ± 0.1 nM for native VIP. The data shown in A and B are plotted using Prism, and each value represents the mean ± S.E. of three experiments. Then, we investigated by confocal microscopy (C1, C2, and C3) how CHO cells expressing wild-type $VPAC_1$ receptors internalized VIP-FITC with time and temperature. When the cell were incubated with 50 nM VIP-FITC at 20°C for 60 min, there was an exclusive plasma membrane labeling (C1). In contrast, incubations at 37°C during 5 (C2) or 60 min (C3) indicated a cytosolic localization of fluorescent VIP-FITC which was persistent in all of the studied 7-μm *xy* focal sections through the cells.

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term (12-h) receptor internalization because of cell-associated disposal of ligands, we instead detected cell surface epitope-tagged VPAC$_1$ receptors. Shown in Fig. 6, right, the epitope-tagged wild-type receptors of nonpermeabilized cells were detected by an intense rhodamine fluorescence when incubated with anti-Flag antibodies and, subsequently, TRITC-labeled sheep antibodies. A sharp reduction in cell surface expression of wild VPAC$_1$ receptors was induced by 50 nM VIP because there were hardly any TRITC-labeled cells compared with control cells (Fig. 6, A and B). The cells shown in Fig. 6, left, display a green fluorescence caused by the presence of GFP at the C terminus of VPAC$_1$ receptor constructs. This GFP labeling represents total cellular expression of VPAC$_1$ receptors. Similar results are obtained with CHO cells expressing either wild-type or mutated VPAC$_1$ receptor constructs C-T(S447A), whereby VIP also induce an internalization of cell surface receptors (C-D). To quantitate this long-term agonist treatment on receptor level, we estimated the B_{max} values of CHO cells expressing wild and C-T(S447A) VPAC$_1$ receptor constructs that have been incubated overnight with and without 50 nM VIP. As shown at the bottom of Fig. 6, VIP induced an 85% decrease in CHO cell surface expression of wild VPAC$_1$ receptor con-

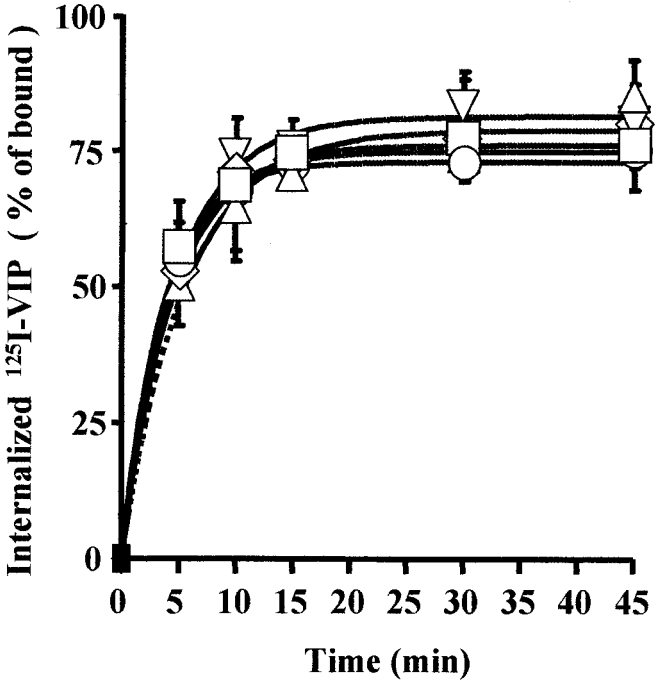
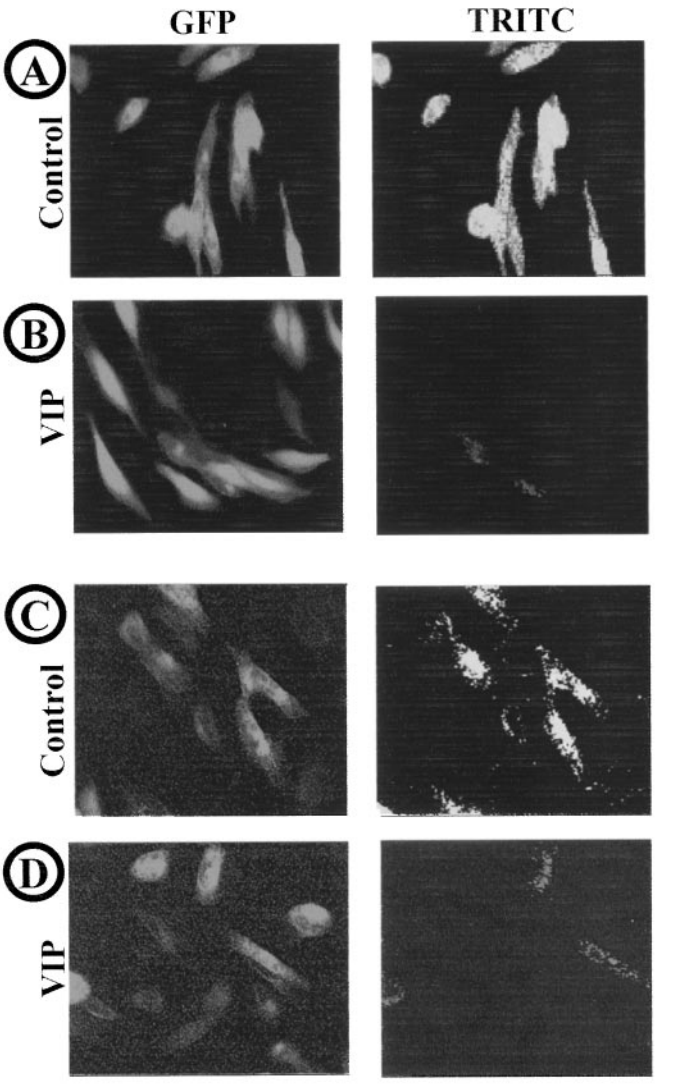


Fig. 5. Internalization of ^{125}I-VIP by CHO cells expressing wild-type or mutated VPAC$_1$ receptors. CHO cells expressing wild-type (□) or mutated VPAC$_1$ receptor IL1/2/3/C-T (△), IL3 (▽), C-T(S447A3) (◇), and C-T(S447A) (○) were incubated at 37°C with ^{125}I-VIP (50 pM) in the absence (total binding) and the presence of 1 μM VIP (nonspecific binding). At the indicated time intervals, the cells were washed and, in parallel, either the reaction was stopped with a 0.5 N NaOH solution or the cells were subjected to radioligand stripping with 0.5 M KSCN solution. In the former case, specific ^{125}I-VIP binding to CHO cells was calculated by subtracting nonspecific values from those of total binding. In the latter case, the amount of specifically internalized ^{125}I-VIP was estimated in a similar manner after cell surface ^{125}I-VIP stripping by KSCN. The data are expressed as a percentage of radioactivity specifically associated with CHO cells after KSCN treatment (internalized receptor pool) to total radioactivity specifically associated with the cells in the absence of KSCN treatment. The data were plotted using Prism and a one-phase exponential association parameter. Each value represents the means ± S.E. of at least three experiments performed in triplicate.



Constructs	B max (fmol/mg of protein) Control	50 nM VIP
Wild-type	300 ± 22	49 ± 11
C-T(S447A)	482 ± 102	63 ± 12

Fig. 6. Internalization of wild-type and mutated VPAC$_1$ receptors in CHO cells after 12-h exposure to VIP. To evaluate long-term receptor internalization, CHO cells expressing wild-type (A and B) or the mutated VPAC$_1$ receptors C-T(S447A3) (C and D) were incubated for 12 h at 37°C in the absence (control) or the presence of 50 nM VIP. All receptor constructs have a Flag epitope in the N-terminal extracellular domain. When receptors were located at the plasma membrane of nonpermeabilized cells, it could be detected by anti-Flag antibodies/TRITC-labeled antibodies as illustrated in right. After an overnight exposure of CHO cells to 50 nM VIP, a significant internalization of VPAC$_1$ receptors occurred because they could be barely TRITC-labeled compared with control cells. Furthermore, in the same field are shown cells labeled with GFP (right) as a result of the presence of GFP at the C terminus of wild-type or mutated VPAC$_1$ receptors C-T(S447A3). This GFP labeling is representative of total cellular expression of these receptors. VIP induced a similar receptor internalization for wild-type and mutated receptors as seen by the extinction of TRITC-labeled versus GFP-labeled cells. This is confirmed by estimating cell surface number of receptor or B_{max} as described under *Materials and Methods*. In the presence of 50 nM VIP, 85% of wild-type or mutated VPAC$_1$ receptors at the CHO cell surface is internalized. The data represent mean ± S.E of three experiments.

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structs in line with a previous study (Gaudin et al., 1996). There was a similar reduction in cell surface expression of C-T(S447A) $VPAC_1$ receptor with exposure to VIP. Thus, down-regulation of $VPAC_1$ receptors is also independent of serine 447, which is a crucial putative phosphorylation site for short-term receptor desensitization.

Phosphorylation of $VPAC_1$ Receptors. To assess whether $VPAC_1$ receptor is phosphorylated in our experimental model and the effect of S447A mutation on phosphorylation, CHO cells were labeled with ^{32}P and $VPAC_1$ receptor was immunoprecipitated. Our data in Fig. 7 indicate that VIP induced the phosphorylation of wild-type $VPAC_1$ receptors expressed in the CHO cells used in our study. The ^{32}P-labeled band migrating at 90 kDa in Fig. 7A corresponded to a phosphorylated Flag-tagged $VPAC_1$ receptor bearing a GFP moiety at its C-terminal tail. VIP at 1 μM induced a 10-fold increase of wild-type $VPAC_1$ receptor phosphorylation over basal values in CHO cells as shown in Fig. 7B. We observed a sharp attenuation of VIP-induced phosphorylation of the mutated $VPAC_1$ receptor C-T(S447A) (Fig. 7B), supporting the idea that serine 447 is a major site of $VPAC_1$ receptor phosphorylation upon VIP treatment.

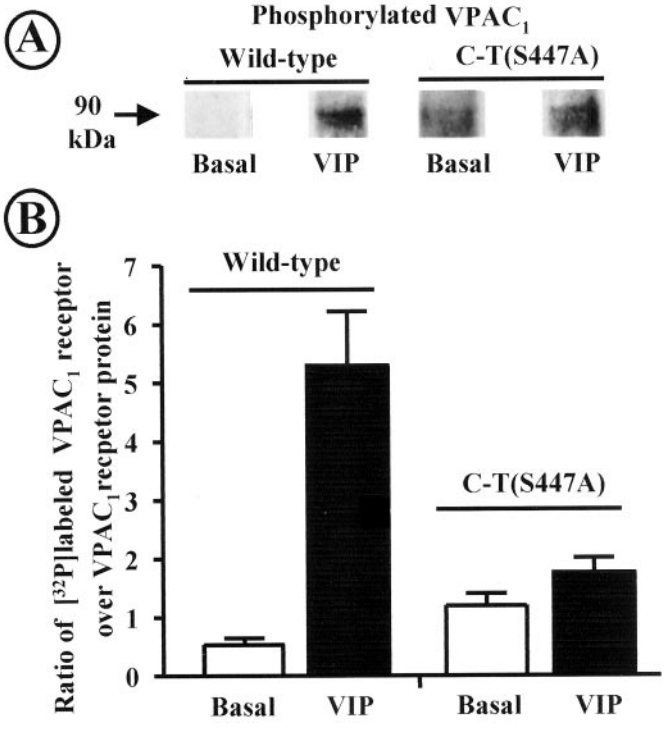

Fig. 7. Phosphorylation of Flag-tagged $VPAC_1$ receptor. The CHO cells expressing wild-type or mutated $VPAC_1$ receptor C-T(S447A) were labeled with [^{32}P]orthophosphate and incubated in the absence (Basal) and in the presence of 1 μM VIP for 10 min. The solubilized cellular proteins were immunoprecipitated, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose as described under *Materials and Methods*. A representative autoradiograph of three blots is shown in A, and it indicates a phosphorylated 90-kDa band corresponding to $VPAC_1$ receptor bearing a GFP protein. The ^{32}P-labeled bands as well as $VPAC_1$ receptor protein detected by an antibody coupled to alkaline phosphatase were scanned and quantitated using Scion Image software. The ratio of ^{32}P-labeled $VPAC_1$ receptor/$VPAC_1$ receptor protein is shown in the histogram of B. Each value represents the means ± S.E. of three experiments. VIP stimulated a 10-fold increase of phosphorylation over basal values for wild-type VPAC1 but was only poorly effective for the mutant C-T(S447A).

Discussion

This site-directed mutagenesis study demonstrates that serine 447 in the C-terminal tail of the human $VPAC_1$ receptor plays a crucial role in the short-term VIP-induced receptor desensitization. This serine, which is a likely GRK-mediated phosphorylation site, further extends the previous data, suggesting that GRKs are involved in desensitizing $VPAC_1$ and certain other related class II G protein-coupled receptors, such as secretin and calcitonin receptors (Shetzline et al., 1998, 2002; Horie and Insel, 2000). In sharp contrast to the above data, serine 447 is not involved in VIP-induced down-regulation or the internalization of $VPAC_1$ receptor, which occurred concomitantly with its desensitization.

Upon a 5-min challenge with 50 nM VIP, wild $VPAC_1$ receptors expressed in CHO cells undergo an important desensitization characterized by a 10-fold rightward shift in the dose-response curve of VIP in stimulating adenylyl cyclase activity in plasma membranes. This rapid desensitization of the recombinant $VPAC_1$ receptor was previously observed for native receptors in cultured human cancer cell lines (Boissard et al., 1986; Robberecht et al., 1989). The rapid desensitization that we observed was independent on VIP-induced cAMP accumulation because desensitization is not mimicked by forskolin. It is also independent on VIP-induced PKA activity because desensitization is not abolished by the selective PKA inhibitor H89 (Fig. 3). This strict independence of cAMP/PKA in agonist-induced desensitization is also observed with the closely related PAC_1 receptor for PACAP and CGRP receptor, another class II GPCR (Aiyar et al., 2000; Dautzenberg and Hauger, 2001). The site-directed mutagenesis of residues in intracellular domains of the $VPAC_1$ receptor corresponding to consensus phosphorylation sites for PKA, PKC, and putative GRK sites clearly indicated that serine 447 in the C-terminal tail is crucial for the rapid VIP-induced receptor desensitization (Fig. 2).

The receptor domain(s) involved in rapid desensitization of other class II receptors for VIP-related peptides have been documented in a few instances, but the specific Ser/Thr residues concerned were rarely determined. For example, the complete desensitization of the rat secretin receptor expressed in CHO cells by 5-min treatment with 10 nM secretin is blunted, at least in part, after deletion of the receptor C-terminal tail (Holtmann et al., 1996). GRK2, GRK5, and, to a lesser extent, GRK3 seem to be involved in the desensitization of the rat secretin receptor expressed in HEK 293 cells (Shetzline et al., 1998). The use of dominant-negative GRK mutant has revealed the clear involvement of GRK2 in calcitonin receptor desensitization (Horie and Insel, 2000). Truncation of a C-terminal domain of the human GRF receptor has also been suggested to decrease receptor desensitization (Gaylinn, 2002). Mutagenesis studies have identified three pairs of serine residues in the C-terminal tail of the GLP-1 receptor that are important for receptor desensitization (Widmann et al., 1997). The C-terminal tail of the glucose-dependent insulinotropic polypeptide receptor has been also shown to be equipped with residues involved in desensitization (Wheeler et al., 1999). Although various GRKs were responsible for the desensitization of those class II receptors (Brubaker and Drucker, 2002; Dong and Miller, 2002), a common feature is the importance of the C-terminal tail of receptors for desensitization. The RSSS sequence,

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which contains the crucial serine 447 in the human VPAC$_1$ receptor, is not conserved in all class II receptors but can be observed in VPAC$_1$ receptors from other species, including pig, rat, and mouse, and a KSSS sequence is present in bovine, human, murine, and rat PAC$_1$ receptors for PACAP (http://www.gpcr.org). Therefore, it may be suggested that this motif and the serine residue adjacent to a basic residue can play a role in the GRK desensitization of VPAC$_1$ and PAC$_1$ receptors. Serine or threonine residues corresponding to serine 447 in human VPAC$_1$ receptor are also observed in rabbit secretin receptor, human, mouse, and rat glucagon receptors, and human glucose-dependent insulinotropic polypeptide receptor, suggesting a more general role in class II receptors. Interestingly, the VPAC$_2$ receptor for VIP does not express the RSSS sequence (Laburthe et al., 2002a), suggesting different ways of receptor desensitization of the two VIP receptor subtypes.

Although the VIP-induced internalization/sequestration of VIP receptors has been described for a long time (Marie et al., 1986; Hejblum et al., 1988), the mechanistic details involved in this process have essentially been documented recently by Shetzline et al. (2002). These authors showed that VPAC$_1$ receptor regulation in HEK 293 cells involves agonist-stimulated, GRK-mediated phosphorylation, β-arrestin translocation, and dynamin-dependent receptor internalization. This is in line with a previous study of various GPCRs supporting a β-arrestin-independent, dynamin-dependent internalization pathway of VPAC$_1$ receptors in the same cells (Claing et al., 2000). In the present study, we have developed a biologically active fluorescent VIP analog and used a recombinant VPAC$_1$ receptor bearing a Flag in its N-terminal extracellular domain and GFP at the C-terminal intracellular domain to follow agonist and receptor internalization. Our data are consistent with a very rapid VIP-induced receptor sequestration in VPAC$_1$-expressing CHO cells. They also showed that the serine residues, which are potential phosphorylation sites for GRKs, PKA or PKC (see Fig. 1), could be mutated into alanine without altering the process of VIP-induced receptor internalization. Of special interest was the observation that the S447A mutant, which no longer desensitizes upon a 5 min VIP treatment, is still able to internalize like the wild-type receptor. Thus, this mutant is useful to delineate the processes of rapid desensitization from internalization. In our hands, the time course of desensitization of wild-type VPAC$_1$ receptor could not be discriminated from that of internalization even at shorter time points than the 5-min VIP treatment (data not shown). In this context, it is not possible to determine whether the reduction in maximal adenylyl cyclase activity observed in all of the short-term desensitization curves is caused by rapid VPAC$_1$ receptor internalization.

VPAC$_1$ receptor desensitization in HEK293 cells would involve VIP-induced, GRK-mediated receptor phosphorylation and subsequent β-arrestin translocation to the receptor (Shetzline et al., 2002), which probably blunts interaction between VPAC$_1$ receptor and Gs protein. Of interest is that the cellular environment o CHO versus HEK293 cells can modify, as in the case of secretin receptors, the kinase specificity in receptor phosphorylation (Dong and Miller, 2002). We found that VIP induced a similar extent of phosphorylation of wild-type VPAC$_1$ receptors in CHO cells as described for HEK293 cells (Shetzline et al., 2002). Moreover, there was a sharp attenuation of phosphorylation with the mutant C-T(S447D) compared with wild-type receptor, suggesting a key role for serine 447 in its phosphorylation. However, further investigations are needed to better understand the role of phosphorylation for short- versus long-term desensitization. These include the stoichiometry of receptor phosphorylation, time course and dose dependence of phosphorylation of the mutants included, and analysis of the other C-terminal sites of potential phosphorylation in this receptor. The role of phosphorylated serine 447 in receptor desensitization was also tentatively investigated using a construct C-T(S447D), whereby aspartic acid would mimic phosphorylated serine 447 and induce a desensitized state. Our data indicate that the pseudophosphorylated VPAC$_1$ C-T(S447D) construct displays normal binding and coupling to adenylyl cyclase compared with wild-type receptors. VIP did not induce any significant short-term desensitization of VPAC$_1$ C-T(S447D) highlighting the idea that the negative charge introduced by aspartate is insufficient by itself to mimic the serine phosphorylation. One explanation is that an aspartate residue has a bulkier volume than a serine residue, which can hinder the mimicking of a pseudophosphorylated state, as shown by Inanami et al. (1998).

If the process of internalization were also dependent on β-arrestin, as shown for many GPCRs (Pitcher et al., 1998; Pierce et al., 2002), it would be difficult to understand why the S447A mutant, which no longer desensitizes, still internalizes. In fact, the behavior of the mutant is consistent with the β-arrestin–independent, dynamin-dependent internalization of the VPAC$_1$ receptor (Claing et al., 2000; Shetzline et al., 2002), which is a pathway taken by some GPCRs, such as muscarinic, serotoninergic, and endothelin B receptors (Claing et al., 2000; Bhatnagar et al., 2001; Delaney et al., 2002). In this context, the S447A VPAC$_1$ receptor mutant may become a unique tool to study receptor internalization independently of rapid desensitization. Finally, the fact that upon VIP treatment of cells for 12 h, the down-regulation of VPAC$_1$ receptor remains unaltered in the S447A receptor mutant suggests that this process may be directly related to receptor internalization rather than the process of rapid desensitization.

In conclusion, this work characterizes a serine residue in the C-terminal tail of the human VPAC$_1$ receptor that is necessary and sufficient to desensitize the receptor upon short-term treatment with its natural agonist VIP. This serine residue is adjacent to a basic residue in an RSSS sequence that is conserved in mammalian VPAC$_1$ receptors and is also observed as a KSSS sequence in the closely related PAC$_1$ receptor for PACAP. This serine may play a critical role in the GRK-mediated desensitization of VPAC$_1$ receptor (Shetzline et al., 2002).

Acknowledgments

We gratefully acknowledge Jean-José Maoret, Anne-Marie Lorinet [Institut National de la Santé et de la Recherche Médicale (INSERM) U 410], and Cécile Pouzet (Institut Fédératif de Recherche 02) for their technical assistance and Dr. Alexaendre Fontayne (INSERM U479) for his helpful suggestions.

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