Site-Directed Mutagenesis of Human Vasoactive Intestinal Peptide Receptor Subtypes VIP1 and VIP2: Evidence for Difference in the Structure-Function Relationship

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ABSTRACT
Vasoactive intestinal peptide (VIP1 and VIP2) receptors belong to the new class II subfamily of G protein-coupled receptors. We investigated here human VIP1 and VIP2 receptors by mutating in their extracellular domains all amino acid residues that are conserved in VIP receptors but are different in other members of their subfamily. They are present in 1) the N-terminal domain, i.e., E36, I43, S64, D132 and F138 in the VIP1 receptor and E24, I31, S53, D116 and F122 in the VIP2 receptor; 2) the second extracellular loop, i.e., T288 and S292 in the VIP1 receptor and T274 and S278 in the VIP2 receptor. These residues were changed to alanine (A), and cDNAs were transfected into Cos cells. For the VIP1 receptor, no specific $^{125}$I-VIP binding could be detected in cells transfected with the E36A mutant, whereas other mutants exhibited dissociation constants similar to that of the wild-type receptor, with the exception of I31A and T274A mutants, for which a 11- and 5-fold increase of $K_d$ was observed, respectively. cAMP production experiments provided evidence that the E36A VIP1 receptor and the E24A VIP2 receptor mutants mediated almost no response upon VIP exposure. For the I31A and T274A mutants of the VIP2 receptor and the S64A mutant of the VIP1 receptor, the EC$_{50}$ values of VIP for stimulating cAMP production were increased 35, 8 and 3 times as compared with that observed for the wild-type receptor, respectively. Immunofluorescence studies indicated that all mutants were normally expressed by Cos cells. These data provide the first evidence for differences in the structure-function relationship of VIP1 and VIP2 receptors.

Within the superfamily of G protein-coupled receptors, there has emerged during the past few years a subfamily that shares the seven membrane-spanning domain topography but has a low overall amino acid sequence homology (<20%) with other members of the superfamily (Ségré and Goldring, 1993; Laburthe et al., 1996). It is now referred to as the class II G protein-coupled receptor family and comprises receptors for a structural family of peptides that includes VIP, pituitary adenylyl cyclase-activating peptide, glucagon, secretin, glucagon-like peptide 1, gastric inhibitory polypeptide, growth hormone-releasing factor and (more unexpectedly) receptors for parathyroid hormone and calcitonin. Recent studies extended this subfamily to 15 members (Laburthe et al., 1996) with the discovery of subtypes of the above-mentioned receptors as well as two new members having an extraordinary long N-terminal domain: the putative EGF module-containing, mucin-like hormone receptor EMR1 (Baud et al., 1995) and the leukocyte activation antigen CD99 (Hamman et al., 1995). Class II G protein-coupled receptors for peptides have homologies ranging between 30% and 50% and, among several common structural properties, have a large N-terminal extracellular domain (>120 amino acid residues) that contains highly conserved amino acids, including numerous cysteine residues and several potential N-linked glycosylation sites (Laburthe et al., 1996).

A human VIP1 receptor consisting of 457 amino acid residues has been cloned from an intestinal library (Couvineau et al., 1994). Taking this human VIP1 receptor, which activates adenylyl cyclase via stimulatory Gs proteins (Couvineau et al., 1986; Couvineau et al., 1995), as a prototype of class II G protein-coupled receptors, we recently provided evidence for an important role of the N-terminal domain for ligand binding with several crucial residues (Couvineau et al., 1995) probably positioned in a tertiary structure maintained by multiple disulfide bonds (Gaudin et al., 1995). We also demonstrated the mandatory role of two glycosylation sites in this domain for correct delivery of the receptor to the plasma membrane.

ABBREVIATION: VIP, vasoactive intestinal peptide.
membranes (Couvineau et al., 1996a). Other functional domains for ligand recognition do exist; our results using VIP1 receptor chimeras from different species showed that a structural determinant for peptide selectivity was made of three nonadjacent amino acid residues in the first extracellular loop and third transmembrane domain (Couvineau et al., 1996b).

A VIP2 subtype of VIP receptors has been cloned recently from rat hypophysis (Lutz et al., 1993), a human lymphoblast cell line (Svoboda et al., 1994) and human placenta (Adamou et al., 1995). The human VIP2 receptor consists of 438 amino acid residues and has 49% homology with the human VIP1 receptor. Like VIP1 receptors, VIP2 receptors mediate activation of adenyl cyclase. However, there is no evidence for any major difference between their pharmacological profiles with respect to recognition of natural agonists of the VIP family of peptides (Lutz et al., 1993; Svoboda et al., 1994; Adamou et al., 1995), in contrast with the relatively high divergence of their amino acid sequences. The anatomical mapping supported the interpretation that VIP1 and VIP2 receptors have complementary tissular distribution in that VIP2 receptors are found in tissues where the VIP1 receptor is absent or is present at low levels (Usdin et al., 1994).

After completion of our studies of the structure-function relationship of the human VIP1 receptor by site-directed mutagenesis of amino acid residues highly conserved in the class II family of G protein-coupled receptors (Laburthe et al., 1996), we considered human VIP1 vs. VIP2 receptors. The rationale of this study was based on the idea that residues that were conserved in VIP1 and VIP2 receptors but were poorly or not at all conserved in other members of the class II family of G protein-coupled receptors may be essential for VIP binding. Because the N-terminal extracellular domain (Couvineau et al., 1995; Gaudin et al., 1995; Holtmann et al., 1995; Vilardaga et al., 1995) and extracellular loops (Gaudin et al., 1995; Du et al., 1997) appear to play a crucial role in VIP binding to the VIP1 receptor, we selected such amino acid residues and mutated them in extracellular domains. This approach allowed us to characterize a new crucial residue (glutamate) in the N-terminal domain of both receptors. Moreover, quite unexpectedly, we also characterized three conserved residues in the VIP1 or VIP2 receptor, the mutation of which decreased the affinity of VIP for a VIP receptor subtype but not for the other subtype. These data provide the first experimental evidence for differences in the structure-function relationship of VIP receptor subtypes.

Materials and Methods

Materials. Enzymes for cloning, sequencing and oligonucleotide-directed mutagenesis were obtained from Promega (Charbonnière, France) or BRL (Life Technologies, Cergy, France) and synthetic oligonucleotides from Eurogentec (Seraing, France). The human VIP1 receptor cDNA was cloned in our laboratory (Couvineau et al., 1994). The human VIP2 receptor cDNA (Svoboda et al., 1994) was a gift from Dr. M. Svoboda and Dr. P. Robberecht (Brussels, Belgium). [α-32P]dATP (1000 Ci/mmol) and other radioactive reagents were obtained from Amersham (Les Ulis, France). Synthetic porcine VIP was purchased from NeoSystem (Strasbourg, France) and culture medium from Gibco (Life Technologies, Cergy, France). 125I-VIP was prepared and purified as described (Laburthe et al., 1987). The monoclonal anti-Tag antibody (Evan et al., 1985) was prepared from ascite fluid after injecting into mice the hybridoma MYC-1-9E10.2 obtained from ATCC (CRL-1729). All other chemicals were of the highest quality commercially available and were purchased from Sigma (Saint-Quentin-Fallavier, France).

Site-directed mutagenesis. The 1.4-kilobase EcoRI fragment containing the entire coding sequence of the human VIP1 receptor (Couvineau et al., 1994) or the 1.3-kilobase EcoRI fragment containing the entire coding sequence of the human VIP2 receptor (Svoboda et al., 1994) was subcloned into the EcoRI site of the pALTER-1 vector, and single-stranded DNA (+ strand) was produced in Escherichia coli JM109. Full-length VIP receptor mutants were generated by oligonucleotide-directed mutagenesis as described (Couvineau et al., 1996a). Identification of the desired mutations was obtained by direct double-strand sequencing of the regions encompassing mutations. Inserts encoding mutant sequences were subcloned in the eucaryote expression vector pcDNA1 Invitrogen, Leek, Netherlands). The wild-type and mutant receptors were all tagged at the C-terminal with a marker dodecapeptide (Tag) as described (Couvineau et al., 1996a).

Transfection of cells. Wild-type and mutant VIP receptors were transfected into Cos-7 cells. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cells were transfected by the electroporation method using an electroporator Express apparatus (Invitrogen) as described (Couvineau et al., 1996a). Briefly, 4 × 106 cells were preincubated in ice for 5 min with 15 µg of salmon sperm DNA used as carrier and 15 µg of wild-type or mutant receptor cDNA constructs in phosphate-buffered saline. After electroporation (330 V, 500 µF, infinite resistance), cells were put on ice for 5 min and then transferred into culture medium containing 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) penicillin-streptomycin before seeding in Petri dishes for binding assay, in 12-well trays for cAMP assay or on glass slides in 24-well trays for immunofluorescence studies. The culture medium was changed 16 h after transfection, and cells were used 48 h after transfection.

Ligand binding assay. The binding properties of wild-type and mutant VIP receptors were analyzed by 125I-VIP binding to transfected cell membranes. Transfected Cos-7 cells were washed twice with cold phosphate-buffered saline. Then they were harvested with a rubber policeman and centrifuged at 3000 rpm for 5 min at 4°C and the cell pellets were incubated for 30 min on ice in a hypotonic 5 mM HEPES buffer, pH 7.4. Thereafter, cells were homogenized as described (Couvineau et al., 1985), and the homogenate was centrifuged at 11,000 rpm for 15 min at 4°C. The pellet was washed with 20 mM HEPES buffer and stored at −80°C until use. This pellet was referred to as the membrane preparation. Membranes (200 µg of protein/ml) were incubated for 60 min at 30°C in 20 mM HEPES buffer, pH 7.4, containing 2% (w/v) bovine serum albumin, 0.1% (w/v) bacitracin and 0.05 or 0.4 nM 125I-VIP (see tables) in the presence of increasing concentrations of unlabeled VIP. The reaction was stopped as described (Couvineau et al., 1985). Specific binding was calculated as the difference between the amount of 125I-VIP bound in the absence and in the presence of 1 µM unlabeled VIP. Binding data were analyzed using the LIGAND computer program ( Munson and Rodbard, 1980). Protein content in membrane preparations was evaluated by the procedure of Bradford (Bradford, 1979) with bovine serum albumin as standard.

cAMP experiments. Transfected Cos-7 cells were grown in 12-well trays as described above. The culture medium was discarded, and attached cells were gently rinsed with phosphate-buffered saline (pH 7). They were then incubated without or with VIP under continuous agitation in 0.5 ml of phosphate-buffered saline containing 2% (w/v) bovine serum albumin, 0.1% (w/v) bacitracin, 0.01 mg/ml aprotinin and 1 mM 3-isobutyl-1-methylxanthine as described (Couvineau et al., 1994). At the end of the incubation (30 min at 25°C) the medium was removed and the cells lysed by 1 M perchloric acid. The cAMP present in the lysate was measured by radioimmunoassay as...
described (Laburthe et al., 1978). Cell number was determined in parallel wells and data reported as picomoles of cAMP per 10^6 cells.

**Confocal laser scanning microscopy.** Transfected cells were grown on 12-mm glass coverslips for 48 h as described above. After washing with phosphate-buffered saline, cells were fixed with 2% (v/v) paraformaldehyde for 15 min and then permeabilized in phosphate-buffered saline containing 0.2% gelatine and 0.075% saponin (PBSGS). After washing with PBSGS, permeabilized cells were incubated for 30 min at room temperature with the mouse monoclonal anti-Tag antibodies (Evan et al., 1985) diluted 1:250. The coverslips were mounted in Glycergel, and selected fields were scanned using a True Confocal Scanner Leica TCS 4D composed of a Leica Diaplan inverted microscope equipped with an argon-crypton ion laser (488 nm) with an output power of 2 to 50 mW and a VME bus computer system coupled to an optical disc for image storage (Leica Lasertchnik GmbH). The emitted light was collected through a long-pass filter on the target of the photomultiplier. Each sample was treated with a kalman filter to increase the ratio signal vs. background. All image-generating and -processing operations were carried out using the Leica CLSM software package.

**Results**

Residues conserved in extracellular domains of VIP1 and VIP2 receptors but poorly or not at all conserved in other members of the class II G protein-coupled receptors were highlighted in the multialignment shown in figure 1. These residues were found in the N-terminal extracellular domain (E36, I43, S64, D132 and F138 in the VIP1 receptor corresponding, respectively, to E24, I31, S53, D116 and F122 in the VIP2 receptor) and the second extracellular loop (T288 and S292 in the VIP1 receptor corresponding, respectively, to T274 and S278 in the VIP2 receptor). All these residues were mutated into alanine by site-directed mutagenesis, and the mutated cDNAs were transfected into Cos cells for subsequent functional studies. Scatchard analysis of competitive inhibition of ^125^I-VIP (0.05 nM) binding to transfected cells by unlabeled VIP gave straight lines for the wild-type VIP1 receptor and for all VIP1 receptor mutants (I43A, S64A, D132A, F138A, T288A and S292A) but one (E36A), for which no specific ^125^I-VIP binding could be detected (table 1). Dissociation constants calculated from Scatchard plots were

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Amino acid sequence alignment of parts of the N-terminal extracellular domains (panel A) and second extracellular loops (panel B) of the human VIP1 and VIP2 receptors with the corresponding regions in other human members of the class II subfamily of G protein-coupled receptors. The following human sequences were aligned: VIP1 receptor (VIP1); VIP2 receptor (VIP2); pituitary adenylate cyclase-activating polypeptide receptor (PACAP); secretin receptor (SEC); grow hormone-releasing factor receptor (GRF); glucagon receptor (GLUC); gastrin inhibitory polypeptide receptor (GIP); glucagon-like peptide 1 receptor 1 (PTH1); parathyroid hormone receptor 2 (PTH2); calcitonin receptor (CAL); putative EGF module-containing, mucin-like hormone receptor (EMR1); leucocyte activation antigen (CD97). References to the cloning and access numbers of all these receptors in DNA data base are available in Laburthe et al., 1996. Amino acid residues are numbered according to the sequence of each individual receptor. Boxes point out residues that are conserved in VIP1 and VIP2 receptors but are poorly or not at all conserved in other receptors of this family. Although they are not conserved in other human receptors of this family, D126 in the VIP1 receptor and D113 in the VIP2 receptor have not been mutated in the present study because they are present in the rat or mouse PACAP receptors (Laburthe et al., 1996).
similar for the wild-type receptor and for all mutants that bound 125I-VIP but one (S64A), for which a significant 3-fold increase of the dissociation constant was observed (table 1).

Because the absence of specific 125I-VIP binding to the E36A mutant might be due to an important decrease in its affinity as compared with the wild-type receptor, we also performed binding studies with a higher concentration of 125I-VIP: 0.4 nM instead of 0.05 nM in standard assay conditions. No specific binding could be detected under this condition with the E36A mutant (not shown). Binding studies were then performed for VIP2 receptor mutants (table 1). Under standard assay conditions, Scatchard analysis gave straight lines for the wild-type receptor and for VIP2 receptor mutants S53A, D116A, F122A and S278A, which had $K_d$ values similar to that of the wild-type VIP2 receptor (table 1).

In contrast, no specific 125I-VIP binding could be detected for mutant E24A or for mutants I31A and T274A under the standard binding assay conditions with 0.05 nM 125I-VIP (not shown). Binding experiments with the three latter mutants were then conducted with a higher concentration of 125I-VIP, 0.4 nM. In such conditions, specific binding could be detected with mutants I31A and T274A (table 1), but not with mutant E24A (not shown). Scatchard analysis of binding data revealed that the $K_d$ values of I31A and T274A mutants were 11 and 5 times higher than the $K_d$ of the wild-type VIP2 receptor measured under the same assay conditions (table 1).

In order to verify that mutants for which no specific 125I-VIP binding to transfected cell membranes could be detected were actually synthesized and delivered to the plasma membrane, we conducted confocal laser microscopy immunofluorescence studies of the tagged mutants. It appeared that the E36A VIP1 receptor mutant and the E24A VIP2 receptor mutant were expressed by Cos cells in the same way as the wild-type VIP1 and VIP2 receptors (fig. 2). This also held true for all other mutants of VIP1 and VIP2 receptors described in the present study (not shown). The presence of intracellularly located receptor in these single plane images (fig. 2) is in accordance with the fact that receptors are in an active phase of synthesis after transfection of their cDNA (Couvineau et al., 1996a).

Further experiments were designed to study VIP-stimulated cAMP production in cells transfected with wild-type and mutated receptors, with special focus on those mutants for which a decreased affinity was measured in binding studies. As shown in figure 3A, VIP (1 μM) promoted cAMP production with similar efficacies in wild-type VIP1 receptor and in all mutants but one (E36A). There was therefore a good correlation between binding data (table 1) and cAMP production. With regard to the E36A VIP1 receptor mutant, VIP displayed a very low efficacy, if any, for stimulating cAMP production. It should be also noted that basal cAMP levels in Cos cells transfected with every mutant were identical to that observed in cells transfected with the wild-type VIP1 receptor (fig. 3A), a result that suggests these mutants were not constitutively activated. We further investigated the S64A VIP1 receptor mutant, which exhibited an increased dissociation constant for VIP (see table 1), by performing dose-response experiments on the effect of VIP in stimulating cAMP production (fig. 4A). It appeared that half-maximal stimulation was obtained for 0.3 ± 0.1 and 0.9 ± 0.1 nM VIP for the wild-type receptor and the S64A receptor mutant, respectively. The shift of potency of VIP in stimulating cAMP production through the S64A mutant (fig. 4A) is therefore identical to the shift of affinity of VIP for binding this mutant (table 1). Figure 3B shows cAMP experiments carried out with wild-type and mutated VIP2 receptors. VIP (1 μM) promoted cAMP production with similar efficacies in wild-type VIP2 receptor and in all mutants but one (E24A).

We further investigated the I31A and T274A mutants, for which an increase of $K_d$ was observed in binding experiments (Table 1), by performing dose-response experiments (fig. 4B). It appeared that the dose-responses of VIP for stimulating cAMP production through the I31A and T274A mutants were shifted to the right as compared with the wild-type receptor. Half-maximal stimulation was obtained for 1.9 ± 0.6, 66 ± 16, and 16 ± 6 nM VIP for the wild-type receptor and the I31A and T274A receptor mutants, respectively. This important shift of potency was similar to the shift of affinity measured in binding studies (table 1). As for the VIP1 receptor mutants, it should be also noted that basal cAMP levels in Cos cells transfected with every mutant were identical to that observed in cells transfected with the wild-type VIP2 receptor (fig. 3A). This result supports the interpretation that these mutants were not constitutively activated.

**DISCUSSION**

The present site-directed mutagenesis study provides new information regarding the structure-function relationship of recombinant human VIP1 and VIP2 receptor subtypes. Although mutagenesis studies do not enable us to determine whether the effects of mutations on receptor phenotypes are direct or are brought about indirectly via allosteric mechanisms (Fong et al., 1995), this work implies for the first time that human VIP receptor subtypes that had been identified through molecular cloning (Couvineau et al., 1994; Svoboda

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**TABLE 1**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Dissociation Constant (nM)</th>
<th>Binding Capacity (pmol/mg of protein)</th>
<th>Construct</th>
<th>Dissociation Constant (nM)</th>
<th>Binding Capacity (pmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIP1 wt</td>
<td>1.4 ± 0.7</td>
<td>4.14 ± 0.33</td>
<td>VIP2 wt</td>
<td>1.8 ± 0.9</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>VIP1 I43A</td>
<td>1.3 ± 0.3</td>
<td>7.07 ± 0.71</td>
<td>VIP2 I31A**</td>
<td>20.2 ± 2.9*</td>
<td>4.1 ± 1.1</td>
</tr>
<tr>
<td>VIP1 S64A</td>
<td>4.4 ± 0.5*</td>
<td>7.52 ± 0.76</td>
<td>VIP2 S53A</td>
<td>1.3 ± 0.4</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>VIP1 D132A</td>
<td>1.7 ± 0.2</td>
<td>1.56 ± 0.31</td>
<td>VIP2 D116A</td>
<td>1.7 ± 0.3</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>VIP1 F138A</td>
<td>1.3 ± 0.9</td>
<td>1.36 ± 0.38</td>
<td>VIP2 F122A</td>
<td>1.4 ± 0.3</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>VIP1 T288A</td>
<td>1.3 ± 0.5</td>
<td>1.22 ± 0.15</td>
<td>VIP2 T274A**</td>
<td>8.5 ± 0.5*</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>VIP1 S292A</td>
<td>3.1 ± 1.1</td>
<td>5.19 ± 0.84</td>
<td>VIP2 S278A</td>
<td>1.0 ± 0.5</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>
et al., 1994), but have a very similar pharmacological profile, do differ in the structure-function relationship with respect to VIP binding. Indeed, the mutation into alanine of I31 and T274 in the human VIP2 receptor results in an important decrease in binding affinity of VIP and in potency of the peptide in stimulating cAMP production, whereas the mutation of the corresponding residues in the human VIP1 receptor does not change its phenotype. The $K_d$ of VIP for binding to I31A and T274A mutants is increased 11-fold and 5-fold as compared with the wild-type VIP2 receptor, respectively. This compares rather well with the shift in potency for stimulating cAMP production, 35-fold and 8-fold increases, respectively. Conversely, the mutation of S64 into alanine in the human VIP1 receptor results in a significant 3-fold decrease in binding affinity of VIP and a 3-fold decrease in potency of the peptide in stimulating cAMP production, whereas the mutation of the corresponding serine residue in the human VIP2 receptor does not alter its phenotype. Note that the rightward shift in potency of VIP in stimulating cAMP production via the three above-described mutants as compared with the corresponding wild-type receptors is not related to decreased expression after transfection into Cos cells. Indeed, Scatchard analysis indicates that these mutants are expressed at a higher level than the wild-type receptors (see table 1).

As mentioned above, the mechanism whereby mutation into alanine results in shifts in the binding affinity of VIP in I31A and T274A VIP2 receptor mutants and S64A VIP1 receptor mutant cannot be determined from the present data. We can hypothesize a direct interaction of the corresponding residues with VIP and/or a change in the global structure of the receptors upon mutation. The fact that all mutants are expressed at high levels in Cos cells and delivered to plasma membranes, like the wild-type receptors, does not favor a major alteration of their structure. Indeed, this would probably lead to the sequestration and degradation of mutated proteins during the chaperone-dependent process of folding and quality control (Hayes and Dice, 1996). Such an alteration of the delivery of human VIP1 receptor mutants has been described previously for receptors that lack consensus N-glycosylation sites in the N-terminal extracellular domain; it resulted in their strict sequestration in the perinuclear endoplasmic reticulum (Couvinaud et al., 1996a). Also, the fair correlation between the shifts in binding affinity of VIP and in VIP potency in stimulating cAMP production for all concerned mutants is not indicative of a global, long-range conformational change. Finally, we should keep in mind that the amino acid residues concerned are not conserved in the class II G protein-coupled receptors and therefore are not likely to participate in a common global architecture of members of this subfamily of receptors (Laburthe et al., 1996).

Fig. 2. Confocal laser scanning microscopic detection after transfection in Cos-7 cells of wild-type and mutated receptors. After permeabilization with saponin, cells were incubated with the anti-Tag antibody, washed and incubated with anti-mouse immunoglobulin G conjugated to fluorescein isothiocyanate as described in "Materials and Methods." The following receptor constructs were shown: wild-type VIP1 receptor (panel A), E36A VIP1 receptor mutant (panel B), wild-type VIP2 receptor (panel C) and E24A VIP2 receptor mutant (panel D).
and threonine residues, the present data provide the first evidence for differences in the structure-function relationship of human VIP receptor subtypes involving the N-terminal extracellular domain and also the second extracellular loop.

Our study also points out a new crucial amino acid residue in the N-terminal extracellular domain of the human VIP1 receptor for VIP binding and subsequent stimulation of cAMP production, i.e., glutamate 36. This further emphasizes the importance of the N-terminal extracellular domain of the human VIP1 receptor for VIP binding with four crucial residues—glutamate 36 (this paper), aspartate 68, tryptophan 73 and glycine 109 (Couvineau et al., 1995)—probably positioned in a tertiary functional structure maintained by multiple disulfide bridges formed by six cysteine residues (Gaudin et al., 1995; Laburthe et al., 1996). The presence of two important acidic residues (glutamate 36 and aspartate 68) in the N-terminal VIP binding domain is consistent with the unusual isoelectric point (>11) of VIP (Said and Mutt, 1970) and with the importance of many basic residues of VIP for biological activity (author’s unpublished results), which suggests the participation of electric charges for VIP binding to receptors under physiological conditions. In that respect, it is worth pointing out that VIP binding to receptors occurs in a narrow range of pH (Amiranoff et al., 1980). The importance of tryptophan 73 is also consistent with previous work suggesting the role of hydrophobic interactions in VIP binding to receptors (Bodanszky et al., 1974).

Finally, this paper provides the first site-directed mutagenesis study of VIP2 receptors. Our study indicates that both the N-terminal extracellular domain (glutamate 24 and isoleucine 31) and the second extracellular loop (threonine 274) of the human VIP2 receptor may be involved in VIP binding. Further studies not within the scope of the present one are certainly needed to document the structure-function relationship of the VIP2 receptor. In particular, it will be interesting to determine whether residues that have previously been reported to be important for VIP binding to the human VIP1 receptor in the N-terminal extracellular domain (Laburthe et al., 1996) are also important for VIP binding to the VIP2 receptor subtype.

In conclusion, the current knowledge indicates that the VIP2 receptor is distinct from the VIP1 receptor in sequence (Lutz et al., 1993; Svoboda et al., 1994; Adamou et al., 1995), distribution (Usdin et al., 1994) and structure-function relationship (this paper). However, no natural or synthetic ligand

Fig. 3. Basal and VIP-stimulated cAMP production in Cos-7 cells transfected with the wild-type and mutant human VIP1 (panel A) or VIP2 (panel B) receptors. After transfection of the cDNAs in Cos-7 cells, cells were grown for 48 h and then incubated without (black bars) or with (open bars) 1 μM VIP as described in “Materials and Methods.” Intracellular cAMP was measured by radioimmunoassay. Results are means ± S.E. of three experiments.

Fig. 4. Dose-effect of VIP in stimulating cAMP production in Cos-7 cells transfected with the wild-type and mutant human VIP1 (panel A) or VIP2 (panel B) receptor. After transfection of the cDNAs in Cos-7 cells, cells were grown for 48 h and then incubated with increasing concentrations of VIP as described in “Materials and Methods.” Intracellular cAMP was measured by radioimmunoassay. Results are means ± S.E. of three experiments. A) Wild-type VIP1 receptor (■); S64A VIP1 receptor mutant (○). The EC50 values were 0.3 ± 0.1 and 0.9 ± 0.1 nM VIP, respectively (n = 3; P < .01). B) Wild-type VIP2 receptor (■); I31A VIP2 receptor mutant (○); T274A VIP2 receptor mutant (△). The EC50 values were 1.9 ± 0.6, 66 ± 16 and 16 ± 6 nM VIP, respectively (n = 3). The differences between each mutant and the wild-type receptor were highly significant (P < .01).
selective for VIP receptor subtypes has yet been described. In this context, the respective physiological roles of VIP1 and VIP2 receptors are still conjectural. The availability of comparative studies of the structure-function relationship of VIP receptor subtypes such as this one will facilitate the development of selective agonists and antagonists and will contribute to a better knowledge of their physiological role.

References


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