We mutated the vasoactive intestinal peptide (VIP) Asp^3 residue and two VPAC_r receptor second transmembrane helix basic residues (Arg^{108} and Lys^{108}). VIP had a lower affinity for R188Q, R188L, K195Q, and K195I VPAC_r receptors than for VPAC_r receptors. [Asn^{3}] VIP and [Gln^{3}] VIP had lower affinities than VIP for VPAC_r receptors but higher affinities for the mutant receptors; the two basic amino acids facilitated the introduction of the negatively charged aspartate inside the transmembrane domain. The resulting interaction was necessary for receptor activation. 1/[Asn^{3}] VIP and [Gln^{3}] VIP were partial agonists at VPAC_r receptors; 2/VIP did not fully activate the K195Q, K195I, R188Q, and R188L VPAC_r receptors; a VIP analogue ([Arg^{16}] VIP) was more efficient than VIP at the four mutated receptors; and [Asn^{3}] VIP and [Gln^{3}] VIP were more efficient than VIP at the R188Q and R188L VPAC_r receptors; 3/the [Asp^{3}] negative charge did not contribute to the recognition of the VIP_{1} antagonist, [AcHis^{1},D-Phe^{2},Lys^{6},Arg^{16},Leu^{27}] VIP (1–7)/growth hormone releasing factor (8–27). This is the first demonstration that, to activate the VPAC_r receptor, the Asp\(^{3}\) side chain of VIP must penetrate within the transmembrane domain, in close proximity to two highly conserved basic amino acids from transmembrane 2.

The neuropeptides vasoactive intestinal polypeptide (VIP)\(^{a}\) and pituitary adenylate cyclase-activating polypeptide (PACAP) contribute to the regulation of intestinal secretion and motility, of the vascular tone, of the exocrine and endocrine secretions, of immunological responses, and to the development of the central nervous system (1–3). The effects of VIP are mediated through interaction with two receptor subclasses named the VPAC\(_{1}\) and VPAC\(_{2}\) receptors; the effects of PACAP are also mediated through interactions with the same receptors, as well as through a selective receptor named PAC\(_{1}\) (3, 4).

VPAC\(_{1}\), VPAC\(_{2}\), and PAC\(_{1}\) receptors are encoded by different genes and expressed in different cell populations in both the central nervous system and peripheral tissues (3, 5, 6). They are preferentially coupled to Go\(_{\alpha}\) proteins that stimulate adenylate cyclase activity. The PAC\(_{1}\) and VPAC\(_{1}\) receptors may stimulate, in addition, inositol trisphosphate synthesis and calcium mobilization (7, 8). This effect is however detected only at high VPAC\(_{1}\) receptor expression levels (8). VIP and PACAP receptors are members of a large family of G protein-coupled receptors, often referred as the GPCR-B family (4, 9), that includes the secretin, glucagon, glucagon-like peptide-1, calcitonin, parathyroid hormone, and growth hormone releasing factor (GRF) receptors. The VIP, PACAP, secretin, and GRF receptors constitute a subfamily based on the homology of the ligands and of the receptors. Each receptor recognizes its own cognate ligand with a high affinity but recognizes at least one other parent peptide with a comparable or a lower affinity (4). Because of the sequence homology of the ligands and the receptors, the information obtained on one receptor-ligand pair can be anticipated to be relevant also in the other systems.

The positioning of the ligand on the receptors is still poorly understood. Investigations of chimeric receptors and mutants have indicated that the large amino-terminal domain (10–13) structured by disulfide bridges (14–16) makes a key contribution to ligand recognition that several other highly conserved residues play a role in the general structure (17, 18) and that creating constitutively active receptors through mutations in the intracellular part of the receptor is possible (19). The amino-terminal part of the ligand is necessary for high affinity binding and for second messenger activation; its deletion in VIP, PACAP, and secretin reduced both the affinity and the intrinsic activity of the peptide (20). The identification of the receptor residues interacting with the amino terminus of the ligand is a prerequisite to model the active form of the receptor and conceive new ligands, preferably non-peptidic, that could be of therapeutic interest. We focused in this work on the human VPAC\(_{1}\) receptors and investigated the contribution of two basic residues located in the second transmembrane helix to ligand recognition and adenylate cyclase activation.

We obtained evidence that both basic residues are important for recognition of the Asp\(^{3}\) of VIP and stabilization of the active VIP-receptor complex conformation. Our results also suggested that a second binding mode, that does not involve recognition of
the VIP Asp<sup>3</sup> residue and does not induce receptor activation, is also possible.

**EXPERIMENTAL PROCEDURES**

**Construction of the Four Mutated Receptors, R188L, R188Q, K195Q, and K195I, and Their Stable Expression in Chinese Hamster Ovary (CHO) Cells**—The human VPAC<sub>1</sub> receptor cDNA was cloned by PCR according to the previously reported sequence (21), using specific primers. Generation of the four mutated receptors was achieved using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla CA) essentially according to the manufacturer's instructions. Briefly, the human VPAC<sub>1</sub> receptor-coding region, inserted into the mammalian expression vector pcDNA3.1 (Invitrogen Corp.), was submitted to 22 cycles of PCR (95 °C for 30 s, 54 °C for 1 min, and 68 °C for 14 min) in a 50-μl reaction volume. The forward and reverse primers were complementary and contained the desired nucleotide changes, flanked on either side by 15 perfectly matched nucleotides (only the forward primers are shown): Arg<sup>188</sup> to Leu, CATATCCCTCATCCTCGTGCCTCCCTCTCTCTCTC; Arg<sup>188</sup> to Gln, GATATCCCTCATCCTCGTGCCTCCCTCTCT; Lys<sup>195</sup> to Ile, GCCGCTGTCTCATACAGTCCGTGGCCCTCCCTCTCTC; Lys<sup>195</sup> to Gln, GCCGCTGTCTCATACAGTCCGTGGCCCTGGCCCTCTCTC. Following PCR, 10 μl were analyzed by agarose gel electrophoresis, and the remaining 40 μl were digested for at least 2 h by 1 μl of DpnI restriction enzyme (Stratagene, La Jolla, CA) to remove the parental methylated DNA. The digested PCR products were transformed into TOP10 One Shot competent Escherichia coli bacterial cells (Invitrogen Corp.). Several colonies verified by agarose gel electrophoresis of miniprep plasmid DNA (22), three were retained and further purified on Qiaquick PCR purification spin columns, and the mutations were checked for by DNA sequencing on an ABI automated sequencing apparatus, using the BigDye Terminator sequencing prism kit from ABI (Perkin-Elmer). Plasmid DNA from one clone for each mutation, containing the correct nucleotide substitutions, was prepared using a miniprep endotoxin-free kit (Stratagene, La Jolla, CA). The complete nucleotide sequence of the receptor coding region was verified by DNA sequencing on an ABI automated sequencing apparatus. The nucleotide sequence of the ligand coding region was also verified by DNA sequencing on an ABI automated sequencing apparatus. The ligand purity was assessed by capillary electrophoresis, and the conformity was verified by electrospray mass spectrometry. The nucleotide sequence of the ligand coding region was also verified by DNA sequencing on an ABI automated sequencing apparatus. The ligand purity was assessed by capillary electrophoresis, and the conformity was verified by electrospray mass spectrometry. The nucleotide sequence of the ligand coding region was also verified by DNA sequencing on an ABI automated sequencing apparatus. The ligand purity was assessed by capillary electrophoresis, and the conformity was verified by electrospray mass spectrometry. The nucleotide sequence of the ligand coding region was also verified by DNA sequencing on an ABI automated sequencing apparatus. The ligand purity was assessed by capillary electrophoresis, and the conformity was verified by electrospray mass spectrometry.

**RESULTS**

**Interaction of VIP Analogues with the Human Wild Type Recombinant VPAC<sub>1</sub> Receptor**—The VPAC<sub>1</sub>, IC<sub>50</sub>, values, measured in [<sup>125</sup>I]-VIP, [<sup>125</sup>I]-Glu<sub>4</sub>VIP, or [<sup>125</sup>I]-VIP, antagonist competition curves on membranes of CHO cells expressing VPAC<sub>1</sub>, R188Q, or R188Q-VPAC<sub>1</sub> receptors, respectively. (Table I)

**Table I**

| IC<sub>50</sub> (in nM) | VPAC<sub>1</sub> | R188Q | K195I |
|----------------------|--------------|-------|-------|
| VIP                  | 3            | 300   | 200   |
| [Arg<sup>16</sup>]VIP| 0.6          | 15    | 20    |
| [Glu<sup>4</sup>]VIP | 100          | 500   | 150   |
| [Asn<sup>1</sup>]VIP | 100          | 5     | 30    |
| [Gln<sup>2</sup>]VIP| 300          | 3     | 15    |
| VIP, agonist         | 2            | 20    | 2     |
| [Asn<sup>1</sup>]VIP, agonist | 20 | 4  | 1    |
| VIP, antagonist      | 4            | 5     | 1     |
| [Asn<sup>1</sup>]VIP, antagonist | 2 | 3  | 0.3  |

VIP behaved as partial agonists on both mutant receptors (Fig. 1). The selected clones were then tested for 125I-VIP binding. Binding was non-significant in all the clones tested. As expected from the binding data, the E<sub>50</sub> values for VIP were 30 to 100-fold higher than at VPAC<sub>1</sub> receptors (Fig. 1). There was no noticeable difference between the R188L and R188Q receptors on one hand and the K195I and K195Q on the other hand.

We then tested the ability of VIP analogues to stimulate adenylate cyclase. [Arg<sup>16</sup>]VIP, [Asn<sup>1</sup>]VIP, and [Gln<sup>2</sup>]VIP were more potent and more efficient than VIP at the R188L and R188Q-VPAC<sub>1</sub> receptors, and [Glu<sup>4</sup>]VIP and the VIP agonist behaved as partial agonists on both mutant receptors (Fig. 1 and results not shown). The results are compared in Table II with the data obtained at wild type receptors with the same peptides. [Arg<sup>16</sup>]VIP was also more potent and more efficient than VIP on the K195Q—(Fig. 1) and K195I-VPAC<sub>1</sub> receptors (data obtained from independent experiments).
not shown). [Glu3] VIP, [Gln3] VIP, and [Asn3] VIP behaved as partial agonists on this construct, and the VIP3 agonists' ability to activate adenylate cyclase through K195Q- and K195I-VPAC1 receptor mutants was barely detectable (Table II).

The VIP3 antagonists' affinity for the mutated receptors was evaluated by comparing VIP, [Arg16] VIP, or VIP1 agonist dose-effect curves in the absence (open circles) and presence (closed circles) of 100 nM VIP3 antagonist were obtained at CHO cell membranes expressing the R188L VPAC1 receptors (top panel), and [Arg16] VIP dose-effect curves in the absence (open circles) and presence (closed circles) of 100 nM VIP3 antagonist were obtained at CHO cell membranes expressing the K195I VPAC1 receptors (bottom panel). The figure is representative of at least three experiments performed in duplicate.

**Fig. 2. Competitive inhibition of adenylate cyclase stimulation by the VIP1 antagonist, PG 97 269.** VIP dose-effect curves in the absence (open circles) and presence (closed circles) of 100 nM VIP3 antagonist were obtained at CHO cell membranes expressing the R188L VPAC1 receptors (top panel), and [Arg16] VIP dose-effect curves in the absence (open circles) and presence (closed circles) of 100 nM VIP3 antagonist were obtained at CHO cell membranes expressing the K195I VPAC1 receptors (bottom panel). The figure is representative of at least three experiments performed in duplicate.

**TABLE II**

| Adenylate cyclase stimulation through VPAC1, R188Q, and K195I-VPAC1 |
|---------------------------------------------------------------|
| The standard deviation of the agonists pD2 (−log EC50) values was always below 0.15 log units, and the standard deviation of the agonists' efficiency (Emax, in % of VIPs) was lower than ± 10%. n.d., not determined. |

| EC50 values (in nM) and Emax (in percent of VIPs) | VPAC1 | R188Q | K195Q |
|-------------------------------------------------|-------|-------|-------|
| VIP                                             | 5 (100%) | 30 (100%) | 200 (100%) |
| [Arg16]VIP                                      | 0.2 (100%) | 10 (150%) | 20 (150%) |
| [Glu3]VIP                                       | 30 (80%) | 200 (20%) | 200 (50%) |
| [Asn3]VIP                                       | 150 (25%) | 4 (170%) | 100 (20%) |
| [Gln3]VIP                                       | 100 (50%) | 4 (150%) | 30 (40%) |
| VIP1 agonant                                    | 5 (80%) | 20 (50%) | ? (≤10%) |
| [Asn3]VIP1 agonant                              | ? (0%) | 5 (160%) | n.d. |

We were surprised that, even though the presence of a negative charge in position 3 was clearly deleterious for recognition of the R188L, R188L, K195Q, and K195I mutant VPAC1 receptors by agonists, the VIP3 antagonist (that also possesses an Asp3 residue) retained a high affinity for the mutated receptors. We therefore synthesized and tested two additional peptides with an Asn3 residue, the Asn3 VIP1 agonist and Asn3 VIP1 antagonist. As shown in Tables I and II, replacing Asp3 by an Asn3 residue reduced the affinity and efficacy of the VIP1 agonist at wild type receptors but increased its affinity and efficacy on the mutated R188Q receptor. In contrast, replacing Asp3 by an Asn3 residue in the VIP1 antagonist did not affect its affinity for the receptors studied in this work; the modified peptide always behaved as a high affinity antagonist (Fig. 4).

**DISCUSSION**

VIP receptors belong to a G protein-coupled receptor family that does not share any sequence homology with rhodopsin or with the β-adrenergic receptor family. Previous work with chimeric receptors (see Refs. 10–13 and 29–34) and cross-linking studies (35–37) led to a general VIP positioning model; the amino-terminal and carboxyl-terminal sequences of VIP and of its analogues seemed to interact with the “7TM” (transmem-
brane) receptor domain, whereas recognition of the central VIP sequence (from positions 6 to 22) depends on the amino-terminal extracellular receptor domain.

In view of the significant sequence homologies between secretin, VIP, PACAP, GRF, glucagon, glucagon-like peptide (7–36), and gastric inhibitory peptide on the one hand and between their receptors on the other hand, we hypothesized that these different peptides recognize "equivalent" binding sites in their respective receptors. The observation that all the aforementioned peptides except glucagon possess an acidic residue (Asp or Glu) in position 3, and that all the receptors (except glucagon’s) possess aligned basic Arg and Lys residues at the extracellular end of TM2, led us to suspect that these residues might interact in the peptide-receptor complex.

Our preliminary results confirmed that, as in secretin receptors (17, 38), the VPAC₁ receptor Arg¹⁸⁸ and Lys¹⁹⁵ were important for VIP recognition; we were unable to obtain significant ¹²⁵I-VIP binding at the mutated receptors, and very high VIP concentrations were necessary to activate the adenylate cyclase. The following results suggested in addition that VIP was unable to stabilize the mutated receptors’ active conformations sufficiently to ensure full receptor activation: [Asn³] VIP, [Gln³] VIP, and [Arg¹⁶] VIP were more efficient than VIP at the R188Q and R188L VPAC₁ receptors, and [Arg¹⁶] VIP was more efficient than VIP at the K195Q and K195I VPAC₁ receptors.

When VIP and its receptor are free, the VIP Asp³ and the receptor Arg¹⁸⁸ and Lys¹⁹⁵ side chains probably form dipole-ion interactions with surrounding water molecules. These favorable interactions are disrupted upon ligand binding; they must be compensated by ligand-receptor interactions to allow high affinity binding. The “uncharged VIP analogues” [Asn³] VIP and [Gln³] VIP had a higher affinity than VIP for the “uncharged receptor mutants” (R188Q, R188L, K195Q, and K195I VPAC₁ receptors), suggesting that the VIP Asp³ and the receptor Arg¹⁸⁸ and Lys¹⁹⁵ side chains were in close proximity in the agonist-receptor complex. The affinity loss that we observed upon replacement of the VIP Asp³, VPAC₁ receptor Arg¹⁸⁸, and VPAC₁ receptor Lys¹⁹⁵ (30- to 100-fold) was however comparatively small and did not support the hypothesis that the Asp negative charge is close enough to the receptor Arg and Lys positive charges to form ionic bonds (40). It is more likely that Asp³, Arg¹⁸⁸, and Lys¹⁹⁵ formed strong hydrogen bonds (i.e. dipole-dipole or ion-dipole interactions); the two receptor basic residues probably participated in the formation of an electrophilic pocket that recognized the negatively charged VIP Asp³ side chain.

[Asn³] VIP and [Gln³] VIP behaved as partial agonists at wild type receptors but were more efficient than VIP at the R188Q

![Fig. 3. Binding to VPAC₁, K195Q-, and R188Q-VPAC₁ receptors.](image)

VIP (closed circles), [Arg¹⁶] VIP (open squares), and [Gln³] VIP (open triangles) competition curves were obtained at wild type (top panel), K195Q- (center panel), or R188Q VPAC (bottom panel) receptors, using ¹²⁵I-VIP, ¹²⁵I-VIP₁ antagonist, or ¹²⁵I-[Gln³] VIP as tracer, respectively. The figure represents the average of three experiments performed in duplicate.

![Fig. 4. Importance of the [Asp³] for VIP₁ agonist and antagonist binding.](image)

VIP₁ agonist (closed squares, left panels), [Asn³] VIP₁ agonist (open squares, left panels), VIP₁ antagonist (closed circles, right panels), and [Asn³] VIP₁ antagonist (open circles, right panels) at wild type (top panels), K195Q- (center panels), and R188Q VPAC₁ receptors (bottom panels), using ¹²⁵I-VIP, ¹²⁵I-VIP₁ antagonist, or ¹²⁵I-[Gln³] VIP as tracer, respectively. The figure represents the average of three experiments performed in duplicate.
and R188L mutant receptors. The incomplete activation of the mutant receptors by VIP might be caused by difficulties in burying the anionic Asp³ side chain deep enough in the (uncharged) mutant receptors’ binding site. This hypothesis is indirectly supported by the observation that replacing the VIP Asp³ or the VPAC₁ receptor Arg¹⁸⁸ or Lys¹⁹⁵ with uncharged amino acids affected the recognition of the VIP₃ agonist (an efficient partial agonist) somewhat less than the recognition of VIP and did not affect binding of the VIP₁ antagonist, a compound that does not induce receptor activation. Due perhaps to steric hindrance between the receptor and the large D-Phe² side chain, the VIP₁ antagonist Asp³ was apparently unable to enter the agonist binding pocket and to trigger receptor activation.

The parathyroid hormone (PTH) receptor belongs to the same receptor family as the VPAC₁ receptor. A molecular model of the PTH-receptor interaction has been developed, based on experimental data from cross-linking studies, spectroscopic investigations of the hormone and receptor fragments, and theoretical structure predictions (39). According to this model, PTH recognizes extracellular receptor domains (the amino-terminal domain and extracellular loops) but penetrates very little if at all inside the compact transmembrane helices bundle. It is tempting to suggest that, like PTH, VIP initially recognizes an extracellular binding site. In a second step, driven and stabilized i.e. by the Asp³-Lys¹⁹⁵/Arg¹⁸⁸ interactions, a transmembrane binding pocket opens and recognizes the agonists’ amino-terminal amino acids, and the receptor activates intracellular G proteins. “Too large” amino-terminal VIP amino acids (D-Phe² instead of Ser² and pCl-Phe⁶ instead of Phe⁶) might prevent the recognition of this activated receptor conformation by steric hindrance; [D-Phe²] and [pCl-Phe⁶] VIP or VIP/GRF analogues usually behave as VIP antagonists (27, 28).

The location of the VIP and secretin “Asp³ binding site” to transmembrane helix 2 was somewhat unexpected; indeed, in the rhodopsin-like β-adrenergic G protein-coupled receptor family, the ligand binding pocket is lined by TMs 3 to 7 and does not involve TMs 1 and 2. It is important to note in this respect that most of the “signature” amino acids that define the β-adrenergic G protein-coupled receptor family, including the proline residues that participate in the formation of the agonist binding pocket, are absent from the secretin receptor family (including VPAC₁ receptors). Our results suggest that (in contrast with the G protein binding site that appears to involve the same intracellular loops in both receptor families) the agonist binding site was located in very different transmembrane regions in the secretin- and β-adrenergic-receptor families. Further studies will be needed to extend this observation and allow the construction of an activated agonist-receptor complex model.

To conclude, our present results suggested that the VIP Asp³ side chain fitted inside the transmembrane helix bundle, in close proximity to TM2 Lys³⁵⁶ and Arg³⁶⁶. This interaction was essential for receptor activation. The VIP₁ antagonist Asp³ residue did not recognize the same binding pocket perhaps because of unfavorable coulombic interactions between the D-Phe² side chain and the receptor.

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Two Basic Residues of the h-VPAC₁ Receptor Second Transmembrane Helix Are Essential for Ligand Binding and Signal Transduction
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