Identification of Key Residues for Interaction of Vasoactive Intestinal Peptide with Human VPAC1 and VPAC2 Receptors and Development of a Highly Selective VPAC1 Receptor Agonist

ALANINE SCANNING AND MOLECULAR MODELING OF THE PEPTIDE*

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The widespread neuropeptide vasoactive intestinal peptide (VIP) has two receptors VPAC1 and VPAC2. Solid-phase syntheses of VIP analogs in which each amino acid has been changed to alanine (Ala scan) or glycine (Gly scan) or substituted by His1, Val5, Arg14, Lys15, Lys21, Leu23, and Ile26 decreased biological activity without altering the predicted structure, supporting that these residues interact with VPAC1 receptor. The interaction of the analog with human VPAC1 receptor is similar to that observed with VPAC1 receptor, with three remarkable exceptions: substitution of Thr11 and Asn28 by alanine increased Kᵢ for binding to VPAC1 receptor; substitution of Tyr22 by alanine increased EC₅₀ for stimulating adenylyl cyclase activity through interaction with the VPAC₂ receptor. By combining 3 mutations at positions 11, 22, and 28, we developed the [Ala₁₁,₂₂,₂₈]VIP analog which constitutes the first highly selective (Kᵢ > 1,000-fold) human VPAC₁ receptor agonist derived from VIP ever described.

The vasoactive intestinal peptide (VIP) is a prominent neuropeptide with wide distribution in both peripheral and central nervous systems and a large spectrum of biological actions in mammals (1, 2). VIP-containing nerves and VIP effects have been described in digestive tract, cardiovascular system, airways, reproductive system, immune system, endocrine glands, and brain (1). Besides its short-term actions on exocrine secretions, hormone release, muscle relaxation, and metabolism (1, 2), VIP has been also characterized as a growth regulator for fetuses and tumor cells and during embryonic brain development (3). There are recent evidences for an important role of VIP in the perception of pain (4) and suppression of inflammation (5). Finally, VIP has been involved in diseases such as the watery diarrhea syndrome and clinical applications of VIP have been already suggested in impotence, asthma, lung injury, a variety of tumors and neurodegenerative diseases (1–3).

VIP belongs to a large family of structurally related peptides (2, 6, 7) that comprises VIP, pituitary adenylate cyclase-activating peptide PACAP-27, and its C-terminal extended form PACAP-38, secretin, glucagon, and glucagon-like peptides 1 and 2, gastric inhibitory polypeptide, peptide histidine methionine amide, growth hormone-releasing factor (GRF), and peptides isolated from the venom of the Gila Monster. VIP and PACAP are the most closely related peptides in terms of structure and function (2, 6). They share two common receptors, VPAC₁ and VPAC₂, which display high affinity for both VIP and PACAP (2, 8). These receptors together with receptors for VIP-related peptides (see above) clearly constitute an original subfamily within the superfamily of G protein-coupled receptors (2, 9, 10). This subfamily referred to as class II (2) also comprises receptors for parathyroid hormone, calcitonin, corticotropin-releasing factor, and the so-called EGF-TM7 receptors (11). Class II family of receptors for peptides display several common properties including large N-terminal extracellular domains containing highly conserved cystein residues, N-terminal leader sequences, and complex gene organization with many introns (2).

Although the structure-function relationship of VIP receptors, including VPAC₁ and VPAC₂, has been recently documented (2, 9, 12–20), the structure-function relationship of VIP itself is still poorly understood. Some old studies carried out before the characterization and cloning of VIP receptor subtypes (21–23) indicated that: (i) the entire sequence of VIP is required for full biological function (21, 22); (ii) VIP-related peptides having significant sequence homologies with VIP such as peptide histidine methionine amide, secretin, GRF, and helodermin behave as low potency VIP agonists (23); (iii) there are important differences between species in the pharmacology of VIP receptors, especially between rodents and humans (2,
VIP analogs were obtained by custom peptide synthesis from receptor was stably expressed in CHO cells as described (24). The human action with human VPAC1 and VPAC2 receptors and also resulted in the development of the most highly selective VPAC1 receptor agonist ever described. Moreover, the contribution of each residue was also analyzed after molecular modeling of all VIP analogs. These studies allowed us to determine key residues for interaction with human VPAC1 and VPAC2 receptors and also resulted in the development of the most highly selective VPAC1 receptor agonist ever described.

**EXPERIMENTAL PROCEDURES**

**Materials—**Enzymes and vectors for cloning were obtained from Promega (Charbonnieres, France). The recombinant human VPAC1 receptor was stably expressed in CHO cells as described (24). The human VPAC1 cDNA (25) was a gift from Dr. M. Svoboda and Dr. P. Robberecht (Brussels, Belgium). Culture medium was obtained from Life Technologies, Inc. (Cergy-Pontoise, France). In addition to VIP analogs synthesized by us (see below), [Ala11,22,28]VIP and [Ala12]VIP, [Ala23]VIP, and [Ala27]VIP for which HATU (29) was used. Syntheses of the different peptides were performed from 0.625 g of Fmoc-PAL-PEG-PS resin. An excess of 4 eq of each amino acid was used. A double coupling was performed when Fmoc-Asn(Trt)-OH, Fmoc-Val-OH, Fmoc-Glu(Xan)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Thr(But)-OH, and Fmoc-Tyr(But)-OH were involved in coupling. Fmoc protection was performed with a solution of piperidine in dimethylformamide in a 2:1 ratio. Final deprotection of the peptides from the resin was performed with a solution of piperidine in dimethylformamide in a 2:1 ratio. The peptides were then purified by addition of cold diethyl ether and dissolved in a mixture containing trifluoroacetic acid, water, thiouanisole, ethylenedinitril, phospho in a 82.5/5/5.2/5.5 (v/v) ratio (30) for 2 h. Peptides were characterized by electrospray mass spectrometry using a Jeol LI 2000 spectrometer.

**Native VIP**

| Analogs | Potencya |
|--------|---------|
| Native VIP | 0.9 ± 0.4 |

a Potency is relative to native VIP (1.00).

**TABLE I**

Biological activity of VIP analogs in cells stably expressing human recombinant VPAC1 receptor

| Amino acid | Position | Substitution | Binding, $K_i$ | Potency | Adenylyl cyclase, EC50 | Potency |
|-----------|----------|--------------|---------------|---------|-----------------------|---------|
| His       | 1        | Ala          | 87 ± 21       | 0.01    | 28 ± 3                | 0.01    |
| Ser       | 2        | Ala          | 0.8 ± 0.6     | 1.12    | 0.4 ± 0.1             | 1.00    |
| Asp       | 3        | Ala          | 55.7 ± 6.6    | 0.02    | 43.2 ± 17.7           | 0.009   |
| Ala       | 4        | Gly          | 0.2 ± 0.1     | 4.50    | 1.2 ± 1.0             | 0.31    |
| Val       | 5        | Ala          | 13 ± 2.2      | 0.07    | 3.3 ± 1.2             | 0.12    |
| Phe       | 6        | Ala          | 193 ± 5       | 0.005   | 163 ± 44              | 0.0002  |
| Thr       | 7        | Ala          | 33 ± 5        | 0.03    | 43 ± 2                | 0.009   |
| Asp       | 8        | Ala          | 16 ± 1        | 0.06    | 18 ± 2                | 0.02    |
| Asn       | 9        | Ala          | 0.5 ± 0.1     | 1.80    | 0.3 ± 0.1             | 1.33    |
| Tyr       | 10       | Ala          | 19 ± 6        | 0.05    | 8.1 ± 1.5             | 0.05    |
| Thr       | 11       | Ala          | 0.5 ± 0.2     | 1.80    | 0.2 ± 0.1             | 2.00    |
| Arg       | 12       | Ala          | 88 ± 12       | 0.01    | 97 ± 20               | 0.004   |
| Leu       | 13       | Ala          | 5.6 ± 0.2     | 0.16    | 1.9 ± 0.7             | 0.21    |
| Arg       | 14       | Ala          | 105 ± 18      | 0.0099  | 44 ± 2.5              | 0.009   |
| Lys       | 15       | Ala          | 36 ± 8        | 0.03    | 7.6 ± 2.2             | 0.05    |
| Gln       | 16       | Ala          | 10 ± 0.5      | 0.90    | 1.0 ± 0.4             | 0.40    |
| Met       | 17       | Ala          | 0.8 ± 0.1     | 1.12    | 0.3 ± 0.2             | 1.33    |
| Ala       | 18       | Gly          | 13 ± 0.5      | 0.70    | 0.9 ± 0.2             | 0.44    |
| Val       | 19       | Ala          | 0.8 ± 0.1     | 1.12    | 0.4 ± 0.3             | 1.00    |
| Lys       | 20       | Ala          | 31 ± 4        | 0.03    | 3.2 ± 1.2             | 0.31    |
| Lys       | 21       | Ala          | 15 ± 2        | 0.06    | 7.5 ± 0.8             | 0.05    |
| Tyr       | 22       | Ala          | 5.8 ± 1.9     | 0.16    | 5.6 ± 1.4             | 0.07    |
| Leu       | 23       | Ala          | 117 ± 19      | 0.008   | 11 ± 2                | 0.04    |
| Gln       | 24       | Ala          | 3.1 ± 0.3     | 0.29    | 0.3 ± 0.1             | 1.33    |
| Ser       | 25       | Ala          | 0.8 ± 0.2     | 1.12    | 0.4 ± 0.2             | 1.00    |
| Ile       | 26       | Ala          | 16 ± 4        | 0.06    | 14 ± 2                | 0.12    |
| Leu       | 27       | Ala          | 0.6 ± 0.1     | 1.50    | 0.3 ± 0.1             | 1.33    |
| Asn       | 28       | Ala          | 1.6 ± 0.1     | 0.56    | 2.9 ± 1.9             | 0.14    |
| Thr/Asn   | 11/28    | Ala          | 0.6 ± 0.2     | 1.50    | 0.5 ± 0.1             | 0.80    |
| Thr/Tyr/Asn | 11/22/28 | Ala      | 7.4 ± 2.1     | 0.12    | 0.4 ± 0.1             | 1.00    |
sively attributed to all amino acids of the sequence.

For each rotation axis, the energy is calculated using the following equation,

\[ E(k) = E(k)_{\text{ref}} + \sum_{j=1}^{n} \sum_{1 \leq j < n} q(E_{\text{vdw}} + E_{\text{elec}} + E_{\text{sol}}) + \sum_{i=1}^{m} q \frac{1}{n} E_{\text{ref}}^{\text{out}} \]

where \( E(k) \) is the energetic component associated to the torsion axis \( k \), between atoms \( u \) and \( u+1 \), with \( E(k)_{\text{ref}} \) as torsional energy, \( E_{\text{vdw}} \) as Van der Waals energy, \( E_{\text{elec}} \) as electrostatic energy, \( E_{\text{sol}} \) as internal solvation energy between atoms \( i \) and \( j \), and \( E_{\text{ref}}^{\text{out}} \) as solvent solvation energy, and \( q \) as an energy quantum equal to the interaction energy between atoms \( i \) and \( j \), divided by the number of torsion axes between atoms \( i \) and \( j \). A structure is then obtained from the linear combination of each residue energy minimum. The energy of this construct is calculated and the residues having the lowest energy are frozen. Then the calculation is started again, keeping the structure of frozen residues constant and testing the 179 pairs of \( \Phi, \psi \) angles on the others. At each step of calculation, \( \mu \) residues are frozen, \( \mu \) varying between 5 and 28. Increasing the \( \mu \) value allows to test the variability of the calculated VIP or VIP analog structures. Calculations were performed on RAMSES (Rapid Analysis Master/Slaves Extensible System), a parallel hardware of 21 tracer Europa Pentium Pro PC cadenced at 180 MHz connected by a 100 Mbytes Network and controlled by a HP Vectra VA Pentium Pro cadenced at 200 MHz. Molecular views were drawn with WinMGM 1.0 (Ab Initio Technology, Obernai, France) as described (33).

Statistical analysis of the predicted structures of VIP and VIP analogs was made using the SICLA program (32). This procedure starts from \( n \times n \) distance tables calculated from the local root mean square distances of all pairs of structures obtained using different \( E_{\text{ref}} \) values. The root mean square distances was calculated by fitting the backbone of 5 amino acids of a structure to the 5 corresponding residues of the reference structure (here, the predicted structure obtained with \( \mu = 5 \)). The root mean square distances of the fragment is attributed to the central amino acid, the window is then moved by 1 residue along the sequence and calculations are repeated. The clustering procedure used in SICLA is an automatic nonhierarchical classification that compares pairs of structures (34, 35). This procedure is described in detail elsewhere (32). From the clustering of structures calculated with different \( \mu \) values, a mean distance between the central structure and the others is obtained. This distance is representative of the peptide structural variability.

**Stable Expression of cDNA Encoding Human VPAC<sub>1</sub> and VPAC<sub>2</sub> Receptors in CHO Cells**—Full-length VPAC<sub>1</sub> or VPAC<sub>2</sub> cDNAs were ligated in the EcoRI site of pcDNA3 (Invitrogen). Both receptors were tagged at the C terminus with a marker dodecapeptide (Tag) as described (14). The recombinant plasmid encoding human VPAC<sub>2</sub> was then harvested with a rubber policeman and centrifuged for 10 min at 2,000 × g. Attached cells were washed three times with phosphate-buffered saline, then harvested with a rubber policeman and centrifuged for 10 min at 2,000 × g. The cell pellet was exposed for 30 min at 4 °C to hypoosmotic 5 mM Hepes buffer, pH 7.4, and a particulate fraction, referred to as the density of VPAC<sub>2</sub> and VPAC<sub>1</sub> receptors did not vary significantly.

**RESULTS**

**Alanine Scanning Analysis of VIP for Interacting with Human VPAC<sub>1</sub> Receptor**—Twenty-six analogs of VIP were synthesized in which each side chain of VIP 1–28 was systematically replaced with alanine. Two other analogs were also synthesized in which glycine was substituted for alanine in position 4 or 18.

**FIG. 1.** Binding assay and adenyl cyclase assay of VIP analogs in CHO cell clone stably expressing human VPAC<sub>1</sub> receptor. A, dose effects of native VIP and some VIP analogs for inhibition of 

\[ ^{125}\text{I}-\text{VIP} \]

binding to membranes from CHO cells expressing VPAC<sub>1</sub> receptor. B, stimulation of adenyl cyclase activity by native VIP and some VIP analogs in membranes from CHO cells expressing VPAC<sub>1</sub> receptor. The data are expressed as the percentage of maximal stimulation above basal obtained with 10 μM native VIP. All data are mean ± S.E. of at least three experiments performed in duplicate. ○, native VIP; □, H1A mutant; ▄, F6A mutant; Δ, K20A mutant; ◊, I26A mutant.
response curves for inhibiting 125I-VIP binding were parallel to binding to cell membranes. For all VIP analogs, the dose-residues also of the central part of the molecule with five crucial basic native VIP.

Substitution at other positions resulted in no change of affinity of less than one log. The most decreased affinity for inhibiting 125I-VIP binding to VPAC1 receptor for some of these VIP analogs with decreased affinity e.g. H1A, F6A, K20A, and I26A VIP analogs. Substitution at other positions resulted in no change of affinity or a small decrease of affinity of less than one log. The most pronounced decreases of affinity occurred with H1A, F6A, R12A, R14A, and L23A analogs for which a 100–200-fold decrease was observed. Other significant decreases of affinity were observed with D3A, V5A, T7A, D8A, Y10A, K15A, K20A, K21A, and I26A analogs, e.g. >10-fold. All VIP analogs were also tested for their ability to stimulate adenylyl cyclase activity in membranes of CHO clone 15 cells expressing the human VPAC1 receptor. The dose-response curves for all analogs parallel that of VIP and 10 μM analogs achieved the same maximal stimulation of enzyme activity as native VIP (not shown). Fig. 1 shows the dose-response curves for stimulating adenylyl cyclase activity in membranes of CHO clone 15 cells expressing the human VPAC1 receptor. The dose-response curves for all analogs paralleled that of VIP and 10 μM analogs achieved the same maximal stimulation of enzyme activity as native VIP (not shown).

In general, there is a good correlation between the EC50 for stimulating enzyme activity and the Ki for inhibiting 125I-VIP binding (see Table I) (Fig. 2). All analogs behaved as VPAC1 receptor agonists with identical or lower potencies than native VIP.

Altogether, the data were consistent with the importance of many residues along the VIP 1–28 sequence. One can notice the particular contributions of the N-terminal 1–8 sequence and also of the central part of the molecule with five crucial basic residues i.e. Arg12, Arg14, Lys16, Lys20, and Lys21. Two hydrophobic residues in the C-terminal part also play a role, i.e. Leu23 and Ile26. A recurrent issue for explaining alanine scanning data for peptide ligand is to determine whether a given substitution by alanine disrupts a specific interaction between peptide and receptor and/or alters the global structure of the peptide. To address this important question, modeling of three-dimensional structure of all VIP analogs was performed.

**Molecular Modeling of VIP and VIP Analogs**—The conformation of native VIP as well as those of all VIP analogs were analyzed using OSIRIS (see “Experimental Procedures”). The first step of this ab initio procedure allows to assign a secondary structure to each residue. Iterative calculations were performed and at each iteration residues are frozen up to the entire sequence, μ varying between 5 and 28 (μ = 5, 7, 9, 11, 12, 16, 20, and 28 residues). This mimics nucleation centers observed during the folding of a protein and was used here to estimate the structural variability of native VIP and each analog. Fig. 3 shows the different conformers obtained for native VIP. The conformational variability is essentially located at the N- and C-terminal extremities, the central domain from Val16 to Asn24 being clearly helical. A statistical analysis was then carried out to assess the structural dispersion shown in Fig. 3. A statistical mean distance (D) based on the local root mean square between all pairs of conformers was calculated using SICLA (see “Experimental Procedures”). The structural dispersion of several VIP analogs is clearly higher than that of native VIP. Indeed, D3A, F6A, T7A, D8A, Y10A, R12A, and K20A VIP analogs exhibit D values >1.5 Å versus 1.2 Å for native VIP. This indicates that the corresponding analogs have a significant change of conformation as compared with native VIP (see below). In contrast, other mutations did not affect this dispersion with D values <1.2 Å, e.g. H1A, S2A, A4G, K15A, Q16A, M17A, A18G, V19A, Y22A, L23A, S25A, I26A, and N28A VIP analogs. These latter analogs have predicted structures which are very similar to that of native VIP (for example, see the L23A analog in Fig. 3). An intermediate behavior was observed for the V5A, T11A, R14A, K21A, N24A, and L27A VIP analogs which exhibited D values between 1.2 and 1.5 Å. For these analogs, we noticed that the dispersion is mostly due to one of the eight conformers corresponding to one peculiar μ value as shown, for example, for the V5A VIP analog (Fig. 3). In this particular example, when μ = 28 the predicted structure of the V5A analog does not fit as well in the N-terminal domain as for the other μ values. We assumed that a single deviation is not significant and we concluded that an intermediate D value is not representative of a structural change in the analog as compared with native VIP. For analogs with D values >1.5 Å, the high dispersions are clearly due to differences of several, if not all, conformers. The D3A, F6A, T7A, D8A, and Y10A mutants exhibit changes in their N-terminal domain as compared with native VIP (Fig. 3). The R12A mutant exhibits a global alteration of the predicted structure due to partial disruption of the central helical domain (Fig. 3). Finally, the K20A mutant exhibits a change in its C-terminal domain as compared with native VIP (Fig. 3).

**Structure-Activity Relationship of VIP for Interacting with Human VPAC1 Receptor**—The 28 residues of native VIP can be classified in three categories with respect to the interaction of VIP with human VPAC1 receptor: (i) residues which can be substituted into alanine or glycine without significant alteration of their binding affinity or biological potency. This category includes Ser2, Ala4, Asn9, Thr11, Leu13, Gin16, Met17, Ala18, Val19, Tyr22, Asn24, Ser25, Leu27, and Asp28. It is quite interesting to note that, compared with native VIP, mutants at these positions do not exhibit significant change in their predicted structure; (ii) residues whose substitution into alanine is associated with a significant alteration of the binding affinity or biological potency of the corresponding analog as well as a change of the predicted analog structure. This category includes Asp3, Phe6, Thr7, Asp8, Tyr10, Arg12, and Lys20. Al-
though we can speculate that the decreased binding affinity of the corresponding mutant is due to its altered structure, the possible direct involvement of the category ii residues in the VIP-VPAC₂ receptor interaction cannot be ruled out; (iii) residues whose substitution into alanine results in a decreased binding affinity or biological potency of the corresponding analog without change of the predicted analog structure. This category includes His³, Val⁶, Arg¹⁴, Lys¹⁵, Lys²¹, Leu²³, and Ile²⁶. These residues are likely to participate in the direct interaction between VIP and human VPAC₁ receptor. The VIP sequence with the three categories of residues is shown in Fig. 4.

It is worth noting that all analogs exhibiting alteration of the predicted peptide structure, as compared with native VIP, have a decreased binding affinity or biological potency. In this context, it was interesting to align the amino acid sequences of human VIP and human PACAP-27 (Fig. 4) since both peptides have the same high affinity for human VPAC₁ receptors (38). It clearly appears that residues belonging to categories ii and iii are identical in VIP and PACAP or highly conserved, e.g. exchanges between valine and isoleucine at positions 5 and 26.

Alanine Scanning Analysis of VIP for Interacting with Human VPAC₂ Receptor: Selectivity of VIP Analogs—The ability of each VIP analog to interact with the recombinant human VPAC₂ receptor expressed in CHO clone 10 cells was analyzed by competitive inhibition of ¹²⁵I-VIP binding to cell membranes. All competitor curves fitted with a monophasic binding profile, consistent with a single binding site (not shown). Fig. 5 shows the competition curves for ¹²⁵I-VIP binding to VPAC₂ receptor for some VIP analogs, e.g. T11A, Y22A, and N28A. Analysis of $K_i$ (Table II) showed that substituting residues at 16 positions of VIP resulted in a $>1$ log decrease of affinity for VPAC₂ receptors. This includes the same 14 positions as for VPAC₁ receptor and two additional spots at positions 11 and 28. Since the corresponding analogs T11A and N28A VIP do not exhibit changes in their predicted structure as compared with native VIP, it can be suggested that Thr¹¹ and Asn²⁸ participate in the direct interaction between VIP and human VPAC₂ receptor. Further differences between VPAC₁ and VPAC₂ receptors can be noticed since some VIP analogs exhibit a much higher decrease in binding affinity for the VPAC₂ receptor than for the VPAC₁ receptor, i.e. D8A, Y10A, and L23A. All VIP analogs were also tested for their ability to stimulate adenylyl cyclase activity in membranes of CHO clone 10 cells expressing the human VPAC₂ receptor. The dose-response curves for all analogs paralleled that of VIP and 10 µM analogs achieved the same maximal stimulation of enzyme activity as native VIP (not shown). The dose-response curves for T11A, Y22A, and N28A analogs are shown in Fig. 5. In general, there is a good correlation between the Ec₅₀ for stimulating enzyme activity and the $K_i$ for inhibiting ¹²⁵I-VIP binding (Fig. 6 and Table II). However, the Ec₅₀ of Y22A VIP for stimulating adenylyl cyclase activity is much higher than its $K_i$ for binding to VPAC₂ receptor (Fig. 5 and Table II). This property is specific for VPAC₂ receptor since the Ec₅₀ and $K_i$ of Y22A VIP at the VPAC₁ receptor are very similar (Table I).

From the above described data, it appears that several analogs discriminate between VPAC₁ and VPAC₂ receptors. They exhibit a much higher binding affinity and/or biological potency for VPAC₁ than for VPAC₂ receptors. The reverse is not true since none of the analogs had a higher affinity for VPAC₂ than for VPAC₁. In this context, we synthesized new analogs with the aim to develop more selective human VPAC₁ receptor agonists. For that purpose, we first combined mutations at positions 11 and 28 which individually resulted in a decreased affinity for VPAC₂ receptor without any change in the affinity for VPAC₁ receptor. The [Ala¹¹,²⁸]VIP analog clearly discriminated between VPAC₁ and VPAC₂ receptors (Tables I and II). Indeed, this VIP analog had the same affinity as native VIP for VPAC₁ receptor whereas it displayed a 44-fold lower affinity than native VIP for VPAC₂ receptor (Fig. 7). Similar data were obtained in the adenylyl cyclase assay (Fig. 7). The [Ala¹¹,²⁸]VIP analog was as potent as native VIP for stimulating enzyme activity via VPAC₁ receptor whereas it was 21-fold less potent than VIP for stimulating enzyme activity via VPAC₂ receptor (Fig. 7). Since we also noted that position 22 in VIP was important for discriminating between VPAC₁ and VPAC₂ receptors with respect to adenylyl cyclase activation (see above), we further synthesized a VIP analog which combines 3 mutations at positions 11, 22, and 28. Quite interestingly, the [Ala¹¹,²²,²⁸]VIP analog was highly selective for VPAC₁ receptor. Indeed, its binding affinity for VPAC₂ receptor and subsequent biological potency in stimulating adenylyl cyclase were decreased by >1,000-fold as compared with native VIP (Fig. 7). In sharp contrast, this analog with a triple mutation retained a binding affinity for VPAC₁ receptor closed to

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**Fig. 3.** Molecular modeling of native VIP and some VIP analogs: structural modifications triggered by alanine substitutions. The figure shows superposition of all subsets of possible conformations. Ribbon representation and superposition of the structures obtained with different $\mu$ values are shown. Green is for $\mu = 5$; yellow for $\mu = 7$; dark blue for $\mu = 9$; pink for $\mu = 11$; purple for $\mu = 12$; green-yellow for $\mu = 16$; orange for $\mu = 20$; light blue for $\mu = 28$. See “Experimental Procedures” for details. The lowest energy conformation is shown in purple. Native VIP is essentially a $\alpha$-helix from Val³ to Asn²⁸. The N-terminal domain (His¹-Ala⁴) is shown on the left, and C-terminal domain (Ser²⁵-Asn²⁸) on the right are less structured with several possible low energy conformations.
that of native VIP (Fig. 7). The same held true for biological response, the triple mutant and native VIP having identical potencies for stimulating adenylyl cyclase activity in VPAC1 receptor-transfected cells (Fig. 7). It is interesting to note that $10^{-8}$ M of this analog which triggers maximal response at VPAC1 receptor is inactive at VPAC2 receptor (Fig. 7). This makes [Ala11,22,28]VIP the most highly selective human VPAC1 receptor agonist derived from VIP ever described.

**DISCUSSION**

Although several studies have long been reported regarding the pharmacology of analogs of VIP and related peptides (40–43), no systematic evaluation of the functional and structural role of every residue of VIP has been yet performed since the cloning of the two VIP receptor subtypes VPAC1 and VPAC2 (44). Moreover most previous studies were performed using VIP receptors from rodents although it is clear that there are important differences between species in the pharmacology of VIP receptors (8, 15). In the present study, we have synthesized a total of 28 single alanine or glycine mutants of VIP and have analyzed both their predicted three-dimensional structure by molecular modeling and their biological properties by binding assay and adenylyl cyclase assay in CHO cell clones expressing recombinant human VPAC1 or VPAC2 receptor. Our data provides new information: (i) they delineate key amino acid residues of VIP which play a role in biological activity for interacting with human VIP receptor subtypes and/or contribute to the three-dimensional structure of the peptide; (ii) they point to the role of specific amino acid residues in VIP for discriminating VPAC1 and VPAC2 receptors; (iii) they provide a rationale for developing a new highly specific agonist of the human VPAC1 receptor by combining three single mutations at key positions of VIP. This new information represents a significant advance over the current knowledge on this widespread neuropeptide.

Analysis of the interaction of the 28 single alanine or glycine mutants of VIP indicates that 14 residues out of 28 in native VIP could not be changed without a significant decrease in affinity for binding to human VPAC1 receptor. These important residues are distributed along the peptide chain from position 1 to 26. This distribution is in good agreement with previous observations indicating that VIP fragments obtained by deletion of the N-terminal, central, or C-terminal domains of VIP retained very low affinity, if any, for VIP receptors (21). In a previous Ala scan of the VIP derivative Ro 23-7059 the binding activity of analogs had been tested in guinea pig or human lung membranes and emphasized the importance of only 6 positions including Asp3, Phe6, Thr7, Tyr10, Tyr22, and Leu23 (22). These data are somewhat different from ours since we have found many other important positions and identified Tyr22 as an unimportant position, at least for the interaction of VIP with VPAC1 receptor. The reasons for these discrepancies probably include several factors: (i) a VIP derivative was used, i.e. Ac-
Biological activity of VIP analogs in cells stably expressing human recombinant VPAC₂ receptor

Table II

| Amino Acid | Position | Substitution | Binding, K_i (nM) | Potency (nM) | Adenylyl cyclase, EC₅₀ (nM) | Potency (a) |
|------------|----------|--------------|-------------------|--------------|-----------------------------|-------------|
| His        | 1        | Ala          | 350 ± 108         | 0.005        | 507 ± 135                   | 0.001       |
| Ser        | 2        | Ala          | 4.2 ± 0.4         | 0.40         | 11 ± 2                      | 0.05        |
| Asp        | 3        | Ala          | 183 ± 40.3        | 0.009        | 32.2 ± 7.2                  | 0.02        |
| Ala        | 4        | Gly          | 2.0 ± 0.4         | 0.85         | 2.5 ± 0.6                   | 0.20        |
| Val        | 5        | Ala          | 22 ± 2.5          | 0.08         | 2.8 ± 0.6                   | 0.20        |
| Phe        | 6        | Ala          | 2233 ± 556        | 0.0008       | 247 ± 18                    | 0.0002      |
| Thr        | 7        | Ala          | 633 ± 62          | 0.003        | 17 ± 2                      | 0.03        |
| Asp        | 8        | Ala          | 508 ± 136         | 0.003        | 138 ± 22                    | 0.004       |
| Asn        | 9        | Ala          | 1.5 ± 0.8         | 1.13         | 0.2 ± 0.1                   | 2.50        |
| Tyr        | 10       | Ala          | 983 ± 24          | 0.002        | 46 ± 9                      | 0.01        |
| Thr        | 11       | Ala          | 55 ± 11           | 0.03         | 2.1 ± 0.8                   | 0.24        |
| Arg        | 12       | Ala          | 230 ± 34          | 0.007        | 36 ± 4                      | 0.01        |
| Leu        | 13       | Ala          | 22 ± 6            | 0.08         | 1.2 ± 0.3                   | 0.42        |
| Arg        | 14       | Ala          | 95 ± 22.7         | 0.02         | 28.6 ± 4.0                  | 0.02        |
| Lys        | 15       | Ala          | 29 ± 3            | 0.06         | 5.6 ± 0.1                   | 0.09        |
| Gln        | 16       | Ala          | 3.5 ± 0.5         | 0.49         | 0.3 ± 0.1                   | 1.67        |
| Met        | 17       | Ala          | 3.4 ± 0.1         | 0.50         | 1.3 ± 0.5                   | 0.38        |
| Ala        | 18       | Gly          | 16 ± 5            | 0.11         | 2.9 ± 1.2                   | 0.17        |
| Val        | 19       | Ala          | 1.5 ± 0.6         | 1.13         | 0.3 ± 0.1                   | 1.67        |
| Lys        | 20       | Ala          | 29 ± 8            | 0.06         | 8.1 ± 0.7                   | 0.06        |
| Lys        | 21       | Ala          | 110 ± 8           | 0.02         | 11 ± 1                      | 0.05        |
| Tyr        | 22       | Ala          | 11 ± 1            | 0.15         | 163 ± 62                    | 0.003       |
| Leu        | 23       | Ala          | 2167 ± 624        | 0.0008       | 89 ± 3                      | 0.006       |
| Asn        | 24       | Ala          | 6.6 ± 3.4         | 0.26         | 6.6 ± 3.2                   | 0.08        |
| Ser        | 25       | Ala          | 3.8 ± 0.5         | 0.45         | 0.3 ± 0.1                   | 1.67        |
| Ile        | 26       | Ala          | 44 ± 13           | 0.04         | 14 ± 3                      | 0.04        |
| Leu        | 27       | Ala          | 6.2 ± 2.0         | 0.27         | 2.8 ± 0.3                   | 0.18        |
| Asn        | 28       | Ala          | 22 ± 8            | 0.08         | 4.8 ± 1.2                   | 0.10        |
| Thr/Asn    | 11/28    | Ala          | 76 ± 9            | 0.02         | 23 ± 3                      | 0.02        |
| Thr/Tyr/Asn| 11/22/28 | Ala          | 2352 ± 53         | 0.0007       | 1222 ± 225                  | 0.0004      |
| Native VIP |          |              | 1.7 ± 0.6         | 1.00         | 0.5 ± 0.1                   | 1.00        |

(a) Potency is relative to native VIP (1.00).

Fig. 6. Correlation between the EC₅₀ values and Kᵢ values determined for native VIP and all single-substituted analogs of VIP in the adenylyl cyclase assay and binding assay in CHO cell clone expressing human VPAC₁ receptor. All analogs are numbered according to the position of the amino acid that was substituted. Wt, native VIP. See Table II and the legend to Fig. 5 for details. Note the Y22A mutant for which the EC₅₀ for stimulating adenylyl cyclase activity is much higher than its Kᵢ for binding.

Important residues in VIP for interacting with human VPAC₁ receptor, are strictly conserved or replaced by homologous residues in VIP-related peptides which behave as VIP agonists. This suggests that these residues may play a similar role in allowing the binding of VIP, PACAP, peptide histidine methionine amide, GRF, and secretin to human VPAC₁ receptor but...
Fig. 7. VIP analogs discriminating between human VPAC<sub>1</sub> and VPAC<sub>2</sub> receptors. Top, dose effects of native VIP and two VIP analogs for inhibition of [125I]VIP binding to membranes from CHO cells expressing VPAC<sub>1</sub>(A) or VPAC<sub>2</sub>(B) receptor. Bottom, stimulation of adenylyl cyclase activity by native VIP and two VIP analogs in membranes from CHO cells expressing VPAC<sub>1</sub>(C) or VPAC<sub>2</sub>(D) receptor. The data are expressed as the percentage of maximal stimulation above basal obtained with 10 μM native VIP. All data are mean ± S.E. of at least three experiments performed in duplicate. ●, native VIP; □,[Ala<sup>11,22,28</sup>]VIP analog; ▲,[Ala<sup>11,22,28</sup>]VIP analog.

altogether are not sufficient to ensure high affinity binding. To further substantiate this issue, it is worth pointing out that (i) we could not find well conserved amino acid residues in VIP-related peptides which do not play a role in the activity of VIP; (ii) conversely, we did identify nonconserved residues which are important for VIP activity at the human VPAC<sub>1</sub> receptor.

At this stage of the Ala scan study of VIP and more generally of other similar studies with any bioactive peptide, the question arises of whether one given residue is important because it directly interacts with the receptor and/or it plays a role in maintaining the three-dimensional structure of the peptide, an issue which is not often addressed. In the present study, the conformations of native VIP and VIP analogs were calculated by molecular modeling using an ab initio approach (31). When this approach was applied to native VIP, the predicted three-dimensional structure of VIP was consistent with a central α-helical domain from Val<sup>β</sup> to Ass<sup>24</sup> and conformational variability essentially located at the N- and C-terminal domains (see Fig. 3). This is in good agreement with previous experimental studies of the VIP structure in methanol/water solutions using nuclear magnetic resonance and circular dichroism which also indicated that the structure of VIP is mostly helical (48) with the existence of a central well defined α-helix, the remaining residues being not ordered (49). Similarly, PACAP38 was shown to have a disordered N-terminal domain followed by a central α-helical structure (50). Interestingly, helodermin, a peptide isolated from the lizard Gila Monster (51), which is highly homologous to VIP (51) and behaves as a VIP agonist (52), has also been shown to exhibit a central α-helix with random coiled N and C terminus (53). With respect to three-dimensional structure, VIP analogs in which amino acid substitution into alanine was associated with alteration of binding affinity and biological potency could be classified into two categories: (i) analogs which exhibited significant change in predicted peptide structure as compared with native VIP. This includes D8A, F6A, T7A, D8A, Y10A, R12A, and K20A mutants. For these analogs, it can be suggested that altered activity is related to altered structure, although we cannot strictly rule out the possibility that the corresponding residues may directly participate in the interaction of VIP with the VPAC<sub>1</sub> receptor; (ii) analogs which exhibited no change in predicted peptide structure as compared with native VIP. This includes H1A, V5A, R14A, K15A, K21A, L23A, and I26A mutants. It is tempting to speculate that the corresponding amino acid residues in native VIP (see Fig. 8) participate in the interaction of the neuropeptide with VPAC<sub>1</sub> receptor. This is in line with other observations: (i) His<sup>1</sup> in VIP (22) as well as His<sup>1</sup> in some VIP-related peptides (54–56) is known to play an important role in biological activity; (ii) the fact that half of the residues falling into the category of candidates for direct interaction with VPAC<sub>1</sub> receptors are basic residues including Arg<sup>14</sup>, Lys<sup>15</sup>, and Lys<sup>21</sup>, is probably significant in view of the fact that site-directed mutagenesis of the human VPAC<sub>1</sub> receptor previously identified several important acidic residues in the receptor for VIP binding, including Glu<sup>36</sup> (16), Asp<sup>68</sup> (12), and Asp<sup>258</sup> (57). This supports the idea that the very basic VIP with an isoelectric point >11 (6) may use some of its basic residues for an electrostatic interaction with acidic residues of the receptor; (iii) finally, the remaining important residues of VIP including Val<sup>8</sup>, Leu<sup>23</sup>, and Ile<sup>26</sup> are highly hydrophobic in consonance with the old idea that VIP, or at least a binding region of VIP, has an hydrophobic environment within the receptor (58). Recent observations support the idea that important residues in the VPAC<sub>1</sub> receptor for binding VIP are indeed hydrophobic including Trp<sup>67</sup>, Trp<sup>70</sup>, and Trp<sup>110</sup> (12).<sup>2</sup> Finally, it is worth pointing out that all analogs which have the same affinity as native VIP for VPAC<sub>1</sub> receptor exhibit no alteration of predicted three-dimensional structure, further validating our molecular modeling approach.

The data obtained from the analysis of the interaction of the 28 single alanine or glycine mutants of VIP with human VPAC<sub>1</sub> receptor are very similar to those obtained with VPAC<sub>2</sub> receptors with some specific differences. The great similarities indicate that VIP binding to VPAC<sub>1</sub> and VPAC<sub>2</sub> receptor is mostly ensured by a common set of crucial amino acids along the VIP peptide sequence. However, two important differences can be noted: (i) some VIP analogs exhibit a much lower binding affinity for the VPAC<sub>2</sub> than for the VPAC<sub>1</sub> receptor, including the D8A, Y10A, and L23A mutants. Two other analogs have no alteration of binding to VPAC<sub>1</sub> receptor whereas they exhibit a significant decrease of affinity for VPAC<sub>2</sub> receptor, <i>i.e.</i> T11A and N28A analogs (see Fig. 5 and Table II). When these two modifications are combined in one single analog,

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the resulting peptide behaves as a VIP agonist which clearly discriminates between human VPAC1 and VPAC2 receptors (see Fig. 7). (ii) The Y22A VIP analog exhibits an increased EC50 for stimulating adenyl cyclase activity through interaction with the human VPAC2 receptor whereas no change was observed at the VPAC1 receptor (see Tables I and II). Altogether, these data indicate that single amino acid substitutions in VIP either alter similarly peptide interaction with the two receptors or elicit a rather weak affinity for the human VPAC1 receptor (see Table I and II). Moreover, it has a rather weak affinity for the human VPAC1 receptor (60), in line with the important differences between species in the pharmacology of VPAC1 receptors (15, 44). The chimeric, substituted peptide [Lys15, Arg16, Leu27]VIP (1–7)/GRF (8–27) has an important selectivity for VPAC1 receptor but its possible interaction with GRP receptors was not directly evaluated (61). In this context, the highly selective human VPAC1 receptor agonist developed in this study by rationale combination of three mutations in VIP itself constitutes henceforth the most operative pharmacological tool derived from VIP for characterizing VPAC1 receptor-mediated events.

In conclusion, the present activity data on the series of VIP analogs in combination with molecular modeling provide the first characterization of the functional properties of the VIP molecule for interaction with the two human VIP receptor subtypes. Key residues for interaction of VIP with human VPAC1 or VPAC2 receptor are highlighted in the ribbon representation of the VIP molecule (Fig. 8). In view of considerable interest of this widespread neuropeptide in health and diseases, this work already provides useful pharmacological tools and paves the way for further development in the design of new analogs and mimetic versions of VIP.

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Total Alanine Scanning of Vasoactive Intestinal Peptide