Creation of a Selective Antagonist and Agonist of the Rat VPAC$_1$ Receptor Using a Combinatorial Approach with Vasoactive Intestinal Peptide 6–23 as Template

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This paper is available online at http://www.molpharm.org

ABSTRACT

We have used combinatorial chemistry with amino acid mixtures (X) at positions 6 to 23 in vasoactive intestinal peptide (VIP) to optimize binding affinity and selectivity to the rat VPAC$_1$ receptor. The most efficient amino acid replacement was a substitution of alanine at position 18 to diphenylalanine (Dip), increasing the displacement efficiency of ^{125}I-VIP by 370-fold. The [Dip^{18}]VIP(6–23) was subsequently used to find a second replacement, employing the same approach. Tyrosine at position 9 was selected and the resulting [Tyr^9,Dip^{18}]VIP(6–23) analog has a K_i value of 90 nM. This analog was unable to stimulate cAMP production at 10^{-6} M but was able to inhibit VIP-induced cAMP stimulation (K_b = 79 nM). The K_i values of [Tyr^9,Dip^{18}]VIP(6–23) using the rat VPAC$_2$ and PAC$_1$ receptors were 3,000 nM and >10,000 nM, respectively. Thus, [Tyr^9,Dip^{18}]VIP(6–23) is a selective VPAC$_1$ receptor antagonist. The C-terminally extended form, [Tyr^9,Dip^{18}]VIP(6–28), displays improved antagonistic properties having a K_i and K_b values of 18 nM and 16 nM, respectively. On the contrary, the fully extended form, [Tyr^9,Dip^{18}]VIP(1–28), was a potent agonist with improved binding affinity (K_i = 0.11 nM) and ability to stimulate cAMP (EC$_{50}$ = 0.23 nM) compared with VIP (K_i = 1.7 nM, EC$_{50}$ = 1.12 nM). Furthermore, the specificity of this agonist to the VPAC$_1$ receptor was high, the K_i values for the VPAC$_2$ and PAC$_1$ receptors were 53 nM and 3,100 nM, respectively. Seven other analogs with the [Tyr^9,Dip^{18}] replacement combined with previously published VIP modifications have been synthesized and described in this work.

Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are neuropeptides with widespread distribution in both the central and the peripheral nervous systems. Besides being a central neurotransmitter, VIP is involved in the nervous control of vascular and nonvascular smooth-muscle activity and endocrine and exocrine secretion (Fahrenkrug, 1993). In addition to a neurotransmitter function, PACAP has been shown to be a neurotrophic factor that plays a role during the development of the brain (Arimura, 1998). These peptides act through three distinct receptors: the PAC$_1$, VPAC$_1$, and VPAC$_2$ receptors. The PAC$_1$ receptor possesses a high-affinity binding site for PACAP, whereas the VPAC$_1$ and VPAC$_2$ receptors show high-affinity binding for both VIP and PACAP. These receptors belong to the secretin receptor family (Tams et al. 1998) that constitutes a subfamily of G protein-coupled receptors, each having seven transmembrane helices.

To investigate the physiological roles of the three different receptors, selective agonists and antagonists are required. All VIP or PACAP antagonists have modifications in the N-terminal part of the peptide, suggesting that the N-terminal part of VIP is responsible for the activation of the receptor. The N-terminal truncated form of PACAP, PACAP 6–38, is an antagonist for the VPAC$_2$ and PAC$_1$ receptors (Robberecht et al., 1992; Dickinson et al., 1997). [Ac-His^1,D-Phe^2,Lys^{15},Arg^{16},Leu^{22}]VIP(3–7)/growth hormone-releasing factor (GRF)(8–27) has been described as the most potent and selective VPAC$_1$ antagonist (Gourlet et al., 1997a) with a K_i value of 15 nM for the rat receptor, and [Lys^{15},Arg^{16},Leu^{22}]VIP(1–7)/GRF(8–27) is described as a selective agonist IC$_{50}$ = 1 nM (Gourlet et al., 1997b). These analogs, however, consist mainly of the homologous peptide GRF, and binding to other homologous receptors for peptides in the glucagon/VIP/secretin peptide family could affect the interpretations of physiological studies using these two analogs. Likewise, [Arg^{16}] chicken secretin is reported as a selective VPAC$_1$ receptor agonist (Gourlet et al., 1996b). The

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ABBREVIATIONS: VIP, vasoactive intestinal polypeptide; PACAP, pituitary adenylate cyclase activating polypeptide; GRF, growth hormone-releasing factor; HEK, human embryonic kidney; CHO, Chinese hamster ovary; Nal-2, β-2-naphthyl-L-alanine; Dip, β,β-diphenyl-L-alanine; S(Bzl), O-benzyl-L-serine; Y(Bzl), O-benzyl-L-tyrosine; Cha, β-cyclohexyl-L-alanine.

IC$_{50}$ values are 1 nM, 10,000 nM, and 3,000 nM for the VPAC$_1$, VPAC$_2$, and PAC$_1$ receptors, respectively, but [Arg^{16}] chicken secretin is also a potent agonist for the secretin receptor. As an alternative to the chimeric approach of homologous peptides, we have used a combinatorial approach with VIP as template. Thus, selectivity toward other homologous receptors with low affinity to VIP is initially preserved. In our laboratory, we have predominantly used rat as the model animal for studying the physiological features of VIP and PACAP. Consequently, rat VPAC$_1$ receptor is used as target for the generation of a selective VIP antagonist and agonist.

Bogan and Thorn (1998) examined 2325 alanine mutants at protein interfaces for which the change in free energy of binding has been measured. They concluded that the free energy of binding is not evenly distributed across protein interfaces; instead, there are hot spots of binding energy made up of a small subset of residues in the interface. O'Donnell et al. (1991) made an alanine scan of VIP and found several side chains important for binding and biological responses. These side chains are probably situated at the ligand-receptor interface and minor chemical changes of these residues could result in an optimized binding. However, we have used a different approach. We assumed that it was most advantageous to optimize poor interactions in the VIP-VPAC$_1$ receptor interface and convert these to hot spots instead of trying to optimize a hot spot that already could be near the limited maximal binding energy. Thus, in our combinatorial approach, we have initially used amino acid mixtures with very different chemical properties at positions not important for binding, with the hope that a few of these positions are at the ligand-receptor interface and therefore a target for optimization of the ligand binding and receptor specificity.

Materials and Methods

Peptide Synthesis. Solid-phase peptide synthesis was performed using the 9-fluorenylmethyloxycarbonyl strategy as described in Ploug et al. (1998). TentaGel S RAM (S = 0.25 mmol/g; RAPP Polymere, Tübingen, Germany) was used as resin and the synthesis was carried out either in single vessels or in a multiple-column peptide synthesizer (Holm and Meldal, 1989; Meldal et al., 1993). Particular to these syntheses was the use of extended reaction times or double couplings for assembly of the Lys-Gln-Met-Ala-Val sequence of the peptides. Coupling of amino acid mixtures was generally carried out as described by Pinilla et al. (1992) using 1.1 Eq of amino acids in total and 1.1 Eq coupling reagent for a minimum of 2 h followed by a double coupling of 30 min or longer. Amino acid mixtures of similar chemical structure or properties (motive mixtures) were preferred to ensure near equimolar incorporation of the individual amino acids. Complete removal of the arginine side chain protecting group (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl or 2,2,5,7,8-pentamethylchroman-6-sulfonyl) usually required a longer trifluoroacetic acid treatment than normally employed. All peptides are amidated in the C terminal and VIP truncated peptides are *N*-acetylated. The peptide identity was verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry obtained using a Fisons TofSpec E instrument. The purity of the peptides was assessed by HPLC performed on a Waters 600 E instrument equipped with a Waters 996 Photodiode Array Detector using Waters Radial Pak or Waters Symmetry RP C-18 column. Peptides devoid of X positions were purified by preparative HPLC with crude product purities lower than ~80%. Verification of amino acid composition and concentration determinations of the final aqueous peptide solutions was done by amino acid analysis using Waters PICOTAG system.

VIP and PACAP 1–27 were purchased from Peninsula Laboratories, Inc., (Belmont, CA). Iodination and RP C-18 HPLC purification of ^{125}I-VIP and ^{125}I-PACAP 1–27 were conducted as described for VIP by Martin et al. (1986).

Cell Lines Used for Receptor Characterization. The coding region of rat VPAC2 or rat PAC1 receptors [obtained from Dr. Anthony Harmar (Lutz et al., 1993) and Dr. Stephen Wank (Wank and Pisegna, 1993), respectively] was subcloned into pcDNA3 from Invitrogen (Leek, The Netherlands). Human embryonic kidney (HEK) 293 cells were transfected transiently by the calcium phosphate precipitation technique (Gorman, 1988). Cells were plated into a 200-mm diameter culture dish (4×10^6 cells/dish). Ten micrograms of receptor cDNA was used for transfection and the cells were harvested 72 h later or seeded to 24-well culture dishes 48 h later. Transfected cells were grown in Eagle's minimum essential medium (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 10% fetal calf serum (Biological Industries) and 0.1% gentamicin. Stable transfection of the rat VPAC$_1$ receptor in CHO cells was as described by Wulff et al. (1997).

General DNA manipulations were performed as described by Sambrook et al. (1989). DNA fragments required for subcloning experiments were gel-purified using Geneclean kit (BIO 101, Inc., KEBO lab, Albertslund, Denmark). Restriction enzymes were purchased from Amersham (Birkerød, Denmark).

Membrane Preparations from CHO and HEK293 Cells. Confluent monolayers of transfected or wild-type CHO and HEK293 cells were washed with 0.1 M PBS and detached from their plastic support using a cell scraper. The cells, solubilized in 20 ml of 25 mM HEPES, 2.5 mM CaCl$_2$, 1.0 mM MgCl$_2$, 50 mg/l bacitracin, pH 7.4 per plate, were disrupted using a Polytron (Ultra-Turrax T25, Janke & Kunkel GMBH, Bie & Berntsen, Rødovre, Denmark) for 30 sec. The homogenate was spun for 20 min at 30,000g at 4°C. The resulting pellet was resuspended in 15 ml of 25 mM HEPES, 2.5 mM CaCl$_2$, 1.0 mM MgCl$_2$, 50 mg/l bacitracin, pH 7.4 per plate. The preparation was aliquoted and stored at -80°C.

Binding Assay. Ten micrograms of membrane protein was incubated at room temperature, for 90 min, in a total volume of 0.15 ml containing 24 mM HEPES, pH 7.4, 2.5 mM CaCl$_2$, 3.0 mM MgCl$_2$, 100 mM NaCl, 0.5 g/l bacitracin, 15 pM ^{125}I-VIP or ^{125}I-PACAP 1–27, and increasing concentrations of unlabeled peptide. Nonspecific binding was determined in the presence of 1 μM VIP. The separation of membrane bound and free radioactivities was achieved by centrifugation at 20,000g for 5 min. The apparent IC$_{50}$ value was estimated from a sigmoid dose-response equation, $Y = \text{Top} + (\text{Bottom} - \text{Top}) / [1 + (X / IC_{50})^P]$, where X is the concentration, Y is the response, and P is the slope factor. The affinity constant (K_i) of the nonlabeled ligand is then calculated using the formula of Cheng and Prusoff (1973): $K_i = IC_{50} / [1 + (D / K_d)]$, where D is the concentration of the labeled ligand and K_d its affinity constant. Iodination, HPLC purification, and binding curves of nonradioactive I-VIP have been made. The K_d value for I-VIP was determined to be 0.42 $\pm$ 0.05 nM and 1.1 $\pm$ 0.3 nM for rVPAC$_1$ and rVPAC$_2$ receptors, respectively; finally, the K_d value for I-PACAP binding to rPAC$_1$ was determined to be 2.9 $\pm$ 0.8 nM.

Intracellular cAMP Assay. CHO or HEK293 cells were seeded at 2×10^5 cells/well into 24-well culture dishes and incubated for 24 h at 37°C with Roswell Park Memorial Institute medium (Biological Industries), 10% fetal calf serum, and 0.2 M L-glutamine. To the CHO cells expressing the VPAC$_1$ receptor, an extra 0.8 mg/ml G418 (geneticin, Life Technologies, Gaithersburg, MD) was added. After two washes with Dulbecco's modified Eagle's medium (Biological Industries), 0.5% newborn calf serum (Biological Industries), and 0.2 M L-glutamine, the cells were incubated with 500 μl of Dulbecco's modified Eagle's medium, 0.5% newborn calf serum, 0.2 M L-glutamine, 0.1 mM 3-isobutyl-1-methylxanthine (Boehringer Ingelheim

Bioproducts Partnership, Ingleheim, Germany), 20 μM H89 (protein kinase inhibitor; Calbiochem, San Diego, CA) for 10 min and for a further 20 min at room temperature with ligand in increasing concentrations (0–10^{-6} M). cAMP was extracted by incubating the cells with 100 μl of 20 mM HCl and neutralized by 2 μl of 1 M sodium acetate. The amount of cAMP produced by the cells was quantified using a cAMP radioimmunoassay kit from Amersham (Paisley, UK). The apparent EC$_{50}$ was estimated from a sigmoid dose-response equation as detailed under *Binding Assay*. The antagonistic properties of the analogs have been determined using the Gaddum equation (Gaddum, 1957): $K_b = [B]/(\text{"dose ratio"} - 1)$, where K_b is the estimate of the binding constant of the antagonist and [B] is the concentration of the antagonist. The "dose ratio" is here determined as the ratio of EC$_{50}$ in the presence of 300 nM antagonist divided by the EC$_{50}$ value without antagonist.

Results

Selection of Template and X Positions. We were interested in a template having a reduced length, which 1) simplifies peptide synthesis and 2) increases the potential bioavailability of an antagonist. Initially, several truncated VIP analogs were tested for the ability to displace ^{125}I-VIP on the rat VPAC$_1$ receptor. The truncated VIP(6–23) was chosen because we expected that removal of the first five residues would convert the peptide to an antagonist (see under *Discussion*). The five residues at the C terminal are of less importance for VIP binding to the VPAC$_1$ receptor and they were therefore omitted to reduce the size of the template. Furthermore, this template has a reasonable ratio of peptide size versus binding affinity ($K_i = 110 \pm 30 \mu$M) to the rat VPAC$_1$ receptor.

The residues important for VIP binding in the 6–23 part of VIP are residues F6, T7, Y10, R14, Y22, and L23 (O'Donnell et al., 1991). These residues were preserved in our 6–23 VIP template together with R12, K15, K20, and K21, which were preserved to ensure high solubility of the peptide. The VIP-VPAC$_1$ receptor interface is not known, but the remaining residues are presumably either 1) poorly fitted residues at the interface or 2) exposed to the solvent. Thus, the positions selected for scanning with a mixture of amino acids (X) were D8, N9, T11, L13, Q16, M17, A18, and V19.

Selection of the First Amino Acid Replacement. A broad amino acid mixture containing various side-chain sizes and hydrophilicity was used initially and the results of the displacement of ^{125}I-VIP in a competitive binding assay are shown in Fig. 1, top. The X replacement at positions 9, 16, and 18 had the most pronounced effect, and four different motive amino acid mixtures, Xa, Xb, Xc, and Xd, with fewer, similar amino acids were used in the second scan at these three positions (Fig. 1, middle). Amino acid mixture Xc was most efficient at all three positions, especially at position 18, where the relative ^{125}I-VIP binding was decreased to 30%. Each of the four different amino acids in the Xc mixture [W, β-benzothienyl-L-alanine, β-2-naphthyl-L-alanine (Nal-2), and β,β-diphenyl-L-alanine (Dip)] was incorporated in positions N9, Q16, and A18 (Fig. 1, bottom), and the A18Dip replacement had a large effect on the displacement of ^{125}I-VIP. The following combinations at positions 9, 16, and 18 were made: [Trp^9,BzThi^{16},Dip^{18}]VIP(6–23), [BzThi^9,BzThi^{16},Dip^{18}]VIP(6–23), [Trp^9,Nal^{16},Dip^{18}]VIP(6–23), [BzThi^9,Nal^{16},Dip^{18}]VIP(6–23) but these triple substitutions all had approximately the same ^{125}I-VIP displacing efficiency as [Dip^{18}]VIP(6–23) (results not shown). Thus, no

additive effects were observed by combination of these residues.

Selection of the Second Amino Acid Replacement. The VIP analog [Dip^{18}]VIP(6–23) was used as second template and a new scan with amino acid mixtures was done at positions 9 and 16 (Fig. 2). The following motive amino acid mixtures were tested: mixture with small amino acids (Xe), polar amino acids (Xf), flexible amino acids (Xg), and hydrophobic amino acids (Xh). The substitution with hydrophobic amino acids at position 9 had a pronounced effect on the ^{125}I-VIP displacement efficiency, but none of the selected amino acid mixtures substituted at position 16 were productive (Fig. 2, top). Several hydrophobic amino acids were substituted at position 9 (Fig. 2, bottom) and the substitution with Tyr, Cha, S(Bzl), and Y(Bzl) were most efficient. The marked difference in displacement efficiency between N9Y and N9F indicates that the hydroxyl group of tyrosine is important for the binding efficiency. We expect that hydro-
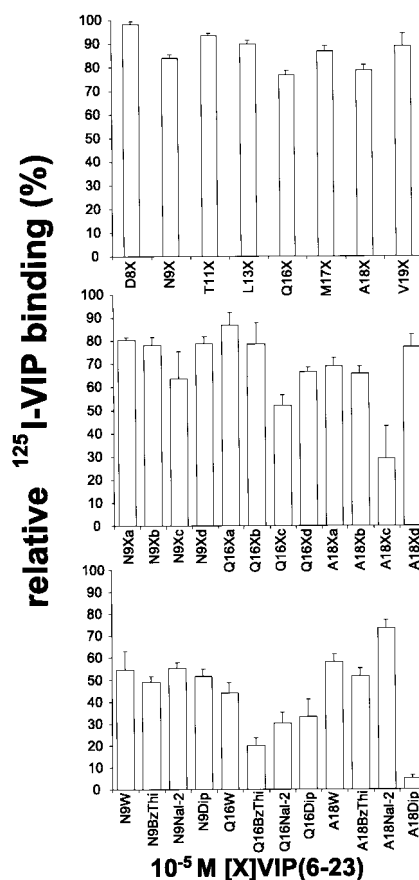
Fig. 1. ^{125}I-VIP displacement efficiency of analogs with amino acid mixtures X or single amino acid substitutions at different positions in VIP(6–23). The effect is measured as relative ^{125}I-VIP binding compared with ^{125}I-VIP binding without analog using membranes of CHO cells expressing the rat VPAC$_1$ receptor. Top, a broad amino acid mixture X [O-benzyl-L-serine (S(Bzl)), O-benzyl-L-tyrosine (Y(Bzl)), O-benzyl-L-*trans*-4-hydroxyproline (Hyp(Bzl)), β-cyclohexyl-L-alanine (Cha), Nal-2, A, E, F, K, L, P, Q, S, W, and Y] is introduced at positions D8, N9, T11, L13, Q16, M17, A18, and V19. Middle, a narrow motive amino acid mixture Xa (A, E, K, L, Q, S), Xb [S(Bzl), Y(Bzl), O-benzyl-L-*trans*-4-hydroxyproline (Hyp(Bzl)), P], Xc [W, β-benzothienyl-L-alanine (BzThi), Nal-2, Dip], or Xd (F, Y, Cha, β-2-thienyl-L-alanine, β-2-thiazolyl-L-alanine) is introduced at positions N9, Q16, and A18. Bottom, single amino acid substitutions are introduced at positions N9, Q16, and A18. The results are expressed in percentage of tracer bound in absence of unlabeled peptide and the mean and S.E.M. values of four determinations are shown.

philic interactions, in general, are more important for specificity of ligand binding compared with hydrophobic interactions. We therefore selected [Tyr^9,Dip^{18}]VIP(6–23) as third template for further modifications of the VIP analog.

Properties of the Modified VIP(6–23) Analog and the C- and N-Terminal Extended Forms. Fig. 3 shows the binding curves of VIP(6–23) and VIP(6–23) analogs with the first [Dip^{18}] and second [Tyr^9,Dip^{18}] selected substitutions. The substitution with Dip at position 18 shows more than 300-fold improvement of the binding affinity, whereas the second substitution was only 3-fold. Further extension of five amino acids at the C-terminal [Tyr^9,Dip^{18}]VIP(6–28) improves the binding affinity by 5-fold and a pronounced increase is seen for the fully extended VIP analog [Tyr^9,Dip^{18}]VIP(1–28), displaying a 16-fold better binding affinity compared with VIP. As shown in Table 1, VIP has approximately the same binding affinity to the VPAC$_1$ and VPAC$_2$ receptors. The VIP analogs, however, have an improved selectivity to VPAC$_1$ compared with the VPAC$_2$ receptor and the $K_{i, VPAC2}/K_{i, VPAC1}$ ratios are 30, 5, and 480 for [Tyr^9,Dip^{18}]VIP(6–23), [Tyr^9,Dip^{18}]VIP(6–28), and [Tyr^9,Dip^{18}]VIP(1–28), respectively. The PAC$_1$ receptor has, as expected, a low affinity to VIP analogs and the $K_{i, PAC1}/K_{i, VPAC1}$ ratios are >100, 300, and 27,000 for [Tyr^9,Dip^{18}]VIP(6–23), [Tyr^9,Dip^{18}]VIP(6–28), and [Tyr^9,Dip^{18}]VIP(1–28), respectively. Table 2 shows that [Tyr^9,Dip^{18}]VIP(6–23) and

[Tyr^9,Dip^{18}]VIP(6–28) are unable to activate the receptor at 1 μM but are able to inhibit the VIP-induced cAMP production with K_b values of 79 ± 7 nM and 16 ± 1 nM, respectively (Table 2). In contrast to the analogs with a truncated N terminal, the fully extended form [Tyr^9,Dip^{18}]VIP(1–28) is 5-fold more potent in stimulating cAMP production (EC$_{50}$ = 0.23 ± 0.01 nM) compared with VIP (EC$_{50}$ = 1.12 ± 0.06 nM) as seen in Fig. 4. The cAMP response data using HEK293 cells transfected with rat VPAC$_2$ or rat PAC$_1$ receptor cDNA are shown in Fig. 5. The VIP-stimulated dose-response curve using VPAC$_2$ receptor-transfected cells has an EC$_{50}$ value of 0.74 ± 0.09 nM. [Tyr^9,Dip^{18}]VIP(1–28) was 100-fold less potent, resulting in an EC$_{50}$ value of 74 ± 5 nM. PACAP 1–27 stimulation on the PAC$_1$ receptor has an EC$_{50}$ value of 0.43 ± 0.01 nM, whereas the EC$_{50}$ value for [Tyr^9,Dip^{18}]VIP(1–28) is > 1 μM at the same receptor. VIP had a slight stimulating effect on untransfected HEK293 cells (EC$_{50}$ = 0.3 ± 0.1 nM), but the maximal stimulation at 10^{-6} M VIP was only 5% of the cAMP response compared with the VPAC$_2$ receptor transfected cells. Binding studies on membranes from HEK293 cells transfected with human VPAC$_1$ cDNA disclosed that VIP and [Tyr^9,Dip^{18}]VIP(1–28) have similar IC$_{50}$ values (data not shown).

Features of the [Tyr^9,Dip^{18}]VIP(6–23) Analog Combined with Previous Reported VIP Modifications. Amino acid substitutions on VIP with rabbit secretin amino acids, [Arg^{16}]VIP and [Leu^{22}]VIP, have been made by Gourlet et al. (1996b, 1998). The [Tyr^9,Arg^{16},Dip^{18}]VIP(6–23) analog shows improved binding to all three receptors (the VPAC$_1$, VPAC$_2$, and PAC$_1$ receptors) by 2.3-, 3.2-, and >2.6-fold, respectively (Table 1) and a minor improvement of the inhibitory properties was seen for the VPAC$_1$ receptor compared with [Tyr^9,Dip^{18}]VIP(6–23) (Table 2). In contrast, the [Tyr^9,Dip^{18},Leu^{22}]VIP(6–23) has a 4.3-fold lower affinity for the rat VPAC$_1$ receptor and a correspondingly high K_b value. The selectivity to the rat VPAC$_2$ receptor was not improved compared with [Tyr^9,Dip^{18}]VIP(6–23). The PACAP(29–38)
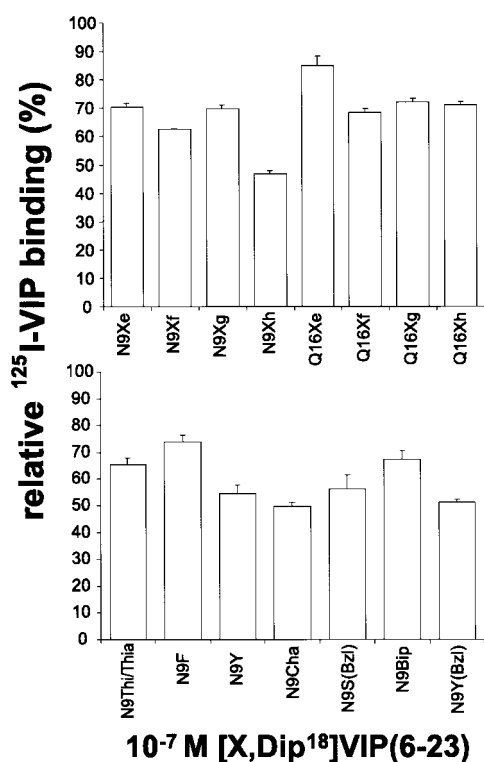
Fig. 2. ^{125}I-VIP displacement efficiency of analogs with amino acid mixtures X or single amino acid substitutions at different positions in [Dip^{18}]VIP(6–23). The effect is measured as relative ^{125}I-VIP binding compared with ^{125}I-VIP binding without analog using membranes of CHO cells expressing the rat VPAC$_1$ receptor. Top, four different motive amino acid mixtures are introduced at positions N9 and Q16: Xe [G, aminoglycine (Gly(NH$_2$)), α-aminoisobutyric acid (Aib), Val], Xf (D, R, H, S), Xg [K, norleucine (Nle), β-Ala, M], and Xh [phenylglycine (Phg), S(Bzl), Cha, β-2-thienyl-L-alanine (Thi)]. Bottom, single-amino-acid substitutions are introduced at positions N9. The results are expressed in percentage of tracer bound in absence of unlabeled peptide. The mean and S.E.M. values of four determinations are shown. Bip, β-biphenyl-L-alanine.
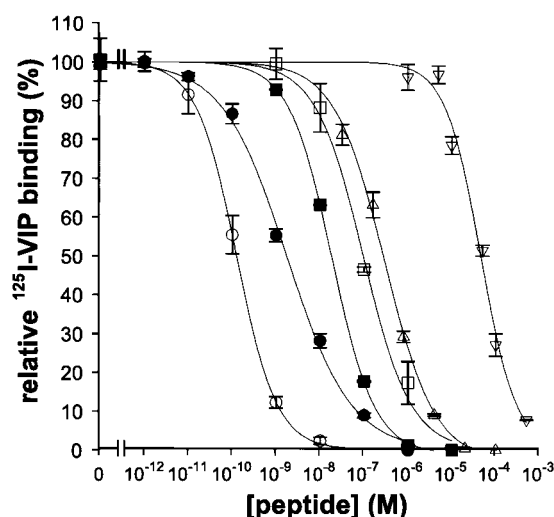
Fig. 3. Competition with ^{125}I-VIP for binding to membrane preparations of CHO cells expressing the rat VPAC$_1$ receptor. The competitors were VIP (●), VIP(6–23) (▽), [Dip^{18}]VIP(6–23) (△), [Tyr^9,Dip^{18}]VIP(6–23) (□), [Tyr^9,Dip^{18}]VIP(6–28) (■), and [Tyr^9,Dip^{18}]VIP(1–28) (○). The results are expressed in percentage of tracer bound in absence of unlabeled peptide. The mean and S.E.M. values of six determinations are shown. Nonspecific binding (<1% of total count of the tracer) was determined in the presence of 1 μM unlabeled peptide.

extension, [Tyr^9,Dip^{18}]VIP(6–28)/(29–38)PACAP, improves the binding by 1.5-, 14-, and 250-fold for the VPAC$_1$, VPAC$_2$, and PAC$_1$ receptors, respectively (Table 1), but the K_b values were 10-, 9-, and 4-fold higher compared with the K_i values, respectively (Table 2). Two unnatural amino acids used previously in VIP, 4Cl-D-Phe^6 and D-Phe^2 (Pandol et al., 1986; Gourlet et al., 1997a), were also tested at the rat VPAC$_1$ receptor. As shown in Table 1, the K_i values of [4Cl-D-Phe^6,Tyr^9,Dip^{18}]VIP(1–28), [4Cl-D-Phe^6,Tyr^9,Leu^{17},Dip^{18}]VIP(1–28), and [Ac-His^1,D-Phe^2,Tyr^9,Dip^{18}]VIP(1–28) are 340-, 490-, and 100-fold higher compared with [Tyr^9,Dip^{18}]VIP(1–28) and all are partial agonists, having apparent EC$_{50}$ values of 80 ± 30 nM, 44 ± 4 nM, and 90 ± 9 nM, respectively (Fig. 4). The neurotensin/VIP chimera (Gozes et al., 1989) has also been combined with the [Tyr^9,Dip^{18}] replacement and the resulting peptide, [Lys^1,Pro^2,Arg^3,Arg^4,Pro^5,Tyr^9,Dip^{18}]VIP(6–28), has 2-fold higher K_i (Table 1) and K_b values compared with [Tyr^9,Dip^{18}]VIP(6–28) and has only low agonist properties [1.7 ± 0.3% compared with VIP (Table 2)].

Discussion

We have chosen to use a combinatorial approach with a truncated VIP, 6–23, as a template to optimize the VIP-VPAC$_1$ receptor binding to create a high-affinity, selective antagonist. The use of a truncated VIP as a template has the advantage, compared with other templates, of having initial binding ability and selectivity toward the VPAC receptors. Truncation of the first five residues was expected to convert the template to an antagonist (PACAP 6–38 is an antagonist for the VPAC$_2$ and PAC$_1$ receptors), whereas the final five residues at the C terminal were less important for VIP binding to the VPAC$_1$ receptor and was therefore omitted to reduce the size of the template. All charged residues were preserved to ensure high solubility of the template but also because charged interactions are strong and able to direct the molecule at long distances; the energy of charged interactions is proportional to a factor of 1/radius (r). The energy of other noncovalent interactions are weaker and typically proportional to a factor of $1/r^6$ (Fersht, 1985). Thus, charged residues are important for the first events in the binding process and for the general solubility of a molecule in a biological environment (e.g., the degree of adsorption to other proteins and surfaces as the lipid membrane). The structure of the VIP-VPAC$_1$ receptor interface is not known, but amino acids that affect ligand binding by an alanine substitution (O'Donnell et al., 1991) are candidates to participate in the interaction at the binding interface. A crucial point in our

TABLE 1

Binding properties of the VIP analogs

K_i values (nanomolar ± S.E.M.) for binding of VIP and VIP analogs to rat VPAC$_1$, VPAC$_2$, and PAC$_1$ receptors. The results shown are the mean and S.E.M. values of three to six determinations.

Peptide	K_i		
	VPAC$_1$R[a]	VPAC$_2$R[b]	PAC$_1$R[b]
	nM		
VIP	1.7 ± 0.3	2.1 ± 0.3	nd
VIP(6–23)	110,000 ± 30,000	N.D.	N.D.
[Dip^{18}]VIP(6–23)	290 ± 30	N.D.	N.D.
[Tyr^9,Dip^{18}]VIP(6–23)	90 ± 20	3,000 ± 200	>10,000
[Tyr^9,Dip^{18}]VIP(6–28)	18 ± 2	96 ± 4	6,000 ± 7,000
[Tyr^9,Dip^{18}]VIP(1–28)	0.11 ± 0.01	53 ± 4	3,000 ± 3,000
[Tyr^9,Arg^{16},Dip^{18}]VIP(6–23)	41 ± 5	900 ± 200	3,900 ± 600
[Tyr^9,Dip^{18},Leu^{22}]VIP(6–23)	400 ± 50	3,400 ± 800	>10,000
[Tyr^9,Dip^{18}]VIP(6–28)/PACAP(29–38)	13 ± 1	7 ± 2	23 ± 2
[4Cl-D-Phe^6,Tyr^9,Dip^{18}]VIP(1–28)	37 ± 1	500 ± 6	2,500 ± 700
[4Cl-D-Phe^6,Tyr^9,Leu^{17},Dip^{18}]VIP(1–28)	54 ± 6	400 ± 200	>10,000
[Ac-His^1,D-Phe^2,Tyr^9,Dip^{18}]VIP(1–28)	11 ± 1	220 ± 30	>10,000
[Lys^1,Pro^2,Arg^3,Arg^4,Pro^5,Tyr^9,Dip^{18}]VIP(6–28)	32 ± 1	200 ± 30	910 ± 40

N.D., not determined.

[a] The displacement curves of the first six peptides are shown in Fig. 3. The slope factor (P) of the displacement curves ranged between 0.8 and 1.1 except for VIP and VIP(6–23), which had 0.7 and 1.2, respectively.

[b] The highest peptide concentration used in the displacement curves for VPAC$_2$ and PAC$_1$ receptors was 10^{-5} M. The slope factor was fixed to have a value of 1 for peptides with K_i above 1 μM.

TABLE 2

Antagonistic properties (K_b) of the peptides

Peptides unable to stimulate cAMP at 10^{-6} M peptide (i.e., peptides having less than 2% response compared with 10^{-6} M VIP) were tested for their ability to inhibit the VIP-induced cAMP response. A dose-response curve for VIP with 300 nM peptide was used to evaluate K_b (see under *Materials and Methods*). The results given are the mean and S.E.M. values of three to six determinations. The ability of the peptides to stimulate cAMP at 10^{-6} M is shown in brackets as percentage of the cAMP production of 1 μM VIP.
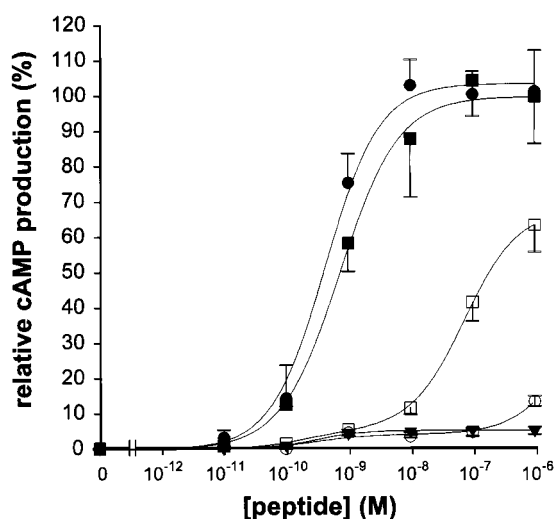
Peptide	K_b		
	VPAC$_1$R	VPAC$_2$R	PAC$_1$
	nM		
[Tyr^9,Dip^{18}]VIP(6–23)	79 ± 7 (0.5%)	>500 (0.0%)	>500 (0.1%)
[Tyr^9,Dip^{18}]VIP(6–28)	16 ± 1 (0.3%)	94 ± 9 (0.0%)	>500 (0.2%)
[Tyr^9,Arg^{16},Dip^{18}]VIP(6–23)	60 ± 5 (0.3%)	N.D.	N.D.
[Tyr^9,Dip^{18},Leu^{22}]VIP(6–23)	>500 (0.4%)	N.D.	N.D.
[Tyr^9,Dip^{18}]VIP(6–28)/PACAP(29–38)	130 ± 60 (0.3%)	61 ± 8	100 ± 50
[Lys^1,Pro^2,Arg^3,Arg^4,Pro^5,Tyr^9,Dip^{18}]VIP(6–28)	30 ± 10 (1.7%)	N.D.	N.D.

N.D., not determined.

strategy of using combinatorial chemistry on VIP was to select positions that were unimportant for binding, but situated at the ligand/receptor interface anyway, and convert these positions to hot spots. The positions selected for combinatorial analysis were D8, N9, T11, L13, Q16, M17, A18, and V19. The first substitution with a broad amino acid mixture showed that the three most effective analogs to displace ^{125}I-VIP were N9X, Q16X, and A18X. The broad X mixture was divided into subsets of similar amino acids and the most efficient motive mixture was divided into single-amino-acid substitutions. The most efficient amino-acid replacement was a substitution of alanine at position 18 to Dip,
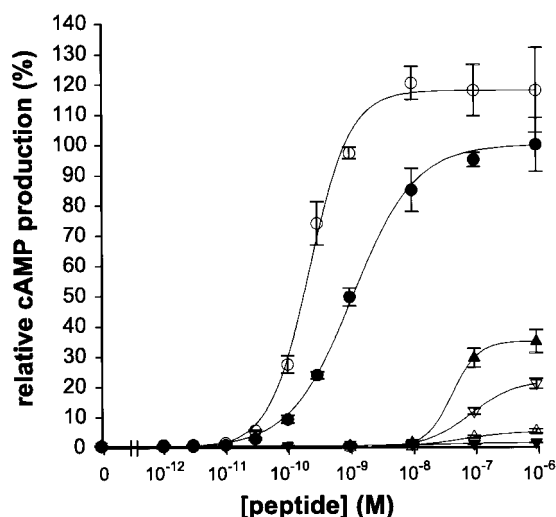
Fig. 4. Dose-effect curves of peptides using CHO cells expressing the rat VPAC$_1$ receptor. The peptides were VIP (●), [Tyr^9,Dip^{18}]VIP(1–28) (○), [4Cl-D-Phe^6,Tyr^9,Dip^{18}]VIP(1–28) (△), [4Cl-D-Phe^6,Tyr^9,Leu^{17},Dip^{18}] VIP(1–28) (▲), [Ac-His^1,D-Phe^2,Tyr^9,Dip^{18}]VIP(1–28) (▽), and untransfected CHO cells stimulated with VIP (▼). The results are the mean and S.E.M. values of four to six experiments and expressed in percentage of cAMP formed by CHO cells expressing the rat VPAC$_1$ receptor stimulated with 10^{-6} M VIP.

Fig. 5. Dose-effect curves of peptides using HEK293 cells transfected with rat VPAC$_2$ or rat PAC$_1$ receptors. The peptides used were VIP (■) and [Tyr^9,Dip^{18}]VIP(1–28) (□) for stimulation of rat VPAC$_2$ transfected cells, PACAP 1–27 (●) and [Tyr^9,Dip^{18}]VIP(1–28) (○) for stimulation of rat PAC$_1$ transfected cells, and finally VIP stimulation of untransfected HEK293 cells (▼). The results are the mean and S.E.M. values of four to six experiments expressed in percentage of cAMP formed by HEK293 cells expressing the rat VPAC$_2$ receptor stimulated with 10^{-6} M VIP.

increasing the displacement efficiency of ^{125}I-VIP by 370-fold (K_i = 290 ± 30 nM). The [Dip^{18}]VIP(6–23) was used as template to find a second substitution using the same approach; a tyrosine at position 9 was selected and the resulting [Tyr^9,Dip^{18}]VIP(6–23) analog had a K_i value of 90 ± 20 nM. This analog was not able to stimulate cyclic AMP production at 10^{-6} M, and the K_b value was determined to be 79 ± 7 nM. The K_i values of [Tyr^9,Dip^{18}]VIP(6–23) at the rat VPAC$_2$ and PAC$_1$ receptors were 3,000 nM and >10,000 nM, respectively, and the K_b values were above 500 nM for both receptors. Thus, [Tyr^9,Dip^{18}]VIP(6–23) is a selective VPAC$_1$ receptor antagonist. The C-terminally extended form, [Tyr^9,Dip^{18}]VIP(6–28), had improved binding (K_i = 18 ± 2 nM) and antagonist properties (K_b = 16 ± 1 nM), but the specificity to the other receptors was diminished. The K_i and K_b values were 96 ± 4 nM and 94 ± 9 nM, respectively, for the rat VPAC$_2$ receptor, whereas K_i and K_b values were 6,000 ± 7,000 nM and >500 nM, respectively, for the PAC$_1$ receptor. In contrast, the fully extended form [Tyr^9,Dip^{18}]VIP(1–28) was a full agonist with improved binding (K_i = 0.11 ± 0.01 nM) and ability to stimulate cAMP production (EC$_{50}$ = 0.23 ± 0.01 nM) compared with VIP (K_i = 1.7 ± 0.3 nM; EC$_{50}$ = 1.12 ± 0.06 nM). Furthermore, the specificity to the VPAC$_1$ receptor was high, because K_i and EC$_{50}$ were severalfold higher for the rat VPAC$_2$ receptor (K_i = 53 ± 4 nM; EC$_{50}$ = 74 ± 5 nM) and for the PAC$_1$ receptor (K_i = 3,000 ± 3,000 nM; EC$_{50}$ > 1 μM).

The VIP/VPAC$_1$ interface is not known; nevertheless, the 480-fold difference in the displacement efficiency of [Tyr^9,Dip^{18}]VIP, comparing the VPAC$_1$ receptor with VPAC$_2$ receptor, suggests that the [Tyr^9,Dip^{18}] modifications are situated at the ligand/receptor interface. Furthermore, the [Tyr^9,Dip^{18}] modification of VIP and VIP(6–23) improves both the agonist and antagonist activity on the VPAC$_1$ receptor. This indicates that the [Tyr^9,Dip^{18}] modifications bind to the same areas of the receptor and do not involve receptor activation.

An observation of our specificity studies was that the C-terminal extensions (24–28 and 24–38) of the [Tyr^9,Dip^{18}]VIP(6–23) analog had only minor effect on the VPAC$_1$ receptor, whereas binding affinity increased by several orders of magnitude on the VPAC$_2$ and PAC$_1$ receptors. In contrast, the N-terminal extension had the reverse effect on the three receptor subtypes. These observations could indicate that the ligand binding sites of the VPAC$_2$ and PAC$_1$ receptors are more similar compared with the VPAC$_1$ receptor, despite the fact that the PAC$_1$ receptor only binds VIP with low affinity. Likewise, the PACAP 6–38 and VIP 6–28 are antagonist for the VPAC$_2$ and PAC$_1$ receptors but not for the VPAC$_1$ receptor (Dickinson et al., 1997), which supports this notion.

Several VIP analogs have been described in the literature and we have tested whether these modifications of VIP would show the same features in combination with the [Tyr^9,Dip^{18}] modification. The homologous peptide secretin from rabbit has an arginine at position 16, and [Arg^{16}]VIP has been shown to improve the binding to the rat VPAC$_1$ and PAC$_1$ receptors (Gourlet et al., 1996b). Improvement of the binding was also seen for the [Tyr^9,Arg^{16},Dip^{18}]VIP(6–23) analog for all three receptors tested, and a slightly lower K_b value was obtained for the VPAC$_1$ receptor compared with [Tyr^9,Dip^{18}]VIP(6–23). Secretin has a leucine at position 22

and the same authors have reported that the Y22L substitution in VIP results in a selective VPAC$_1$ receptor agonist. They showed that [Leu^{22}]VIP had a slightly lower IC$_{50}$ value with the rat VPAC$_1$ receptor compared with VIP, but binding to the rat VPAC$_2$ receptor was reduced resulting in an IC$_{50, \text{VPAC2}}$/IC$_{50, \text{VPAC1}}$ ratio of 270 (Gourlet et al., 1998). As seen in Table 1, [Tyr^9,Dip^{18},Leu^{22}]VIP(6–23) has a 4.3-fold lower affinity to the VPAC$_1$ receptor, but the $K_{i, \text{VPAC2}}$/$K_{i, \text{VPAC1}}$ ratio was not improved compared with [Tyr^9,Dip^{18}]VIP(6–23).

The addition of the C-terminal PACAP extension (PACAP 28–38) to VIP- or VIP fragment has been shown to increase the affinities for the rat PAC$_1$ receptor by more than 100-fold without affecting the binding to the rat VPAC$_1$ receptor (Gourlet et al., 1996a). Similarly, [Tyr^9,Dip^{18}]VIP(6–28)/(29–38)PACAP improved the binding by 1.5-, 14-, and 250-fold for the VPAC$_1$, VPAC$_2$, and PAC$_1$ receptors, respectively. PACAP(6–38) is an antagonist for the VPAC$_2$ and PAC$_1$ but not for the VPAC$_1$ receptor. [Tyr^9,Dip^{18}]VIP(6–28)/(29–38)PACAP, however, was able to bind to all three receptors with the approximately the same K_i (7–23 nM) and was shown to be an antagonist for all three receptors. Thus, [Tyr^9,Dip^{18}]VIP(6–28)/(29–38)PACAP is probably able to fully antagonize the action of PACAP, because PACAP is an agonist for VPAC$_1$, VPAC$_2$, and PAC$_1$. However, the K_b value of [Tyr^9,Dip^{18}]VIP(6–28)/(29–38)PACAP was 4- to 10-fold higher than the K_i value for the same receptors. This discrepancy between binding and antagonistic properties is probably caused by the highly charged feature of the 29–38 extension (6 of 10 residues are positively charged), which could result in different solubility or kinetic properties in the two different assay conditions.

[4Cl-D-Phe^6,Leu^{17}]VIP is described as a competitive antagonist for the action of VIP (Pandol et al., 1986). Likewise, [Ac-His^1,D-Phe^2,Lys^{15},Arg^{16},Leu^{17}]VIP(3–7)/GRF(8–27) is reported as a selective VPAC$_1$ receptor antagonist (Gourlet et al., 1997a), whereas [Lys^{15},Arg^{16},Leu^{17}]VIP(3–7)/GRF(8–27) is reported as a selective VPAC$_1$ receptor agonist (Gourlet et al., 1997b). Thus, 4Cl-D-Phe^6 or D-Phe^2 could be general modifications, which convert a VPAC$_1$ receptor agonist to an antagonist. However, as shown in Fig. 5, the 4Cl-D-Phe^6 or D-Phe^2 substitution in the agonist [Tyr^9,Dip^{18}]VIP(1–28) was not able to convert these analogs to antagonists. [4Cl-D-Phe^6,Tyr^9,Dip^{18}]VIP(1–28), [4Cl-D-Phe^6,Tyr^9,Leu^{17},Dip^{18}]VIP(1–28) and [Ac-His^1,D-Phe^2,Tyr^9,Dip^{18}]VIP(1–28) were partial agonists with more than 100-fold lower binding affinity.

Conclusion

C- and N-terminal truncated VIP, amino acids 6–23, has been used as template to generate a selective rat VPAC$_1$ receptor antagonist and agonist. Factors that can be crucial for the usability of these analogs are 1) the overall charge distribution is preserved, 2) the size of the antagonist is diminished by 10 residues, and 3) selectivity for VPAC$_1$ in vivo is presumably better than that of chimerical analogs made by homologous peptides.

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