Development of Simplified Vasoactive Intestinal Peptide Analogs with Receptor Selectivity and Stability for Human Vasoactive Intestinal Peptide/Pituitary Adenylate Cyclase-Activating Polypeptide Receptors

Hisato Igarashi, Tetsuhide Ito, Samuel A. Mantey, Tapas K. Pradhan, Wei Hou, David H. Coy, and Robert T. Jensen

Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland (H.I., T.I., S.A.M., T.K.P., W.H., D.H.C., R.T.J.); and Peptide Research Laboratories, Department of Medicine, Tulane University Health Sciences Center, New Orleans, Louisiana (D.H.C.)

Received April 28, 2005; accepted June 28, 2005

ABSTRACT

Vasoactive intestinal peptide (VIP) is a widespread neurotransmitter whose physiological and pathophysiological actions are mediated by two receptor classes, VIP/pituitary adenylate cyclase-activating polypeptide (VPAC)1 and VPAC2. VIP is a 28-amino acid peptide that is rapidly degraded and simplified; metabolically stable analogs are needed. In this study, we use information from studies of the VIP pharmacophore for VPAC1/VPAC2 to design nine simplified VIP analogs that could have high affinity and selectivity for each VPAC or that retained high affinity for both VPACs and were metabolically stable. From binding studies of their abilities to directly interact with hVPAC1 (T47D cells, hVPAC1-transfected cells) and hVPAC2 (Sup T1- and VPAC2-transfected cells) and to stimulate adenylate cyclase in each, two analogs [(Ala2,8,9,11,19,24,25,27,28)VIP and (Ala2,8,9,11,19,24,25,27,28)VIP] were found to have >2000- and >600-fold selectivity for hVPAC1. None of the nine analogs had hVPAC2 selectivity. However, two simplified analogs [(Ala2,8,9,16,19,24)VIP and (Ala2,8,9,16,19,24,25)VIP] retained high affinity and potency for both hVPACs. 125I-[Ala2,8,9,16,19,24,25]VIP was much more metabolically stable than 125I-VIP. The availability of these simplified analogs of VIP, which are metabolically stable and have either hVPAC1 selectivity or retain high affinity for both hVPACs, should be useful for exploring the role of VPAC subtypes in mediating VIPs’ actions as well as being useful therapeutically and for exploring the usefulness of VIP receptor imaging of tumors and VIP receptor-mediated tumor cytotoxicity.

Vasoactive intestinal peptide (VIP) is a widely distributed neurotransmitter that is thought to play an important role in a number of physiological and pathological processes (Dockray, 1994; Gozes and Brenneman, 2000). VIP is proposed to play a role in a number of disease states (Gozes and Furman, 2004), including a role in growth of cancer cells (Moody, 1996; Moody et al., 2003; Gozes and Furman, 2004), various central nervous system disorders (Gozes and Brenneman, 2000; Dogrukol-Ak et al., 2004; Gozes and Furman, 2004), various inflammatory disorders such as rheumatoid arthritis (Gozes and Brenneman, 2000), and various immunological disorders (Delgado et al., 2004), and a role has been proposed for VIP in treatment of asthma (Groneberg et al., 2001), impotence (Sandhu et al., 1999; Kalsi et al., 2002), and for treatment of septic shock (Kalsi et al., 2002; Delgado et al., 2004), central nervous system disorders (Gozes and Brenneman, 2000; Dogrukol-Ak et al., 2004), and diabetes (Yung et al., 2003).

In almost all cases, which VIP receptor subtype is mediating the action of VIP in these various conditions, is unclear. The actions of VIP are mediated by two receptor subtypes (VPAC1 and VPAC2), which have different pharmacology and distributions (Dockray, 1994; Harmar et al., 1998). VIP has high affinity for both VIP receptor subtypes and therefore does not discriminate between the two VIP receptor subtypes (Harmar et al., 1998). Furthermore, VIP is a 28-amino acid peptide that undergoes rapid degradation in vivo with a

ABBREVIATIONS: VIP, vasoactive intestinal peptide; VPAC, VIP-PACAP receptors (for nomenclature see Harmar et al., 1998); hVPAC1, human VPAC1 receptor subtype; hVPAC2, human VPAC2 receptor subtype; IBMX, 3-isobutyl-1-methyl xanthine; BSA, bovine serum albumin; PACAP, pituitary adenylate cyclase-activating peptide; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high-performance liquid chromatography; Ro 25-1533, Ac-His-Ser-Asp-Ala-Val-Phe-Thr-Glu-Asn-Tyr-Thr-Lys-Leu-Arg-Lys-Gln-Nle-Ala-Ala-Lys-cyclo[Lys-Tyr-Leu-Asn-Asp]-Leu-LysLysGly-Gy-Thr-NH2.
half-life less than 1 min (Domschke et al., 1978). Therefore, simplified VIP analogs that retain high affinity and have selectivity for one VIP receptor subtype, especially if metabolically stable, could be of value in investigating VIP's roles in physiological or pathological states as well as its use as a possible therapeutic agent. Furthermore, a metabolically stable, simplified VIP analog that retained high affinity for both VIP receptor subtypes could be useful for imaging tumors overexpressing VIP receptors as well as possibly for VIP receptor-directed antitumor treatment.

Recently, we (Igarashi et al., 2002a,b) and others (Nicole et al., 2000) have performed alanine scanning as well as D-amino acid scanning of VIP (Igarashi et al., 2002a,b) to define the VIP pharmacophore for the human VPAC1 and human VPAC2 receptors. These studies (Nicole et al., 2000; Igarashi et al., 2002a,b) provided information that could be helpful in the design of a simplified VIP analog that had either selective high affinity for one VPAC or that retained high affinity for both and yet might be metabolically stable. The latter point is supported by results of a previous study that demonstrated a polyalaninated VIP analog with high affinity for VPAC1 that was much more metabolically stable than VIP (Igarashi et al., 2002b). However, in that study (Igarashi et al., 2002b), the selectivity of this VIP analog for VPAC1 (analog 2 in the present study) was not determined. Therefore, in the present study we used an analysis of these study results (Nicole et al., 2000) to study we used an analysis of these study results (Nicole et al., 2000) to determine how much potential there is for this further improvement of an already potent and metabolically stable compound. Therefore, in the present study we used an analysis of these study results (Nicole et al., 2000) to study we used an analysis of these study results (Nicole et al., 2000) to determine how much potential there is for this further improvement of an already potent and metabolically stable compound.

Materials and Methods

Materials. PANC1 human pancreatic cancer cells and Sup T1 human lymphoblastoma cells were obtained from American Type Culture Collection (Manassas, VA). Porcine VIP was from Bachem Biosciences (King of Prussia, PA); basal medium Eagle amino acid mixture, basal medium Eagle vitamin solution, fetal bovine serum, and Lipofectamine transfection reagent were from Invitrogen (Carlsbad, CA); Genetecin (G418 sulfate) was from Mediatech (Herndon, VA); bacitracin, soybean trypsin inhibitor, 3-isobutyl-1-methylxanthine (IBMX), and alunina were from Sigma-Aldrich (St. Louis, MO); AG50W-X4 resin was from Bio-Rad (Hercules, CA); bovine serum albumin (BSA) fraction V was from MP Biomedicals (Irvine, CA); [125I]-VIP (2200 Ci/mmol) and [125I]-PACAP-1-27 (2200 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Boston, MA); Na125I (2200 Ci/mmol) and [2-H]adenine (22 Ci/mmol) were from GE Healthcare (Piscataway, NJ); 1,3,4,6-tetrahydro 3o,6-o-diphenylglycouril was from Pierce Chemical (Rockford, IL); HEPES was from Roche Diagnostics (Indianapolis, IN). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, and Ham's F-12K medium were from Biofluids (Rockville, MD). The standard incubation solution contained 24.5 mM HEPES, pH 7.45, 98 mM NaCl, 6 mM KCl, 2 mM KH2PO4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM CaCl2, 1 mM MgCl2, 1% (w/v) BSA, 0.2% (w/v) soybean trypsin inhibitor, 1% (v/v) amino acid mixture, and 1% (v/v) essential vitamin mixture.

Preparation of Peptides. Multiple alanine-substituted VIP analogs (VIPCA-A-1 to VIPCA-A-9, Table 1 and Fig. 1) were synthesized using standard solid phase methods as described previously (Sasaki and Coy, 1987; Igarashi et al., 2002a). Homogeneity of the peptides was assessed by thin layer chromatography and analytical reverse-phase HPLC with the purity >97% for each peptide.

Construction of PANC1 Cells Stably Transfected with hVPAC2-R and hVPAC1-R. Construction of the human VPAC1 receptor (hVPAC1-R) and human VPAC2 (hVPAC2-R) receptor expression vector and construction of hVPAC1-R or hVPAC2-R stably transfected PANC1 cells (hVPAC1-R or hVPAC2-R/PANC1 cells) was described previously (Ito et al., 2001; Igarashi et al., 2002a).

Cell Culture. The hVPAC1-R/PANC1 cells and hVPAC2-R/PANC1 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics, and 300 μg/ml G418. Sup T1 human lymphoblastoma cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum, and 1% (v/v) antibiotics, and were maintained in incubators at 37°C in an atmosphere of 5% CO2 and 95% air. T47D human breast cancer cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum, and 1% (v/v) antibiotics, and were maintained in incubators at 37°C in an atmosphere of 5% CO2 and 95% air.

TABLE 1

Affinity of VIP and simplified multialaninated VIP analogs designed to be selective hVPAC1-R agonists

<table>
<thead>
<tr>
<th>Proposed hVPAC-R-Selective Agonist (Analog No.)</th>
<th>Binding Affinity (IC50)</th>
<th>hVPAC-R Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hVPAC1-R T47D</td>
<td>1.6 ± 0.1</td>
<td>6.9 ± 0.3</td>
</tr>
<tr>
<td>hVPAC1-R PANC1</td>
<td>1.0 ± 0.1</td>
<td>105 ± 14</td>
</tr>
<tr>
<td>hVPAC2-R T47D</td>
<td>7.3 ± 0.2</td>
<td>105 ± 14</td>
</tr>
<tr>
<td>hVPAC2-R PANC1</td>
<td>9.5 ± 0.2</td>
<td>105 ± 14</td>
</tr>
</tbody>
</table>

The indicated cell type was incubated with 75 pM [125I]-peptide and various concentrations of the unlabelled alanine-substituted VIP analog as described in legends to Figs. 2 and 3 and under Materials and Methods. The IC50 was the concentration causing half-maximal inhibition of the saturable binding caused by 1 μM VIP calculated using the curve-fitting program Kaleidagraph. In each experiment, each value was determined in duplicate; values given are means ± S.E.M. from at least three separate experiments. The selectivity was calculated using the mean value of the IC50 in the two different VPAC cells.
Preparations of $^{125}$I-Ro 25-1553, $^{125}$I-Ro 25-1553 (a cyclic VIP analog selective for VPAC$_2$-R) (O’Donnell et al., 1994a,b; Gourlet et al., 1997b) at a specific activity of 2200 Ci/mmol was prepared by a modification of the methods described previously (Zhou et al., 1989). In brief, 0.8 $\mu$g of 1,3,4,6-tetrachloro 3a,6a-diarylpyrenecoumarin in chloroform was transferred to a vial, dried under a stream of nitrogen, and washed with 100 $\mu$L of 0.5 M KH$_2$PO$_4$, pH 8.0. To this vial, 20 $\mu$L of 0.5 M KH$_2$PO$_4$, pH 8.0, 8 $\mu$L of peptide in 4 $\mu$L of water, and 2 $\mu$L (20 $\mu$L) of Na$_2$H$_2$O$_2$ were added, mixed gently, and incubated at room temperature for 6 min. The incubation was stopped by the addition of 100 $\mu$L of distilled water. The iodination mixture was applied to a Sep-Pak C18 (Waters, Milford, MA), and free $^{125}$I$_2$ was eluted with 200 $\mu$L of sequential elutions ($\times$10) with 60% acetonitrile in 0.1% trifluoroacetic acid. The radiolabeled peptides were eluted with 200 $\mu$L of sequential elutions ($\times$10) with 60% acetonitrile in 0.1% trifluoroacetic acid. The two or three fractions with the highest radioactivity were combined and purified on a reverse-phase, high-performance liquid chromatography with a Vydac C18 column (0.46 $\times$ 25 cm). The column was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid ($\sqrt{v}$) from 16 to 60% acetonitrile in 60 min, and 1-mL fractions were collected and assayed for radioactivity and receptor binding. The pH of the pooled fractions was adjusted to 7 using 0.2 M Tris, pH 9.5, and radioligands were stored in aliquots with 0.5% bovine serum albumin (w/v) at $-20^\circ$C. $^{125}$I-[Ala$^2$,8,9,11,19,24,25]VIP (VIP analog 2; Table 1; Igarashi et al., 2002a) and $^{125}$I-[Ala$^2$,8,9,16,20,24,25]VIP (VIP analog 8; Table 1) were prepared using the same procedures.

Binding Studies. Binding of $^{125}$I-VIP to hVPAC$_2$-R/PANC1 and T47D cells, and binding of $^{125}$I-PACAP(1-27) to hVPAC2-R/PANC1 were performed by incubation in standard incubation solution containing 0.5% (w/v) bacitracin for 60 min at room temperature and as described previously (Ito et al., 2000; Igarashi et al., 2002a,b). To assess VPAC-R affinities in Sup T1 human lymphblastoma cells, binding study was performed using $^{125}$I-Ro 25-1553 in standard incubation solution containing 0.5% (w/v) bacitracin for 60 min at 37°C, because $^{125}$I-VIP and $^{125}$I-PACAP(1-27) were rapidly degraded in these cells even with protease inhibitors present. The separation of bound from free radioactivity was obtained by centrifugation of cells through 2% (w/v) BSA in standard incubation solution containing 0.05% (w/v) bacitracin for 60 min at 37°C. The pH of the pooled fractions was adjusted to 7 using 0.2 M Tris, pH 9.5, and radioligands were stored in aliquots with 0.5% bovine serum albumin (w/v) at $-20^\circ$C. $^{125}$I-[Ala$^2$,8,9,11,19,24,25]VIP (VIP analog 2; Table 1; Igarashi et al., 2002a) and $^{125}$I-[Ala$^2$,8,9,16,20,24,25]VIP (VIP analog 8; Table 1) were prepared using the same procedures.

The pH of the pooled fractions was adjusted to 7 using 0.2 M Tris, pH 9.5, and radioligands were stored in aliquots with 0.5% bovine serum albumin (w/v) at $-20^\circ$C. $^{125}$I-[Ala$^2$,8,9,11,19,24,25]VIP (VIP analog 2; Table 1; Igarashi et al., 2002a) and $^{125}$I-[Ala$^2$,8,9,16,20,24,25]VIP (VIP analog 8; Table 1) were prepared using the same procedures.

Results

Development of Mutialanine-Substituted VIP Analogs That Were Designed to Have a High Affinity for Human VPAC$_1$-R or VPAC$_2$-R (Fig. 1). In a previous study (Igarashi et al., 2002a), we demonstrated a simplified multialanine analog of VIP (analog 2; present study) could be synthesized that retained high affinity for rat, guinea pig, and human VPAC$_1$-R. This analog also had increased stability compared with VIP for the hVPAC$_1$-R (Igarashi et al., 2002a). However, no studies were performed on the selectivity or stability of this analog with hVPAC$_2$-R. In the present study, we examined the VPAC-R selectivity of this analog and synthesized additional simplified, multialanine analogs in an attempt to develop a highly selective VIP analog for hVPAC$_1$-R and hVPAC$_2$-R that might also be metabolically stable for either or both receptors.

Using the results of alanine scanning of VIP to define the amino acids essential for high-affinity interaction with the hVPAC$_1$-R (Igarashi et al., 2002a) and the hVPAC$_2$-R (Igarashi et al., 2002b), we synthesized eight additional multialanine VIP analogs in an attempt to develop a highly selective VIP analog for hVPAC$_1$-R and hVPAC$_2$-R. Using the results of alanine scanning of VIP to define the amino acids essential for high-affinity interaction with the hVPAC$_1$-R (Igarashi et al., 2002a) and the hVPAC$_2$-R (Igarashi et al., 2002b), we synthesized eight additional multialanine VIP analogs that, based on single alanine substitutions, should retain high affinity for the hVPAC$_1$-R or the hVPAC$_2$-R. The simplest, multialanine-substituted analog VIP analog 8; Table 1) was based on the VIP pharmacophore of the hVPAC$_2$-R. In each case, the multialanine-substituted analogs were made by making combinations of analogs in which, when alanine was substituted alone in a given position, there was a -10-fold decrease in affinity. For the hVPAC1 (Igarashi et al., 2002a) and hVPAC2 receptor (Igarashi et al., 2002b) (Fig. 1).

Affinity of Multialanine-Substituted VIP Analogs Designed to Be hVPAC$_1$-R Agonists for hVPAC$_2$-R- Containing Cells. Multialanine VIP analogs 1 and 3 to 6 retained high affinity for the hVPAC$_1$-R, similar to VIP analog 2 (Fig. 2; Table 1). The simplest, multialanine VIP analog (VIP analog 4; Fig. 1; [Ala$^2$,8,9,11,19,24,25]VIP) had a 6- to 7-fold decrease in the affinity compared with VIP, for the hVPAC$_1$-R-containing cells. When two more alanine sub-
stitutions were added in positions 25 and 28, which resulted in the VIP analog 2, it had an equal affinity to VIP for the hVPAC1-R, similar to that reported previously (Igarashi et al., 2002a). An additional alanine replacement of either valine19 (analog 3) or Lys21 (analog 4), Tyr22 (analog 5) or Ile26 (analog 26) had variable effects on hVPAC1-R affinity (Fig. 2, top; Table 1). Analogs 3, 5, and 6 had a 2- to 17-fold decrease in the affinity for the hVPAC1-R compared with VIP and for a 2- to 9.9-fold decrease the exact fold decrease is shown. The position of the alanine substitutions for each analog (labeled Ala) is shown and unchanged amino acids from VIP are indicated by “-“.. VIP analogs 1 to 6 were designed to possibly function as simplified high-affinity hVPAC1-R agonists, and VIP analogs 7 to 9 were designed to function as simplified, possibly high-affinity, hVPAC2-R agonists.

Affinity of Multialanine-Substituted VIP Analogs Designed to Be hVPAC2-R Agonists for hVPAC1-R- or hVPAC2-R-Containing Cells. We synthesized three multialaninated VIP analogs that should retain high affinity for the hVPAC2-R (VIP analogs 7–9; Fig. 1) and determined their abilities to interact with hVPAC1-R receptors (Fig. 3, top; Table 1) and hVPAC2-R-containing cells (Fig. 3, bottom; Table 1). VIP analog 7 with six alanine substitutions (i.e., for Ser2, Asp8, Asn9, Gln16, Val19, and Asn24; Fig. 1) as well as VIP analog 8, which had an additional alanine substitution for Ser25 (Fig. 1), had a similar high affinity to VIP for the hVPAC2-R (Fig. 3, bottom; Table 1). However, when two additional alanine substitutions for Lys20 and Lys21 were added to analog 8, the resultant analog 9 had a 3- to 8-fold decrease in affinity for hVPAC2-R compared with VIP (Fig. 3, bottom; Table 1). We also determined their affinities for hVPAC1-R-containing cells (Fig. 3, top; Table 1) and found that analog 7 and analog 8 showed a 2- to 3-fold and a 5- to 7-fold decrease, respectively, in affinity for hVPAC1-R compared with VIP. In contrast, analog 9 had a 50-fold decrease in the affinity compared with VIP for hVPAC1-R (Fig. 3, top; Table 1). In terms of VPAC-R selectivity, each of these 3 analogs (7–9) had a 2- to 4-fold higher affinity for hVPAC2-R than hVPAC1-R, whereas VIP had a 3.7-fold higher selectivity for hVPAC1-R than hVPAC2-R (Table 1; Fig. 3).
Potency of Multialanine-Substituted VIP Analogs Designed to Be hVPAC₁-R Agonists for hVPAC₁-R or hVPAC₂-R Activation. Activation of adenylate cyclase is the principal intracellular mediator of the action of VPAC₁ and VPAC₂ receptors (Zhou et al., 1989; Ulrich, II et al., 1998; Nicole et al., 2000). To investigate whether these multialanminated VIP analogs functioned as agonists and if so, their potency and efficacy for hVPAC₁-R and hVPAC₂-R activation, we first determined the ability of VIP analogs 1 to 6, which were hVPAC₁-R-preferring by binding studies, to stimulate cAMP generation in hVPAC₁-R- and hVPAC₂-R-containing cells (Fig. 4; Table 2). Each of the six analogs had similar efficacy to VIP in stimulating cAMP generation through the hVPAC₁-R (Fig. 4, top). VIP had a high potency for stimulating cAMP generation in hVPAC₁-R-containing cells with EC₅₀ values of 2.5 to 2.7 nM and caused a maximal stimulation with 100 nM concentration (Table 2; Fig. 4). Except for VIP analogs 1 and 4, which showed a 2- to 4-fold decrease in potency for activating VPAC₁-R compared with VIP, each of the other 4 VPAC₁-R-preferring analogs (2, 3, 5, and 6) retained a high potency for the VPAC₁-R (Fig. 4, top; Table 2).

VIP had a high potency for stimulating cAMP generation via the hVPAC₂-R, with an EC₅₀ of 5 nM and caused maximal stimulation at 100 to 300 nM concentration (Fig. 4, bottom). Three of the VPAC₁-R-preferring analogs (1, 3, and 4) had sufficient potency so that their efficacy could be determined and was shown to be equal to VIP at the VPAC₂-R (Fig. 4, bottom).
VIP analogs 1 to 6 had $>$90-fold lower potency than VIP for stimulating cAMP generation in hVPAC$_2$-R cells. Analog 5 had the lowest potency, demonstrating $>$3000-fold lower potency than VIP (Fig. 4, bottom; Table 2). In terms of their selectivity for activating hVPAC$_1$-R or hVPAC$_2$-R, VIP analogs 1 to 6 had $>$100-fold higher selectivity for hVPAC$_1$-R over hVPAC$_2$-R, whereas VIP had a 2.0-fold selectivity for hVPAC$_1$-R over hVPAC$_2$-R (Table 2). Based on potency, VIP analog 5 had the greater selectivity for stimulating cAMP generation, having a 15,600-fold higher selectivity for hVPAC$_1$-R over hVPAC$_2$-R (Table 2).

Potency of Multialanine-Substituted VIP Analogs (Analogs 7–9) Designed to Have Higher Affinity for the hVPAC$_2$-R for hVPAC$_1$-R or hVPAC$_2$-R Activation. VIP analogs 7 to 9 had equal efficacy to VIP for activating either hVPAC$_1$-R (Fig. 5, top) or hVPAC$_2$-R cells (Fig. 5, bottom). Each of these three analogs had a similar potency to VIP for stimulating cAMP generation in VPAC$_1$-R cells (Fig. 5, top; Table 3). In contrast, none of these three analogs had higher potency than VIP for stimulating cAMP generation in hVPAC$_2$-R cells (Fig. 5, bottom; Table 3). In terms of relative potency for VPAC-R activation, none of these three analogs had selectivity for hVPAC$_2$-R over hVPAC$_1$-R (Table 3).

Stability of VIP and Multialaninated VIP Analogs Incubated with hVPAC$_1$-R/PANC1 Cells or hVPAC$_2$-R/PANC1 Cells. In a previous study (Igarashi et al., 2002a), we demonstrated analog 2 ([Ala$^{2,8,9,11,19,24,25,27,28}$]VIP was
more resistant to degradation than VIP by hVPAC₁-R/PANC1 cells. However, in the present study, we demonstrate the VIP analog 2 has less than a 200-fold selectivity for the hVPAC₁-R (Table 1) and thus would not be useful for in vivo receptor studies attempting to localize both hVPAC₁-R and hVPAC₂-R in tumors. It is unknown whether the other multialaninated analogs that retained high affinity for both hVPAC-R subtypes described in the present study (Tables 1 and 2) and could thus be useful for in vivo receptor characterization of both VPAC-R subtypes, were also resistant to degradation. To address this question, we prepared ¹²⁵I-analog 8 (¹²⁵I-Ala²,⁸,⁹,¹⁶,¹⁹,²⁴,²⁵]VIP) and ¹²⁵I-analog 2 (¹²⁵I-Ala²,⁸,⁹,¹¹,¹⁹,²⁴,²⁵,²⁷,²⁸]VIP) and compared the amount of degradation during an incubation with hVPAC₁-R/PANC1 cells or hVPAC₂-R/PANC1 cells to that seen with ¹²⁵I-VIP. As seen in Fig. 6, left, more than 70% of ¹²⁵I-VIP was degraded during incubation with hVPAC₁-R/PANC1 cells, whereas only 20% ¹²⁵I-analog 8 was degraded. As reported previously (Igarashi et al., 2002a), no degradation of ¹²⁵I-analog 2 was seen under the same conditions, suggesting that analog 8 had a greater stability than VIP incubated with hVPAC₁-R/PANC1 cells, but it was less metabolically stable than analog 2. Figure 6, right, demonstrates that more than 70% of ¹²⁵I-VIP was degraded during an incubation with hVPAC₂-R/PANC1 cells; however, only 20% of ¹²⁵I-analog 8 was degraded, showing that analog 8 also had a greater stability than VIP during incubation with hVPAC₂-R/PANC1 cells.
TABLE 2

Potency of VIP and multialaninated VIP analogs designed to be hVPAC1-R agonists for stimulating cAMP generation in hVPAC1-R or hVPAC2-R-containing cells

<table>
<thead>
<tr>
<th>Proposed hVPAC1-R Agonist</th>
<th>Potency for cAMP Generation (EC50)</th>
<th>hVPAC1-R Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM</td>
<td>fold</td>
</tr>
<tr>
<td>VIP</td>
<td>2.5 ± 0.1</td>
<td>2</td>
</tr>
<tr>
<td>VIP analog 1</td>
<td>10.0 ± 1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>2</td>
<td>2.9 ± 0.3</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>2.9 ± 0.2</td>
<td>6.5</td>
</tr>
<tr>
<td>4</td>
<td>1.1 ± 0.9</td>
<td>7.3</td>
</tr>
<tr>
<td>5</td>
<td>0.68 ± 0.07</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>3.2 ± 0.3</td>
<td>15</td>
</tr>
</tbody>
</table>

Discussion

The purpose of the present study was to attempt to identify simplified VIP analogs that either have high selectivity and high affinity for one subtype of VIP receptor or that are metabolically stable and retained high affinity for both VIP receptor subtypes. In a previous study (Igarashi et al., 2002a), we identified a simplified analog of VIP, [Ala²,8,9,11,19,24,25,27,28]VIP (analog 2 in present study), which retained high affinity for hVPAC1 and which was synthesized after analyzing the VIP pharmacophore for VPAC1 in human, rat, and guinea pig. In the present study, we confirmed that [Ala²,8,9,11,19,24,25,27,28]VIP has a similar high affinity to VIP for the human VPAC1 in both native hVPAC1-containing cells (T47D cells) and hVPAC1-transfected PANC1 cells; however, we further demonstrate this simplified VIP analog has relatively low selectivity (<170-fold) for hVPAC1 over hVPAC2. Therefore, [Ala²,8,9,11,19,24,25,27,28]VIP does not fulfill our goal of identifying a highly selective agonist for either VPAC. Furthermore, even though it is metabolically stable (Igarashi et al., 2002a), its lower affinity for hVPAC2 (100–500 nM) makes it unsuitable as a high-affinity ligand for both VPACs, our second goal. In the present study, we have successfully identified simplified VIP analogs that have high selectivity and retain high affinity for the hVPAC1. Two of the five new, simplified VIP analogs, [Ala²,8,9,11,19,24,25,27,28]VIP (analog 5) and [Ala²,8,9,11,19,24,25,27,28]VIP (analog 6), had >2400-fold and 600-fold higher binding affinities, respectively, for hVPAC1 over hVPAC2 receptors. Furthermore, each of these two analogs was a fully efficacious agonist at the VPAC1 receptor, and analog 5 had >15,000-fold greater potency for activating the hVPAC1 compared with the hVPAC2, and analog 6 had >1200-fold greater potency for hVPAC1 over hVPAC2. This discrepancy between potency and binding affinity for the VPAC1 compared with native VIP is probably at least partially due to their greater metabolic stability, because each of these analogs has multialaninated substitutions similar to analogs 8 and 2 in the present study and analog 2 in a previous study (Igarashi et al., 2002a), each of which has enhanced metabolic stability. These results demonstrate that both the simplified VIP analogs 6 and 5 may have greater selectivity than the 65-fold selectivity of [Leu²²]VIP for hVPAC1 over hVPAC2 (Gourlet et al., 1998; Bhargava et al., 2002) and at least comparable and maybe greater than the widely used VPAC1-selective agonist (Gourlet et al., 1997a) [Lys¹⁵, Arg¹⁶, Leu²⁷]VIP(1-7)-GRF(8-27), which is reported to have a 53- to 169-fold selectivity for rVPAC2 over rVPAC2 transfected into Chinese hamster ovary cells in one study (Ito et al., 2000), but a 15,000-fold selectivity in another study (Gourlet et al., 1997a), and a 300- to 30,000-fold selectivity in hVPAC1/hVPAC2-containing cells (Gourlet et al., 1997a; Igarashi et al., 2002b). In contrast to our results with the hVPAC1, none of the three simplified VIP analogs synthesized, based on the analysis of the VIP pharmacophore for the VPAC2 (Igarashi et al., 2002b), demonstrated selectivity for the hVPAC2. However, two of these three simplified VIP analogs ([Ala²,8,9,11,16,19,24,25,27,28]VIP (analog 7) and [Ala²,8,9,11,16,19,24,25,27,28]VIP (analog 8)) retained high affinity (5.6 and 7.1 nM) for the hVPAC2, in contrast to each of the six simplified VIP analogs (analogs 1–6) designed to have high affinity for VPAC1, each of which had low affinity for hVPAC2 (468 to >30,000 nM). These results demonstrate that using the designed strategy applied in the present study, simplified analogs of VIP could be made which retained high affinity for hVPAC2. The lack of selectivity of these two high-affinity simplified VIP analogs (analogs 7 and 8) for VPAC2 is in contrast to findings in other studies using different strategies that have reported finding peptides that have selectivity for VPAC2. The VIP-related peptide, helodermin is reported to have 15-fold higher affinity for hVPAC2 over hVPAC1 (Gourlet et al., 1997b). Ro 25-1553, a cyclic analog of VIP with a lactam ring (O’Donnell et al., 1994a), is reported to have 75- to 600-fold selectivity for hVPAC2 over hVPAC1 (Gourlet et al., 1997b; Ito et al., 2000; Moreno et al., 2000; Igarashi et al., 2002a), 246- to 4300-fold for rVPAC2 over rVPAC1 (Ito et al., 2000), and hexanoyl [Ala¹⁹, Lys²⁷,28]VIP(1-7)-GRF(8-27)VIP (analog 8) to have an 800-fold selectivity for hVPAC2 over hVPAC1 (Langer et al., 2004). A review of the previous studies of the VIP pharmacophore for the VPAC1 and VPAC2 (Nicole et al., 2000; Igarashi et al., 2002a, b) provides some insights into why the approach used in the study probably resulted in selective VPAC1 but not selective VPAC2 agonists. In these previous studies (Gourlet et al., 1998; Nicole et al., 2000; Igarashi et al., 2002a, b) using alanine scanning to identify the VIP pharmacophore for VPACs, a number of amino acids, particularly Thr¹¹, Tyr²², Asn²⁴, Leu²⁷, and Asn²⁸ were more important for VPAC2 than hVPAC1 affinity. In these studies (Nicole et al., 2000; Igarashi et al., 2002a, b) and others (Gourlet et al., 1998), the
TABLE 3

Potency of VIP and multialaninated VIP analogs designed to be hVPAC2-R agonists for stimulating cAMP generation in hVPAC1-R- or hVPAC2-R-containing cells

See legend to Table 2 for cAMP details and methods of calculating values. In each experiment, each value was determined in duplicate; values given are means ± S.E.M. from at least three separate experiments. The selectivity was calculated using the mean value of EC50 in the two different VPAC-containing cells.

<table>
<thead>
<tr>
<th>Proposed HVPAC1-R Agonist</th>
<th>Potency for cAMP Generation (EC50)</th>
<th>hVPAC1-R/T47D</th>
<th>hVPAC1-R/PANC1</th>
<th>hVPAC2-R/SUP T1</th>
<th>hVPAC2-R/PANC1</th>
<th>hVPAC2-R Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIP</td>
<td></td>
<td>2.5 ± 0.1</td>
<td>2.7 ± 0.3</td>
<td>5.2 ± 0.5</td>
<td>5.0 ± 0.3</td>
<td>0.51</td>
</tr>
<tr>
<td>VIP analog 7</td>
<td></td>
<td>0.79 ± 0.11</td>
<td>7.9 ± 0.7</td>
<td>50.1 ± 6.5</td>
<td>11.5 ± 1.8</td>
<td>0.14</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>1.0 ± 0.1</td>
<td>7.2 ± 0.6</td>
<td>26.3 ± 3.2</td>
<td>25.1 ± 1.5</td>
<td>0.16</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>2.2 ± 0.2</td>
<td>3.8 ± 0.3</td>
<td>25.1 ± 3.8</td>
<td>7.6 ± 1.1</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Fig. 5. Abilities of VIP and multialaninated VIP analogs designed to be high-affinity hVPAC2-R agonists to stimulate cAMP accumulation in hVPAC1-R-containing (top) or hVPAC2-R-containing (bottom) cells. cAMP was performed as described in Fig. 4 legend and under Materials and Methods. Results are expressed as the percentage of the maximal stimulation of cAMP accumulation caused by 1 μM VIP. In each experiment, each value was determined in duplicate; values given are means ± S.E.M. from at least three separate experiments.
presence of Tyr\textsuperscript{22} in VIP was much more important for high affinity for VPAC\textsubscript{2} than VPAC\textsubscript{1}, with the result that a single substitution of alanine in position 22 of VIP had the most profound effect on VIP’s ability to interact with each VPAC subtype of any single alanine substitution. In our study, analog 5 ([Ala\textsuperscript{2,8,9,11,18,22,24,25,27,28}]VIP), which had the
greatest selectivity for hVPAC1, was the only simplified analog to contain this substitution. The Ala substitution was not included in the other five proposed VPAC-selective agonists (analogs 1–4 and 6) because it has been shown to cause a 4-fold decrease in hVPAC1 affinity (Igarashi et al., 2002a,b), which we wanted to avoid if possible. The single substitution of alanine for Thr or Leu in VIP also caused a >15-fold decrease in VPAC2 affinity with minimal changes in VPAC3 affinity (Igarashi et al., 2002a,b). Both of these substitutions were included in each of the six proposed simplified VPAC1-selective agonists; however, they only resulted in a 35- to 196-fold selectivity in analogs 1 to 4. Furthermore, the single substitution of Ala for Met, Lys, Ile or Asn in VIP caused only a 2.5- to 7.3-fold decrease in VPAC2 affinity, with minimal effects on VPAC1 affinity (Igarashi et al., 2002a,b). Likewise, when they were included in the simplified proposed VPAC1-selective agonists (analogs 2–4 and 6), they generally had only a modest effect on increasing VPAC1 selectivity. In contrast to the results with VPAC1 in studies of the VIP pharmacophore for the VPAC2 (Nicole et al., 2000; Igarashi et al., 2002a,b), no single alanine substitution or p-amino acid substitution in VIP resulted in much greater decrease in affinity for the hVPAC1 than the hVPAC2. VIP itself had a 4-fold greater affinity for hVPAC1 than VPAC2. Single alanine substitution for Glu or Lys in VIP resulted in a 3- to 4-fold greater decrease in affinity for VPAC1 than VPAC2 (Igarashi et al., 2002a,b). However, inclusion of these substitutions in analogs 7 to 9 resulted in only a 3- to 4-fold decrease in affinity of the substituted VIP analogs (analogs 7–9) for VPAC1, with the result these simplified analogs had nearly equal affinity for both hVPAC1 and hVPAC2 and therefore were not selective.

The second goal of this study was to attempt to identify a simplified VIP analog that retained high affinity for each VPAC subtype and that also might be metabolically stable. Such VIP analogs could be particularly useful for imaging of tumors overexpressing VIP receptors or for VIP receptor-directed antitumor treatment. Previous studies (Bryant et al., 1976; Domschke et al., 1978) have demonstrated VIP is rapidly degraded in vivo, having a half-life less than 1 min (Bryant et al., 1976; Domschke et al., 1978) in human It has been proposed (Bryant et al., 1976; Domschke et al., 1978) that VIP is primarily degraded by being cleaved primarily at Ser and Thr residues to yield the major products VIP(1-25) and VIP(26-28) and the minor products VIP(1-7) and VIP(8-28). All of these products are either active or have very low affinity for the VPAC receptors (Bolin et al., 1995). Furthermore, another study (O’Donnell et al., 1991) suggests an alanine replacement of Val in VIP may increase VIP’s resistance to degradation. O’Donnell et al. (1991) investigated the effect of alanine substitutions into the VIP analog Ac-Lys, Nle, Ile, Val, Thr VIP on the duration of bronchodilator activity and reported that the substitution of Val by an increased the duration of effect by more than 17 times. We therefore anticipated that our analogs, which all had with substitutions at positions 8, 19, 25, and/or 26 of VIP, should have enhanced stability. This possibility was further supported by our previous study (Igarashi et al., 2002a) in which we found that the multialaninated [Ala2,8,9,11,19,24,25,27,28]VIP (analog 2) was much more resistant than VIP to degradation. A number of our results support the conclusion that we successfully achieved the second aim in the present study. First, in binding studies both analog 7 ([Ala2,8,9,11,19,24,25]VIP) and analog 8 ([Ala2,8,9,11,19,24,25]VIP) retained high affinity for both VPAC and VPAC2. Second, both analogs 7 and 8 were fully efficacious agonists at both VPACs, and each retained high potency for activating each VPAC and stimulating adenylate cyclase activity. Third, radiolabeled analog 8 demonstrated none to minimal degradation by hVPAC1 or hVPAC2 cells, whereas 125I-VIP was degraded >70% by both, demonstrating analog 8 was metabolically stable with cells containing both VPAC subtypes. Furthermore, with VPAC-containing cells, [Ala2,8,9,11,19,24,25]VIP (analog 8) was only slightly less metabolically stable than 125I-[Ala2,8,9,11,19,24,25]VIP (analog 5) has high affinity and >2000-fold selectivity for hVPAC1 over hVPAC2. No selective VPAC2 agonists were identified. However, [Ala2,8,9,11,19,24,25]VIP (analog 8) had high affinity and potency for both VPAC subtypes and was much more metabolically stable than VIP in cells containing each VPAC subtype. These simplified, metabolically stable analogs should be useful for investigating the role of VPAC1 in biological and pathological processes, for enhanced imaging of tumors overexpressing VIP receptors using VIP receptor scintigraphy (Virgolini, 1997; Thakur et al., 2000, 2004; Rao et al., 2001; Bhargava et al., 2002) as well as for possible VIP receptor-directed antitumor treatment for tumors overexpressing VPACs (Gotthardt et al., 2004; Moody et al., 2004; Ou et al., 2005).

In conclusion, analyzing the results of studies of the VIP pharmacophore for high-affinity interaction with the hVPAC1 and hVPAC2 (Nicole et al., 2000; Igarashi et al., 2002a,b), we synthesized nine simplified, polyalaninated analogs of VIP to attempt to develop high-affinity VIP analogs that were either selective for one of the two VPAC subtypes or that functioned as high-affinity agonists for each VPAC and that would be metabolically stable. Our results demonstrate that [Ala2,8,9,11,19,24,25,27,28]VIP (analog 5) has high affinity and >2000-fold selectivity for hVPAC1 over hVPAC2. No selective VPAC2 agonists were identified. However, [Ala2,8,9,11,19,24,25]VIP (analog 8) had high affinity and potency for both VPAC subtypes and was much more metabolically stable than VIP in cells containing each VPAC subtype. These simplified, metabolically stable analogs should be useful for investigating the role of VPAC1 in biological and pathological processes, for enhanced imaging of tumors overexpressing VIP receptors using VIP receptor scintigraphy (Virgolini, 1997; Thakur et al., 2000, 2004; Rao et al., 2001; Bhargava et al., 2002) as well as for possible VIP receptor-directed antitumor treatment for tumors overexpressing VPACs (Gotthardt et al., 2004; Moody et al., 2004; Ou et al., 2005).

References


