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Effect of positive charge in VIP $^{16}\gamma$-glutamyl diamino derivatives on hVPAC1 and hVPAC2 receptor function

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Abstract: Increase of VPAC receptor s binding to the $^{16}\gamma$-glutamyl diaminopropane vasoactive intestinal peptide (VIP-DAP) agonist, a vasoactive intestinal polypeptide (VIP) structural analogue containing a positive charge at position 16, has confirmed the importance of a positive charge at this site. By investigating the effect of distance from the peptide backbone Cα of a positive charge in position 16, data are reported here concerning: (i) a novel chemical method used for the synthesis of a new family of $^{16}\gamma$-glutamyl diamine VIP derivatives differing among them for single carbon atoms and including diaminoethane (VIP-DAE2), diaminopropane (VIP-DAP3), diaminobutane (VIP-DAB4), diaminopentane (VIP-DAP5), and diaminohexane (VIP-DAH6); (ii) functional characterization of these compounds on human VPAC1 and VPAC2 receptors. In more detail, the EC50 and IC50 values, when measured as a function of the alkylic chain length, show in more detail, that the use of VIP-DAB4 derivative changes the IC50 but not the EC50, thus indicating on hVPAC2 receptor an unexpected relationship between binding and activity that differs from that obtained on hVPAC1.

Keywords: glutamate polyamination; chemical synthesis; VIP diamino derivatives; human VIP receptors function

INTRODUCTION

The vasoactive intestinal polypeptide (VIP) is a 28-aminoacid long peptide that serves the function of hormone, neurotransmitter, and immunomodulator in mammals and other vertebrates [1]. VIP exerts its biological effects by interaction with two distinct natural class II G protein-coupled receptors (GPCRs) VIP/PACAP 1 (VPAC 1) and VIP/PACAP 2 (VPAC 2), which transduce the ligand signal through the activation of different enzymatic effector systems, such as adenylate cyclase, phospholipase C, and iNOS [2–4].

Work is more advanced on the mechanism of ligand binding and activation of class I GPCR, that use relatively small molecules as ligands. In contrast, fewer results are available in the case of class II GPCRs, peptide receptors that have much larger ligands and that exhibit greater conformational flexibility. The detailed signaling mechanism of VIP receptors and their physiological role are currently investigated. The only structural information available on VIP and its related peptides has been mainly obtained by CD and NMR analyses [5].

Structure–activity studies, performed on a number of analogues and VIP fragments, demonstrated that full action of VIP is critically dependent upon integrity of the entire molecule [6–8]. More precisely, the *N*-terminal helix is known to be critical for the high affinity binding and human VPAC1 (hVPAC1) and human VPAC2 (hVPAC2) discrimination [9–12]. Concerning the VIP central region, Robberecht and coll. demonstrated the unexpected importance of the aminoacid at position 16 in this region on its interaction with the receptor *N*-terminal domain [13]. In order to investigate the effect of a positive charge at position 16 on VPAC receptors activity we used the transglutaminase (TGase) to modify the primary structure of VIP by poliaminating Gln^{16} with a variety of amines of different carbon chain length and positive charge, both in rats and humans [14–16]. The functional characterization of three polyaminated VIP derivatives has demonstrated their ability to act as 'super' (VIP_{Dap}) or 'weak-full' (VIP_{Spd} and VIP_{Spm}) agonists on VPAC receptors [17]. Recently, the stability of VIP_{Dap} analogue has been evaluated in experiments of

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limited proteolysis [18]. The data obtained have shown that this peptide has a structural stability higher than VIP, associated to an increased biological activity on cell systems [19]. The role played by the different VIP residues in the recognition and activation of VIP natural receptors has been revised in the light of peptide agonist docking on to the *N*-terminal ectodomain of hVPAC1 receptor, how recently established by photoaffinity, NMR, and molecular modeling studies [20].

In this paper, we plan to evaluate on hVPAC1 and hVPAC2 functional activity the effect of the distance of the positive charge from VIP Cα in position 16 by progressively increasing by single carbon atom the alkyl chain of the VIP $^{16}\gamma$-glutamyl diamino conjugated. To obtain the appropriate molecular modification of VIP, we replaced the original enzimatic, TGase-based, method with a novel chemical, faster, more practical, and less expensive protocol to synthesize the five different VIP γ-glutamyl derivatives used in this paper.

MATERIALS AND METHODS

Chemical Synthesis of VIP-$^{16}\gamma$-Glutamyl Derivatives

Materials and general procedures. All solvents were purchased from Carlo Erba (Rodano, Milan, Italy). Extraction solvents were dried over sodium sulfate. Solvents used for reactions were dried over 3 Å molecular sieves. All the solvents were filtered and degassed prior to use. Reagent grade materials were purchased from Bachem (Bubendorf, Switzerland), Inalco-Novabiochem (Milan, Italy) and from Aldrich (Milan, Italy) and were used without further purification. Thin layer chromatography was performed on precoated silica gel Durasil-25 UV_{254} (Macherey-Nagel, Germany) plates. The compounds were detected on thin layer chromatography plates by UV light and ninhydrin. Molecular weights of final peptides were assessed by electrospray ionization mass spectrometry (ESI-MS) on a ThermoFinnigan LCQ Ion-Trap. Aminoacid analyses were carried out using PITC methodology (Pico-Tag, Waters-Millipore, Waltham, MA). Lyophilized samples of peptides (50–100 pmol) were placed in heat-treated borosilicate tubes (50 × 4 mm), sealed, and hydrolyzed using 200 µl of 6 N HCl containing 1% phenol in the Pico-Tag work station for 1 h at 150 °C. A Hypersil ODS column (250 × 4.6 mm, 5 µm particle size) was employed to separate the PITC-aminoacid derivatives. Reversed–phase purification was routinely performed on a Waters Delta-Prep 4000 system equipped with a Waters 484 multiwavelength detector on a Vydac C_{18} silica (15–20 µm, 22 × 5000 mm) high performance liquid chromatography (HPLC) column. The operational flow rate was 60 ml/min. Homogeneity of the products was assessed by analytical reversed-phase HPLC using a Vydac C_{18} column (5 µm, 4.6 × 250 mm); UV detection at 220 nm, flow rate 1 ml min^{-1}. The column was connected to a Rheodyne model 7725 injector, a Waters 600 HPLC system, a Waters 486 tunable absorbance detector set to 220 nm, and a Waters 746 chart recorder.

Unconventional aminoacids synthesis. These compounds (**4a–4e**) were prepared following the procedure reported in Figure 1. H-Glu-OBzl (**1**) was dissolved in 9% Na_2CO_3 and a solution of *N*-(9-fluorenylmethoxycarboniloxy)succinimide (Fmoc-Osu) in DMF was added dropwise. The obtained Fmoc-Glu-OBzl [21] (**2**) was condensed with the appropriate *tert*-butyl aminoalkylcarbamate [NH_2-$(CH_2)_n$-NH-Boc where $n = 2$, 3, 4, 5 or 6] using DCC/HOBt as coupling reagents to obtain intermediates **3a–3e**. Hydrogenation with 5% palladium on activated carbon (Pd/C) in methanol produced the desired final aminoacid derivatives **4a–4e** that were purified by silica gel column chromatography and crystallization.

Fmoc-Glu-OBzl (2). H-Glu-OBzl (**1**) (3.0 g, 0.0126 mol) was suspended in 25 ml of 9% Na_2CO_3 and cooled in ice water. A solution of Fmoc-OSu (4.7 g, 0.014 mol) in 25 ml of DMF was then added dropwise and the mixture was stirred at room temperature (r.t.) for 5 h. The solvent was evaporated and the residue, diluted with water, was acidified to pH 2 with 2 N H_2SO_4. Chloroform was then added and the collected organic phase was dried on anhydrous Na_2SO_4

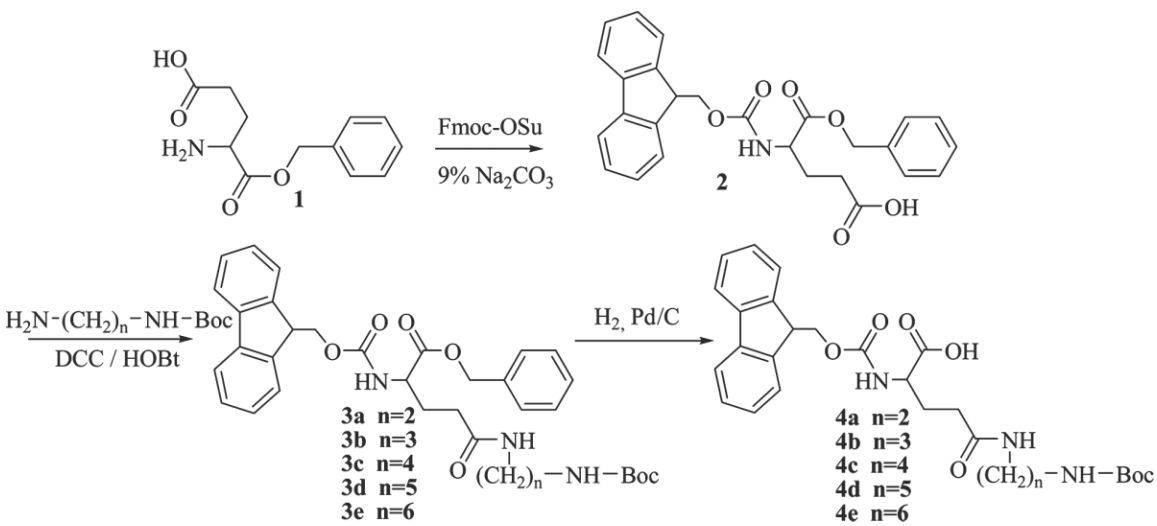

Figure 1 Procedure for the synthesis of unnatural residues **4a–4e**.

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and evaporated. Crystallization from *n*-hexane generated the desired pure product as a white solid: yield 5.5 g (95%).

Fmoc-Glu(NH(CH$_2$)$_2$NHBoc)-OH (4a). DCC (0.6 g, 2.9 mmol) was added portionwise to an ice-cold solution of Fmoc-Glu-OBzl (**2**, 1.2 g, 2.6 mmol) and HOBt (0.4 g, 2.9 mmol) in DMF (25 ml). After completing the addition, the reaction mixture was stirred at 0 °C for 15 min and then (2-aminoethyl)carbamic acid *tert*-butyl ester (0.42 g, 2.6 mmol) was added. The solution was stirred at r.t. overnight; DCU was filtered off and the solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate and washed with 5% $NaHCO_3$, 10% citric acid, and brine. The organic phase was dried on anhydrous Na_2SO_4 and evaporated *in vacuo*. The intermediate Fmoc-Glu[NH(CH$_2$)$_2$NHBoc]-OBzl (**3a**) was precipitated with diethyl ether and dissolved in methanol (40 ml). The obtained solution was hydrogenated at 2 atm at r.t. for 30 min in presence of 5% Pd/C (130 mg). After completion of the reaction, the mixture was filtered on celite and the solvent was removed *in vacuo*. The pure product **4a** was crystallized from diethyl ether/*n*-hexane: yield (calculated on two steps) 0.95 g, 73%. mp 133–135 °C. ESI-MS (MW 511.6): 512.2 [M + H$^+$]. Elemental analysis: calcd for $C_{27}H_{33}N_3O_7$: C, 63.39%; H, 6.50%; N, 8.21%; obtained: C, 63.52%; H, 6.51%; N, 8.03%.

Using the above described procedure for the preparation of **4a**, the following additional derivatives (**4b**–**4e**) were synthesized using as starting material Fmoc-Glu-OBzl (**2**) and (3-amino-propyl)-carbamic acid *tert*-butyl ester, (4-amino-butyl)-carbamic acid *tert*-butyl ester, (5-amino-pentyl)-carbamic acid *tert*-butyl ester and (6-amino-hexyl)-carbamic acid *tert*-butyl ester, respectively:

Fmoc-Glu(NH(CH$_2$)$_3$NHBoc)-OH (4b). Yield 70%. mp 114-116 °C. ESI-MS (MW 525.6): 526.4 [M + H$^+$]. Elemental analysis: calcd for $C_{28}H_{35}N_3O_7$: C, 63.98%; H, 6.71%; N, 7.99%; obtained: C, 63.78%; H, 6.84%; N, 7.97%.

Fmoc-Glu(NH(CH$_2$)$_4$NHBoc)-OH (4c). Yield 75%. mp 110-112 °C. ESI-MS (MW 539.6): 540.8 [M + H$^+$]. Elemental analysis: calcd for $C_{29}H_{37}N_3O_7$: C, 64.55%; H, 6.91%; N, 7.79%; obtained: C, 64.61%; H, 6.89%; N, 7.81%.

Fmoc-Glu(NH(CH$_2$)$_5$NHBoc)-OH (4d). Yield 77%. mp 104-106 °C. ESI-MS (MW 553.6): 554.5 [M + H$^+$]. Elemental analysis: calcd for $C_{30}H_{39}N_3O_7$: C, 65.08%; H, 7.10%; N, 7.59%; obtained: C, 64.90%; H, 7.12%; N, 7.61%.

Fmoc-Glu(NH(CH$_2$)$_6$NHBoc)-OH (4e). Yield 73%. mp 94-96 °C. ESI-MS (MW 567.7): 568.6 [M + H$^+$]. Elemental analysis: calcd for $C_{31}H_{41}N_3O_7$: C, 65.59%; H, 7.28%; N, 7.40%; obtained: C, 65.40%; H, 7.31%; N, 7.38%.

Peptide synthesis. Compounds **I–V** were prepared by solid phase peptide synthesis on 0.1 mmol of a Rink amide MBHA resin (0.78 mmol/g substitution grade) using a Milligen 9050 peptide synthesizer. Fmoc was employed as α-amino protecting group and TBTU/HOBt/NMM in DMF/*N*-methylpyrrolidone (NMP) (1/1) were applied to the coupling reactions. Four times excess of each aminoacid was used and double coupling and extended coupling time were employed when Val^{19}, Ala^{18}, Met^{17}, Lys^{15}, Phe^{6}, Val^{5}, Ala^{4} and unnatural derivatives **4a–4e** were incorporated; capping was accomplished with 20% acetic anhydride in DMF. Cleavage of *N*-Fmoc protecting group, using 25% piperidine (v/v) in DMF/NMP (1/1), was monitored at each stage by measuring the absorbance of the liberated *N*-(9-fluorenylmethyl)piperidine. Protected peptidyl resins were treated with 20 ml of TFA/triethylsilane/H_2O (90/7/3) at r.t. for 2 h; the mixture was filtered and the resin washed with TFA/CH_2Cl_2 (1/1). The combined filtrate and washings were evaporated and the free peptides, **I–V**, precipitated with diethyl ether. The crude material was purified by preparative HPLC on a Vydac C_{18} column (15–20 μm, 22 × 5000 mm) employing the following conditions: eluent A, 0.1% TFA (v/v) in water; eluent B, 0.1% TFA(v/v) in acetonitrile; linear gradient 0–35% B over 60 min. Homogeneity of the products was assessed by analytical reversed-phase HPLC using a Vydac C_{18} column (5 μm, 4.6 × 250 mm) employing the following conditions: eluent A, 0.05% TFA (v/v) in water; eluent B, 0.05% TFA(v/v) in acetonitrile; linear gradient 0–40% B over 35 min. Structural verification of the final desired peptides **I–V** was achieved by aminoacid analysis and mass spectrometry (Tables 1 and 2).

Molecular Modeling

Molecular models of the aminoacids glutamine, arginine, glutamate and their glutamyl derivatives have been created with the Insight II package (Accelrys, San Diego, CA). The Builder module and its tools have been used to modify the side chain by adding the appropriate number of C atoms and hydrogen atoms. The amino and carboxyl terminals were blocked with an acetyl group at the *N*-term and an amidic group at the *C*-term to avoid terminal charge effects. Finally, an energy minimization procedure has been applied to optimize the geometry, according to the standard settings of the optimize option of the package. Distances also have been measured with the package tools.

CHO Cell Line Culture

The recombinant Chinese Hamster Ovary (CHO) cells expressing the rat or human recombinant VPAC1 and VPAC2 receptors were prepared in Prof. P. Robberecht's laboratory. Cells were maintained in α-minimal essential medium (α-MEM), supplemented with 10% FCS, 2 mM L-glutamine, 100 μg ml^{-1} penicillin and 100 μg ml^{-1} streptomycin with an atmosphere of 95% air and 5% CO_2 at 37 °C. Geneticin (0.4 mg ml^{-1}) was maintained in the culture medium of the stock culture. Subcultures prepared for membrane purification were done in a medium without geneticin.

Membrane Preparation, Ligand Binding (IC50), and Adenylate Cyclase Activity (EC50) Determination

An appropriate number of CHO cells was harvested with a rubber policeman and pelleted by low speed centrifugation, the supernatant was discarded and the cells were lysed in 1 mM $NaHCO_3$ and quick freezing in liquid nitrogen. After thawing, the lysate was centrifuged at 4 °C for 10 min at 400 × g and the supernatant was further centrifuged at 20 000 × g for 10 min. The final pellet was resuspended in 1 mM $NaHCO_3$ and used immediately as a crude membrane preparation. [^{125}I]VIP (specific radioactivity: 0.7 mCi mmol^{-1}) was used as tracer for the identification of both rat or human VPAC1 receptors; [^{125}I]Ro 25–1553 (specific radioactivity: 0.8 mCi mmol^{-1}) was used as tracer for labelling both rat and human

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Table 1 Sequence and MS analysis of VIP analogues **I–V**

Comp[a]	Sequence	MW	
		Calcd[b]	Found
I	HSDAVFTDNYTRLRKQ[$(CH_2)_2NH_2$]MAVKKYLNSILN	3368.9	3369.2
II	HSDAVFTDNYTRLRKQ[$(CH_2)_3NH_2$]MAVKKYLNSILN	3382.9	3383.7
III	HSDAVFTDNYTRLRKQ[$(CH_2)_4NH_2$]MAVKKYLNSILN	3397.0	3398.0
IV	HSDAVFTDNYTRLRKQ[$(CH_2)_5NH_2$]MAVKKYLNSILN	3411.0	3411.5
V	HSDAVFTDNYTRLRKQ[$(CH_2)_6NH_2$]MAVKKYLNSILN	3425.1	3425.8

[a] Compound.
[b] Calculated.

Table 2 Analytical data of VIP analogues **I–V**

Comp[a]	Aminoacid analysis[b]												HPLC[c]
	H	S	D	A	V	F	T	Y	R	L	K	I	
I	1.03	1.81	4.65	1.96	1.94	0.96	1.89	1.87	1.81	2.84	2.94	0.89	19.1
II	0.98	1.90	4.85	1.90	2.02	0.94	1.91	1.94	1.96	2.93	2.83	0.91	19.3
III	1.01	1.85	4.93	2.07	1.89	1.04	1.94	1.99	1.84	2.87	2.97	0.94	19.9
IV	0.86	1.82	4.99	1.86	1.98	0.96	1.99	1.85	1.85	3.01	2.91	0.99	20.2
V	0.85	1.84	4.87	2.01	1.87	1.03	2.01	1.95	1.92	3.02	3.02	1.01	20.1

[a] Compound.
[b] The method used for hydrolysis does not allow the recovery of methionine, while asparagine is completely converted to aspartic acid.
[c] Retention time for the following conditions: reversed phase, Vydac C_{18} column (5 μm, 4.6 × 250 mm) and the following gradient system: A, 0.1% TFA in CH_3CN; B, 0.1% TFA in H_2O; linear gradient from 0% A–100% B to 60% A–40% B over 25 min, UV detection at 220 nm, flow rate 1 ml/min.

VPAC2 receptors [22]. The binding of labelled ligands to CHO purified membranes was performed as described [9]; in all the cases the nonspecific binding was defined as the residual binding in the presence of 1 μM VIP. Competition curves were carried out by incubating membranes and tracer in the presence of increasing concentrations of unlabelled peptides. Peptide potency was expressed as IC50 value, i.e. as the peptide concentration required for half maximal inhibition of tracer binding. In detail, the binding was performed at 37 °C in a buffer containing 20 mM Tris-maleate (pH 7.4), 2 mM $MgCl_2$, 0.1 mg ml^{-1} bacitracin and 1% BSA; 3–30 protein was used per assay. The bound was separated from the free? radioactivity by filtration through glass fiber filters GF/C presoaked for 24 h in 0.1% polyethyleneimine and rinsed three times with a 20 mM phosphate buffer (pH 7.4) containing 1% BSA. Adenylate cyclase activity was determined by a previously published technique [23]. Membrane proteins (3–15 mg) were incubated in a total volume of 60 μl containing 0.5 mM [γ-^{32}P]ATP, 10 μM GTP, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM cAMP, 1 mM theophylline, 10 mM phosphoenolpyruvate, 30 μg ml^{-1} pyruvate kinase, and 30 mM Tris-HCl at a final PH of 7.5. The reaction was initiated by membrane addition and was terminated after a 12-min incubation at 37 °C by adding 0.5 ml of stopping buffer (0.5% SDS, 0.5 mM ATP, 0.5 mM cAMP, 20 000 cpm of [8^3H] cAMP). cAMP was separated from ATP by two successive chromatographies on Dowex 50-WX8 and neutral alumina.

Statistical Analysis

The data were expressed as the mean ± standard error of the mean (SEM) obtained from three separate experiments. Each experiment included eight samples, each sample being performed in triplicate. One-way analysis of variance (ANOVA) was used to assess statistical significance between means. Differences between means were considered significant when $p < 0.05$ using the Bonferroni post-test. All statistical analyses were performed with the GraphPad Instant software (Intuitive Software for Science, San Diego, CA).

RESULTS AND DISCUSSION

Unnatural Aminoacids – Chemical Synthesis

The unnatural aminoacid derivatives **4a–4e**, employed for the chemical synthesis of VIP analogues **I–V**, have been prepared following the procedure reported in Figure 1. Commercially available H-Glu-OBzl (**1**) was converted to the corresponding Fmoc derivative (**2**) by means of Fmoc-OSu in 9% Na_2CO_3 and DMF solution. The obtained Fmoc-Glu-OBzl (**2**) was reacted with the appropriate *tert*-butyl aminoalkylcarbamate [NH_2–$(CH_2)_n$–NH–Boc where $n = 2$, 3, 4, 5 or 6] using

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DCC/HOBt as coupling reagents to give intermediates **3a–3e**. Hydrogenation with 5% palladium on activated carbon (Pd/C) in methanol, at r.t. for 30 min, removed the N^{α}-benzyl ester protecting group furnishing the desired final Fmoc aminoacid derivatives, **4a–4e**, that were purified by silica gel column chromatography and crystallization. Some difficulties have been encountered in the synthesis of 28 residues peptides (**I–V**) primarily because of the presence of aminoacids (such as Arg, Met and Tyr) that are prone to undergoing side reactions during either the coupling steps or the final deprotection and also because of sequences that may be considered problematic (Ala^4-Val^5-Phe^6 and Met^{17}-Ala^{18}-Val^{19}). Peptide synthesis was carried out on a continuous-flow Milligen 9050 peptide synthesizer using a Rink amide MBHA resin. Fmoc was employed as α-amino protecting group utilizing two pre- and post-column UV detectors to measure the absorbance of the solution flowing through it obtaining a feedback monitoring of each step of the synthetic process. Several protocols have been used, differing in employed coupling reagents and reaction times; the quality of the syntheses was examined following the incorporation of every five residues via HPLC and ESI-MS of the crude product obtained by cleavage of an aliquot of peptidyl resin with TFA/triethylsilane/H_2O (90/7/3). The following protocol, optimized with the synthesis of native VIP sequence, gave the best results: (i) four times excess of each residue was employed, TBTU/HOBt/NMM as coupling reagents in DMF/NMP (1/1); (ii) double coupling and extended coupling times were employed for Val^{19}, Ala^{18}, Met^{17}, Lys^{15}, Phe^6, Val^5, Ala^4 and unnatural derivatives **4a–4e**; (iii) extended time for Fmoc removal (15 min instead of 7 min) was used when slow Fmoc removal was observed; (iv) capping was accomplished for every four residues with 20% acetic anhydride in DMF. Finally, the cleavage of the peptides from the resin was effected using a mixture of TFA/triethylsilane/H_2O (90/7/3). All the final compounds, purified by RP-HPLC to greater than 98% purity, gave satisfactory aminoacids analyses and were characterized by ESI-MS (Tables 1 and 2).

Structural Models of Diamino Glutamates Used in VIP Modification

The structural model of both the different neo-synthesized diamino glutamates and the lateral chain of glutamine, glutamate, and arginine are reported in Figure 2. In more detail, the distance of the positive charge from the Cα at VIP position 16, is reported in Table 3.

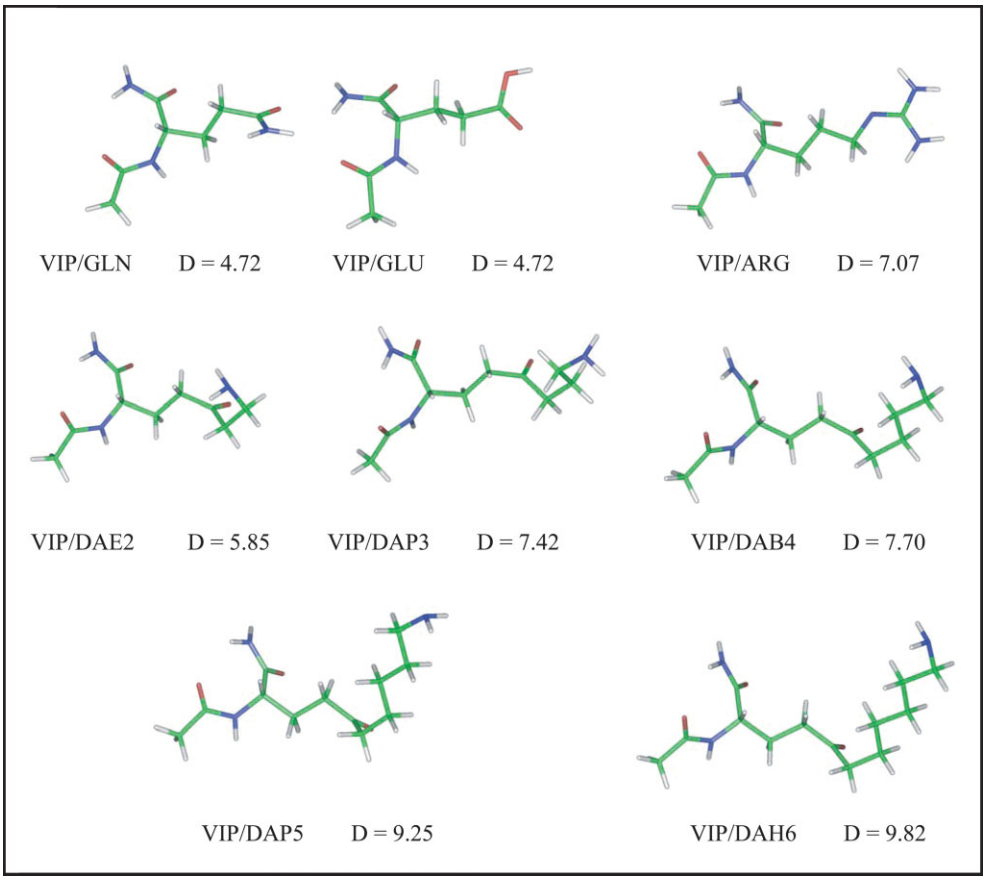

Figure 2 Structural models of the neo-synthesized VIP derivatives interacting with human VPAC1 and VPAC2 receptors. D = Distance (Å) of Z^+ from $^{16}C\alpha$.

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Table 3 IC50 and EC50 values of some VIP analogues, polyaminated at position 16 with polyamines of different length, following binding to hVPAC1 or hVPAC2

Aminoacid at Vip position 16	Distance (Å) of polyamine Z^+ from $^{16}C\alpha$	hVPAC1		hVPAC2	
		IC50 [nM]	EC50 [nM]	IC50 [nM]	EC50 [nM]
GLN	4.72	1.63 ± 0.26	0.87 ± 0.01	1.93 ± 0.26	1.17 ± 0.03
GLU (Z^-)	4.72	10.81 ± 0.27	4.03 ± 0.21	10.21 ± 0.27	5.35 ± 0.07
ARG	7.07	0.61 ± 0.27	0.54 ± 0.08	2.32 ± 0.27	3.08 ± 0.04
γGLU[a]-DAE2	5.85	0.85 ± 0.26	0.69 ± 0.10	3.55 ± 0.27	1.67 ± 0.04
γGLU[a]-DAP3	7.42	0.68 ± 0.26	0.46 ± 0.09	3.42 ± 0.27	1.57 ± 0.04
γGLU[a]-DAB4	7.70	1.22 ± 0.27	0.66 ± 0.11	3.19 ± 0.28	2.35 ± 0.05
γGLU[a]-DAP5	9.25	0.85 ± 0.26	0.45 ± 0.09	2.57 ± 0.27	1.08 ± 0.06
γGLU[a]-DAH6	9.82	0.86 ± 0.26	0.43 ± 0.14	2.06 ± 0.26	0.86 ± 0.06

[a] γGLU- : γglutamyl-.

Effect of VIP $^{16}\gamma$-Glutamyldiamino Derivatives Z^+ on VIP Receptors Functional Activity

In Table 3 are reported the EC50 and IC50 values for hVPAC1 and hVPAC2, respectively, as a function of the distance of positive charge from VIP Cα at position 16. In particular, for hVPAC1 the EC50 and IC50 patterns are remarkably similar, in that the receptor activity mirrors the ligand binding events (Figure 3, left panel). Different results were, in contrast, obtained for hVPAC2 (Figure 3, right panel): EC50 and IC50 patterns are not overlapping because the IC50 of VIP-DAB4 does not reflect its EC50.

The polyamination of Gln16 side chain significantly modulates the ability of VIP to bind and stimulate the hVPAC1 receptor functional activity [17]. This finding supports Robberecht's data indicating the critical role played by the presence of the positively charged arginine at position 16 of VIP polypeptide chain [13]. In addition, the possibility that the side chain length could play an important role in modulating the receptor binding ability and activity is supported by the IC50 and EC50 data, demonstrating a better VIP-Dap functional activity in comparison with VIP, VIP-SPD, and VIP-SPM. Previous work has also showed that in comparison with VIP, VIP-DAP3 possesses a better functional and biological activity on hVPAC1 receptor, probably as a mirror of its higher structural stability [18,19]. The possible existence in the receptor of different dynamic conformational states corresponding to different states of activation [24,25], allows us to hypothesize that the presence at position 16 of a positive charge, associated with possible hydrophobic interactions and a definite side chain length, could be effective in stabilizing a conformational state corresponding to the highest binding affinity with or without change in receptor activity. The hypothesis that a positive charge at the position 16 of VIP could play an important role in the ligand–receptor recognition mechanism, is also supported by the published data on the charge distribution in VPAC receptor aminoacid sequence [17].

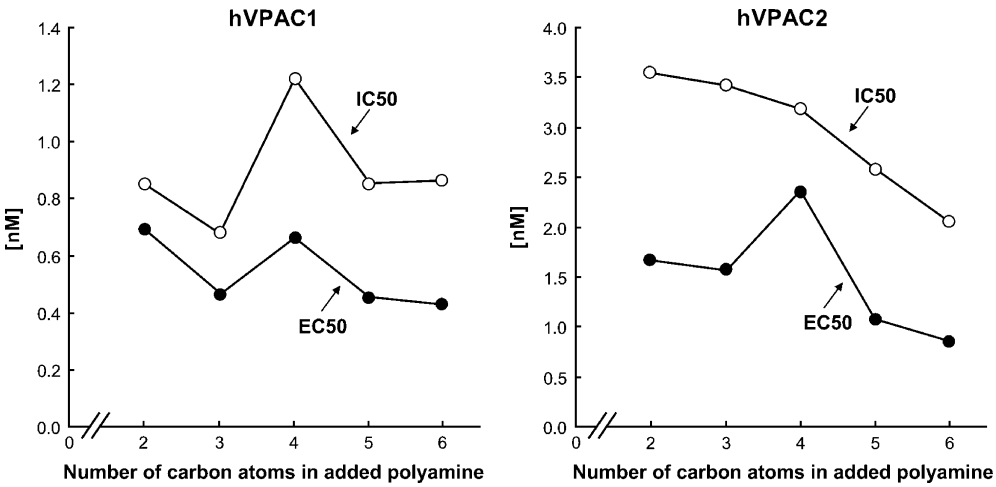

Figure 3 IC50 and EC50 patterns produced by VIP analogues polyaminated at position 16 with polyamines of different lengths.

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In this paper, we have reported data showing a novel chemical method for the synthesis of a family of VIP analogues (five distinct diamino derivatives different among them for a single carbon atom), aiming at analyzing the effects of the positive charge distance from the VIP Cα at position 16 on VIP receptor activity. For the synthesis of these compounds, a chemical technique was substituted for our previous enzymatic method with TGase: this novel technique is faster, less expensive, and more flexible for the pharmacological analysis. The chemical modification is performed, before the synthetic step, on a glutamic acid residue that is then inserted in the polypeptide chain sequence at position 16. In contrast, in the enzymatic TGase catalyzed synthesis the VIP is first incubated with the enzyme in the presence of the diamines to allow the formation of the VIP-γ-derivative, the latter being then separated from the unmodified VIP by HPLC and purified.

The analysis of the atomic distances evaluated by using computational models of the lateral chains (Figure 2), has shown for VIP-DAP3 a distance of of 7.42 Å for the positive charge Z^+ from the Cα at position 16. Starting from this distance, we have analyzed the effect of Z^+ on the hVPAC1 and hVPAC2 receptors in a range of about 4 Å, i.e. from the 5.85 Å of VIP-DAE2 to the 9.82 Å of VIP-DAH6. By analyzing the functional effects (IC50 and EC50) of the neo-synthesized analogues on VIP receptors as a function of the progressive increase in length of the lateral chains, a significant difference was found between hVPAC1 and hVPAC2 receptors. In particular, a comparable pattern of EC50 and IC50 was clearly detectable for the hVPAC1 receptor with a clear peak appearing when Z^+ was at a distance of 7.70 Å (see VIP-DAB4). In this case, the ligand binding to hVPAC1 significantly ($p < 0.05$) changes as a distance function of VIP-DAP3 (7.25 Å) and VIP-DAP5 (9.25 Å) with 80 and 40% IC50 increase, respectively. In line with this finding, the EC50 increase was higher than 40% for these two homologues (Table 3 and Figure 3). In contrast, hVPAC2 shows a progressive IC50 decrease as function of carbon chain length increase. On the other hand, on the same receptor the EC50 pattern differs for the presence of a peak at the same 7.70 Å distance (VIP-DAB4) as in the case of hVPAC1 receptor, with a 50% (VIP-DAP3) and a 118% (VIP-DAP5) increase in EC50, respectively (Table 3 and Figure 3). Furthermore, on hVPAC2 the IC50 of VIP-DAH6 appears comparable with that of VIP, although there is a significant increase (26%) of functional activity (EC50), showing for the first time that the occurrence of a positive charge in position 16 can generate a better agonist of hVPAC2 receptor. This finding suggests that a further increase in the length of lateral chain could results in a superagonist production. At present, the syntheses of additional $^{16}\gamma$-glutamyl diamino VIP derivatives, possessing different lengths and compositions, are in progress for a more complete analysis of their functioning.

Taken together, all these data suggest: (i) the possibility to discriminate hVPAC1 from hVPAC2 on the basis of their different IC50, but not EC50, patterns; (ii) a different structural interaction between the VIP-DAB4 ligand and the two distinct receptor domains involved in ligand binding and functional activation of both receptors, the activation module of hVPAC2 receptor acting probably independent from the binding module; (iii) the possibility that position 16 of the VIP, even if actually used in the best hVPAC1 agonists and antagonists when substituted with arginine, could be used, if appropriately modified, in agonists and/or antagonists of hVPAC2 receptor.

CONCLUSIONS

We have synthesized a novel family of VIP analogues including five distinct diamino derivatives differing among them for a single carbon atom (see above) aiming at analyzing the effects of the positive charge distance from the Cα at position 16 on receptor activity. For the synthesis of these compounds, a novel chemical technique was used in place of our previous enzymatic method with TGase. This technique was found to be faster, less expensive, and more flexible for pharmacological analysis. By investigating the effect of the positive charge distance from the peptide backbone Cα, we have reported here the data concerning the functional characterization of these $^{16}\gamma$-glutamyl diamino VIP derivatives with human VPAC1 and VPAC2 receptors. More precisely, we have found that the EC50 and IC50 analysis, when evaluated as function of their alkylic chain length, shows that the VIP-DAB4 derivative alters IC50 but not EC50, thus indicating with hVPAC2 receptor an unexpected relationship between binding and activity that differs from that obtained with hVPAC1.

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