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European Journal of Pharmacology 485 (2004) 307–316
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α-Helical structure in the C-terminus of vasoactive intestinal peptide: functional and structural consequences

Satomi Onoue[a,b,*], Asami Matsumoto[a], Yumiko Nagano[c], Keiichi Ohshima[d], Yuki Ohmori[e], Shizuo Yamada[e], Ryohei Kimura[e], Takehiko Yajima[b], Kazuhisa Kashimoto[a]

[a] *Health Science Division, Itoham Foods Inc., 1-2-1 Kubogaoka, Moriya, Ibaraki 302-0104, Japan*
[b] *Faculty of Pharmaceutical Sciences, Toho University, Funabashi, Chiba 274-8510, Japan*
[c] *Department of Gastroenterology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan*
[d] *Applied Genome Informatics Division, Shizuoka Cancer Center Research Institute, Sunto-gun, Shizuoka 411-8777, Japan*
[e] *School of Pharmaceutical Sciences and COE21, University of Shizuoka, Shizuoka 422-8526, Japan*

Received 6 October 2003; accepted 18 November 2003

Abstract

The conformational properties of vasoactive intestinal peptide (VIP) include the N-terminal randomized structure and the C-terminal long α-helical structure. We have previously observed that the N-terminal random coil structure plays a crucial role in the receptor-selectivity. Here, to clarify how the formation of the α-helix plays a role in its biological functions, we chemically synthesized VIP analogues modified at the C-terminus, mid-chain, and N-terminus of the α-helical region, and evaluated the relationship between their α-helical contents and their biological activities including relaxant effects on murine stomach and receptor-binding activities. VIP and VIP-(1–27) showed equipotent biological activities with 48% and 50% α-helical content, respectively, each of which corresponds to 14 amino acid residues. VIP-(1–26) was 10% and threefold less potent in relaxant and binding activities, respectively, compared with VIP, and its 49% α-helical content resulted in 13 residues involved in the α-helix. Further truncation from 25 to 21 resulted in decrease in the α-helical content from 43% to 29%, corresponding residues from 11 to 6, the relaxant activity from 72% to 4%, and the affinity to the membrane from 60-fold to over 10^4-fold less potency. In addition, disruption of the mid-chain and the N-terminus in the α-helical stretch by oxidation of Met^{17} and deletion of Thr^{11} also inhibited biological activities. These findings suggest that the presence of α-helical structure forming in 14 amino acid residues between position 10 and 23 in VIP is essential to its biological functions and the C-terminal amino acid residues between position 24 and 27 are requisite for this α-helical formation.

Keywords: Binding activity; Chemical synthesis; CD (Circular dichroism); Helical structure; Relaxant activity; VIP (vasoactive intestinal peptide) derivative

1. Introduction

Vasoactive intestinal peptide (VIP) is one of the major peptide transmitters in the central and peripheral nervous systems, being involved in a wide range of biological properties in organisms including metabolic processes, exocrine and endocrine secretions, cell differentiation, and smooth muscle relaxation (Delgado et al., 1999; Onoue et al., 2001; Said, 1991; Sun and Ganea, 1993). VIP acts through interaction with two subclasses of G protein-coupled receptors, VPAC1 and VPAC2 (Rawlings and Hezareh, 1996; Vaudry et al., 2000). These VIP-preferring receptors are widely expressed in tissues and cell lines including aorta, lung, brain, stomach, heart, and insulin-secreting cell lines (Inagaki et al., 1996; Vaudry et al., 2000).

VIP belongs to the glucagon/secretin peptide family, which includes glucagon (Unger et al., 1978), secretin (Jorpes, 1968), growth hormone-releasing factor (Brazeau et al., 1982; Rivier et al., 1982), and pituitary adenylate cyclase-activating polypeptide (PACAP) (Miyata et al., 1989). These are short, linear peptides consisting of 27–39 amino acid residues that show significant sequence homology (Vaudry et al., 2000). For example, VIP, an octacosapeptide, has 68% sequence homology with PACAP-(1–27) (PACAP27) (Miyata et al., 1989; Said and

* Corresponding author. Health Science Division, Itoham Foods Inc., 1-2-1 Kubogaoka, Moriya, Ibaraki 302-0104, Japan. Tel.: +81-297-45-6311; fax: +81-297-45-6353.
E-mail address: onoue@fureai.or.jp (S. Onoue).

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doi:10.1016/j.ejphar.2003.11.046

Mutt, 1970). Previous structural analyses including circular dichroism (CD), nuclear magnetic resonance (NMR), and theoretical calculations suggested that there are remarkable similarities in the secondary structures among the glucagon/secretin peptide family, featuring random coil structures at the N-terminuses and long α-helical structures at the C-terminuses (Braun et al., 1983; Clore et al., 1986; Gronenborn et al., 1987; Theriault et al., 1991; Wray et al., 1993; Wray et al., 1998). In the case of VIP, the α-helical region was deduced to extend from approximately residue position 10 to its C-terminus, the end of which was varied depending on the analytical methods and conditions used (Bodanszky and Bodanszky, 1986; Bodanszky et al., 1974; Fournier et al., 1988; Haghjoo et al., 1996; Robinson et al., 1982; Theriault et al., 1991).

We previously investigated the effects of VIP/PACAP chimeric peptides on receptor binding, activation of adenylate cyclase, attenuation of nitric oxide synthase and neurite outgrowth in PC12 cells, demonstrating that the random coil structures at the N-terminuses of VIP and PACAP are necessary to exhibit these biological activities and specificities for the recognition of each specific receptor (Onoue et al., 2002; Onoue et al., 1999; Onoue et al., 2001). On the other hand, the role of the long α-helical structure in the C-terminus of VIP has not been fully elucidated. Other investigators showed that excessive amounts of VIP-(10–28) (Turner et al., 1986) and PACAP-(6–27) (Vandermeers et al., 1992), corresponding sequences to the α-helical components for VIP and PACAP, competitively inhibited bindings of VIP and PACAP to VPAC1/2 receptors and PAC1 receptor (PACAP-specific receptor), respectively, suggesting that the α-helical components of VIP and PACAP strongly influence their biological functions.

Herein, in order to define the role of the long α-helical structure at the C-terminus of VIP, we synthesized VIP derivatives in which the α-helical region was site-specifically modified, and investigated the effects of these chemical modifications on their biological activities and formation of secondary structures. We demonstrate here a novel structure–activity relationship of VIP showing that the long α-helical structure is important for its biological activity.

2. Material and methods

2.1. Peptide synthesis

VIP and its derivatives (Table 1) were synthesized on a MBHA (*p*-methylbenzhydrylamine)-resin by the solid-phase *t*-Boc strategy employing optimum side chain protection as reported previously (Merrifield, 1969). Peptides were removed from the resin by HF treatment, and the cleaved products were purified using C-18 column chromatography (Fuji Silysia, Aichi, Japan). The purity (>98%) was checked by reverse-phase HPLC (high performance liquid chromatography) using a TSK-gel ODS-120T column (Tosoh, Tokyo, Japan), and the homogeneity was assessed by amino acid analyses using an amino acid analyzer L-8500 (Hitachi, Tokyo, Japan). Their molecular weights were confirmed on a matrix-assisted laser desorption ionization-time of flight mass spectrometer (Kratos, Manchester, UK).

2.2. Tissue preparation and tension recordings

The whole stomachs were dissected from 6- to 10-week-old male ICR mice (Japan SLC, Shizuoka, Japan). One end of the muscle strip was attached via an inelastic wire to an isometric force transducer (model TB-611T; Nihon Kohden, Tokyo, Japan) connected to a four-channel signal recorder (model R-64M; Rikadenki, Tokyo, Japan). The stomach was bathed under a load of 1 g in Krebs–Ringer solution (pH 7.4) containing 118.1 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$

Table 1
Peptide sequences of synthetic VIP analogues

Peptides	5									10								15								20								25			
VIP	H	S	D	A	V	F	T	D	N	Y	T	R	L	R	K	Q	M	A	V	K	K	Y	L	N	S	I	L	N									
Shortened analogues																																					
VIP-(1–27)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–										
VIP-(1–26)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–											
VIP-(1–25)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–												
VIP-(1–24)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–													
VIP-(1–23)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–														
VIP-(1–22)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–															
VIP-(1–21)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–																
Methionine-oxidized																																					
[Met(O)^{17}]VIP-(1–25)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	M*	–	–	–	–	–	–	–	–												
Leucine-substituted																																					
[Leu^{17}]VIP-(1–23)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	L	–	–	–	–	–	–														
[des-Thr^{11}, Leu^{17}]VIP-(1–23)	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	L	–	–	–	–	–	–														

All peptides were synthesized by the solid-phase strategy employing optimal side-chain protection and amidated at their C-terminuses. The oxidized methionine at position 17 in [Met(O)^{17}]VIP-(1–25) is designated by an asterisk.

and 11.1 mM glucose. The bath was bubbled continuously with 95% O_2 and 5% CO_2 at 37 °C, and the custom-made glass organ bath was flushed with Krebs–Ringer solution. Prior to starting the experiment, the muscle strips were allowed to stabilize in the bath containing saline for 15–20 h and Krebs–Ringer solution for an additional 2 h. The difference in tension between the relaxant activities against carbachol (3×10^{-5} M)-induced contraction before and after loading VIP (10^{-6} M) was regarded as 100% active tension.

2.3. Circular dichroism analysis of synthetic VIP-related peptides

For circular dichroism (CD) analysis, each peptide was dissolved in either 20 mM Tris–HCl (pH 7.4) or 50% (v/v) methanol. CD spectra were baseline-corrected and smoothed using the algorithm provided by the manufacturer, and they were recorded at room temperature in a Jasco model J-720 spectropolarimeter (Jasco, Tokyo, Japan) with a cell path length of 10 mm. Each sample was scanned five times in the wavelength range of 180–400 nm. Ellipticity was calculated as mean residue ellipticity [θ] (° cm^2 $dmol^{-1}$). The secondary structures of synthetic peptides were evaluated from CD spectra using four independent methods established by Chen et al. (1972), Greenfield and Fasman (1969), and software packages for analyzing the CD spectra including k2d (Andrade et al., 1993) and CDPro (Sreerama and Woody, 2000).

2.4. [^{125}I]VIP binding assay in rat lung

Male Sprague–Dawley rats at 9–10 weeks of age (Japan SLC, Shizuoka, Japan) were sacrificed by taking the blood from descending aorta under anesthesia with pentobarbital, and the lung tissues were perfused with 0.9% saline and removed. The tissues were homogenized in 19 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 250 mM sucrose, 5 mM $MgCl_2$ and 0.1% bacitracin with a Polytron homogenizer, and the homogenate was centrifuged at 30,000 × g for 20 min. The pellet was finally suspended in ice-cold 25 mM Tris–HCl buffer containing 5 mM $MgCl_2$, 0.1% bacitracin and 0.2% BSA and used in [^{125}I]VIP binding assay. All steps for the tissue preparation were performed at 4 °C. All the procedures used in the present study were conducted according to the guideline approved by the Institutional Animal Care and Ethical Committee of University of Shizuoka.

[^{125}I]VIP binding assay was performed by a modification of the procedure described by Leroux et al. (1984). Briefly, the membrane fractions (20–40 μg protein) prepared from rat lung tissues were incubated with 0.3 nM [^{125}I]VIP (PerkinElmer Life Sciences, Boston, MA, USA) in a total volume of 500 μl of 25 mM Tris–HCl buffer containing 5 mM $MgCl_2$, 0.1% bacitracin and 0.2% BSA. Incubation was carried out for 3 h at 4 °C. The reaction was terminated by rapid filtration (Cell Harvester, Brandel, Gaithersburg, MD, USA) through Whatman GF/C glass fiber filters presoaked in 0.1% polyethyleneimine solution for 1 h, and the filters were rinsed three times with 2 ml of ice-cold buffer. Tissue-bound radioactivity was determined in a gamma counter. The specific binding of [^{125}I]VIP was determined experimentally from the difference between counts in the absence and presence of 3 μM unlabeled VIP. In a typical study, specific binding was 70.8 ± 2.6% (mean ± S.E.M., n = 6) of total binding of [^{125}I]VIP at the concentration (0.50 nM) close to the K_d value (0.60 nM). All assays were conducted in duplicate.

The ability of VIP and its shortened analogues to inhibit the specific binding of [^{125}I]VIP (0.3 nM) was estimated from the IC_{50} values, which are the molar concentrations of unlabeled drug necessary to displace the 50% specific binding of [^{125}I]VIP (determined by log probit analysis).

2.5. Statistical evaluation

Statistical evaluation was performed by the Student's t-test or one-way analysis of variance (ANOVA) along with pairwise comparison by the Fisher's least significant difference procedure. P values less than 0.05 were considered to be significant in all the analyses.

3. Results

3.1. Relaxant effects of VIP and its C-terminally shortened derivatives on murine stomach

We synthesized VIP, a 28 amino acid peptide, and seven C-terminally truncated VIP analogues with a range from 27 to 21 amino acid residues, in which the C-terminuses were amidated as well as VIP (Table 1). Since it is well-established that both VIP and PACAP exert inhibitory effects on the motility of gastric muscle through VIP-preferring VPAC2 receptors in many mammals (Inagaki et al., 1996; Parkman et al., 1997; Reubi et al., 2000; Robberecht et al., 1998), we investigated the relaxant effects of synthetic VIP analogues on the carbachol (3×10^{-5} M)-induced submaximal contraction of murine stomach. VIP at the concentration of 10^{-6} M significantly inhibited the effect of contraction and this relaxant effect was 1.4-fold higher than that of scopolamine butylbromide (10^{-6} M), an anticholinergic agent (data not shown). When we tested C-terminally truncated VIP derivatives, the degrees of these relaxant effects were remarkably weakened in proportion to the number of composing amino acid residues (Fig. 1A). At the concentration of 10^{-6} M, both VIP-(1–22) and VIP-(1–21) showed little relaxant activity, whereas other VIP-related analogues in which the number of amino acid residues exceeded 22 (residue number ≧ 23) exhibited potent relaxant effects compared with VIP. Both VIP-(1–27)

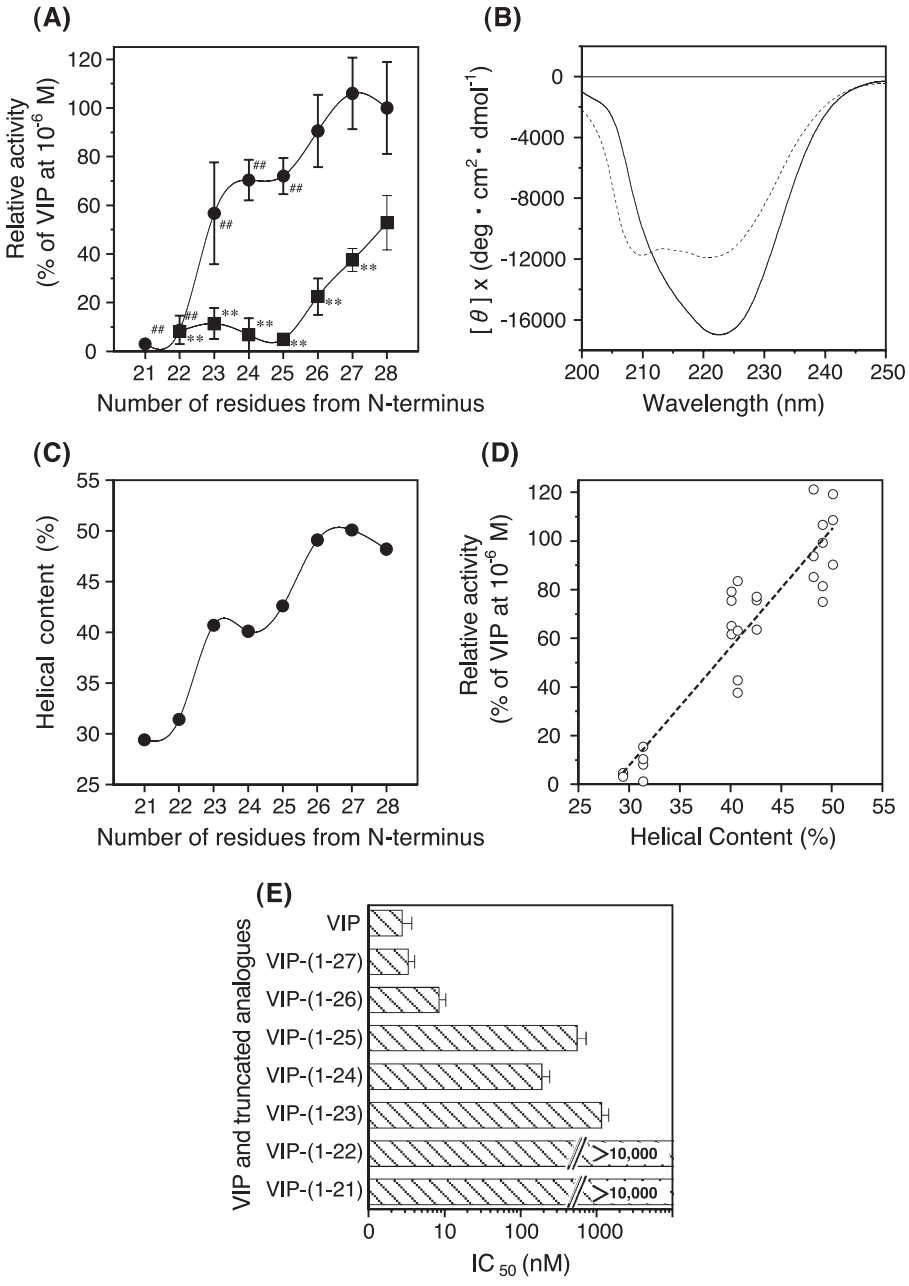


Fig. 1. Effects of VIP and its C-terminally shortened analogues on the carbachol-induced contraction of murine stomach, the formation of secondary structures, and their binding activity to rat lung membrane. (A) The relaxant effects of VIP and its shortened analogues at the concentration of 10^{-6} M (●) and 10^{-8} M (■) against the carbachol-induced contraction of murine stomach in vitro. The relaxant effects are represented as a percentage of VIP (10^{-6} M). Mean values with SD bars ($n>3$). $^{\#\#}P<0.01$ or $^{**}P<0.01$ compared with VIP at the same concentration. (B) CD spectra of synthetic VIP and VIP-(1–22). The measurements of VIP (solid line) and VIP-(1–22) (dashed line) were carried out in 50% methanol/20 mM Tris–HCl buffer (pH 7.4). (C) α-Helical contents calculated based on the CD data for VIP and its shortened analogues. The α-helical content of each peptide was estimated according to the formula established by Chen and Yang. (D) Correlation between relaxant activities and α-helical contents in VIP and its C-terminally shortened analogues. The correlation coefficient between the α-helical content and the relaxant effect at the concentration of 10^{-6} M was estimated to be $r=-0.93$ ($P<0.01$). (E) Inhibitory effects (IC_{50} values) of VIP and its C-terminally shortened analogues on specific [^{125}I]VIP binding to the rat lung. Rat lung homogenate was incubated with [^{125}I]VIP (0.3 nM) and various concentrations of unlabeled peptides at 4 °C for 3 h, and their IC_{50} values were measured from the displacement curves of specific [^{125}I]VIP binding. Data represents the mean ± SE for seven (VIP) and three to five (shortened analogues) determinations.

and VIP-(1–26) showed almost equal relaxant activities with VIP. VIP-(1–25), VIP-(1–24), and VIP-(1–23) had 60–70% activities of VIP. At the final concentration of 10^{-8} M, the relaxant effect of VIP was reduced to half the activity at 10^{-6} M, and the C-terminally truncated analogues including even VIP-(1–27) and VIP-(1–26) resulted in a significant decrease in the relaxant effect by VIP in proportion to the peptide length. Hence, these results indicate that the C-terminal region in VIP has a strong link to its biological functions.

3.2. Secondary structures of VIP and its C-terminally shortened analogues

In order to clarify the relationship between relaxant effects and secondary structures of the C-terminally shortened analogues, we used CD spectral analysis to investigate the effects of truncation at the C-terminus of VIP on the formation of secondary structure. A previous report indicated that the CD spectrum of the β-turn structure displayed an intense positive band at 206 nm and a weak negative band at 228 nm, and that of the α-helical structure showed an intense positive band at 192 nm and two extreme negative bands at 209 and 222 nm (Greenfield and Fasman, 1969). CD spectra of VIP and VIP-(1–22) in 50% methanol/20 mM Tris–HCl buffer (pH 7.4) exhibited the existence of a combination of short β-turn and long α-helical structures in both peptides (Fig. 1B). The formula established by Chen et al. (1972) was commonly used for the determination of the α-helical contents in peptides/proteins, based on the mean residue helicity at 222 nm (Liu et al., 1996; Park et al., 2000; Tanaka et al., 1999). According to this calculative method, VIP and VIP-(1–22) were estimated to contain 48% and 31% α-helical structure, corresponding to 14 and seven residues in the 28 and 22 amino acid peptides, respectively. The α-helical contents in the C-terminally truncated analogues gradually decreased in proportion to the number of composing amino acid residues (Fig. 1C). VIP-(1–27) and VIP-(1–26) showed 50% and 49% α-helical content similar to VIP, corresponding to 14 and 13 residues, respectively. The α-helical contents of VIP-(1–25), VIP-(1–24), and VIP-(1–23) ranged from 43% to 40%, and their corresponding residues were 11, 10, and 9, respectively. VIP-(1–21) showed 29% α-helical content similar to VIP-(1–22), corresponding to six residues. These results indicate the important role of C-terminal moieties in the formation of the α-helical structure. The contents of β-turn structure in VIP and its truncated analogues were calculated to the narrow range between 8.2% and 13.0% by CDPro software (Sreerama and Woody, 2000), indicating no significant correlation with the number of composing amino acid residues (data not shown). Remarkably, the α-helical contents in the C-terminally truncated analogues displayed tight correlation with their relaxant activities at the concentration of 10^{-6} M (Fig. 1D), and this tendency was also applicable to the α-helical contents estimated by Greenfield and Fasman method (Greenfield and Fasman, 1969), k2d (Andrade et al., 1993), and CDPro (data not shown).

3.3. Binding activity of VIP and its C-terminally shortened analogues

VIPergic fibers are present in the airway and lung, and VIP appears to be the major peptide transmitter of the nonadrenergic noncholinergic inhibitory component of autonomic innervation of the lung, which expresses VIP-preferring receptors (Busto et al., 1999; Said, 1989). The RT-PCR experiment indicated the expression of mRNA for VPAC2 receptor in rat lung, but not for VPAC1 receptor (data not shown). We measured the binding activity of VIP and its shortened analogues to rat lung membrane using [^{125}I]VIP binding assay. VIP, VIP-(1–27), VIP-(1–26), VIP-(1–25), VIP-(1–24) and VIP-(1–23) inhibited specific [^{125}I]VIP binding in rat lung in a concentration-dependent manner and their IC_{50} values were 2.81, 3.35, 8.39, 533, 184 and 1100 nM, respectively (Fig. 1E). Thus, the IC_{50} values for VIP analogues increased in proportion to the number of truncated residues in the C-terminus, and VIP-(1–23) showed approximately 400-fold lower binding affinity to VIP receptors compared to that of VIP. The deletion of six and seven C-terminal residues markedly reduced or abolished VIP receptor binding activity as shown by IC_{50} values (>10,000 nM) of VIP-(1–22) and VIP-(1–21). These results were consistent with the observation of the strong correlation between α-helical content and relaxant activity in VIP and its truncated analogues (Fig. 1D). The amino acid residues from positions 7 to 10 were suggested to serve as the initiation site for the formation of the α-helix (Fournier et al., 1988). Thus, according to the C-terminal truncation experiments including α-helical contents (Fig. 1C), relaxant activities (Fig. 1A), and binding activities (Fig. 1E), we deduced that the stretch of 14 amino acid residues from positions 10 to 23 in VIP is important for the formation of α-helix, which correlates with the relaxant and binding effects.

3.4. Chemical modification of VIP in the middle-part of the α-helix

VIP and PACAP27 are closely related neuropeptides in terms of sequence, solution structure and physiological functions. The oxidation of Met^{17} in PACAP yielded a Met(O) (methionine sulfoxide) residue, which resulted in a significant decrease of biological activities along with the induction of conformational changes (Kitada et al., 1991). Therefore, in order to clarify if there are any similar substitutional effects for VIP, we synthesized [Met(O)^{17}]-VIP-(1–25) (Table 1). VIP-(1–25) was used for this modification instead of VIP in order to exclude the effect of a short random coiled structure yielded by the existence of three amino acid residues in the C-terminus. CD spectral analysis revealed a marked conformational change by replacement of Met for Met(O) at position 17 in VIP-(1–25) (Fig. 2A). Although both VIP-(1–25) and [Met(O)^{17}]VIP-(1–25) indicated the presence of α-helical structures in the solution, the α-helical content of [Met(O)^{17}]VIP-(1–25) was calculated to be half that of VIP-(1–25) (Fig. 2B). In addition to the influence on the solution structure, this modification also had a significant inhibitory effect against the relaxant activity of VIP-(1–25) at the concentration of 10^{-6} M (Fig. 2C); the substitution of Met by Met(O) led to a 40% decrease from the relaxant activity of VIP-(1–25).

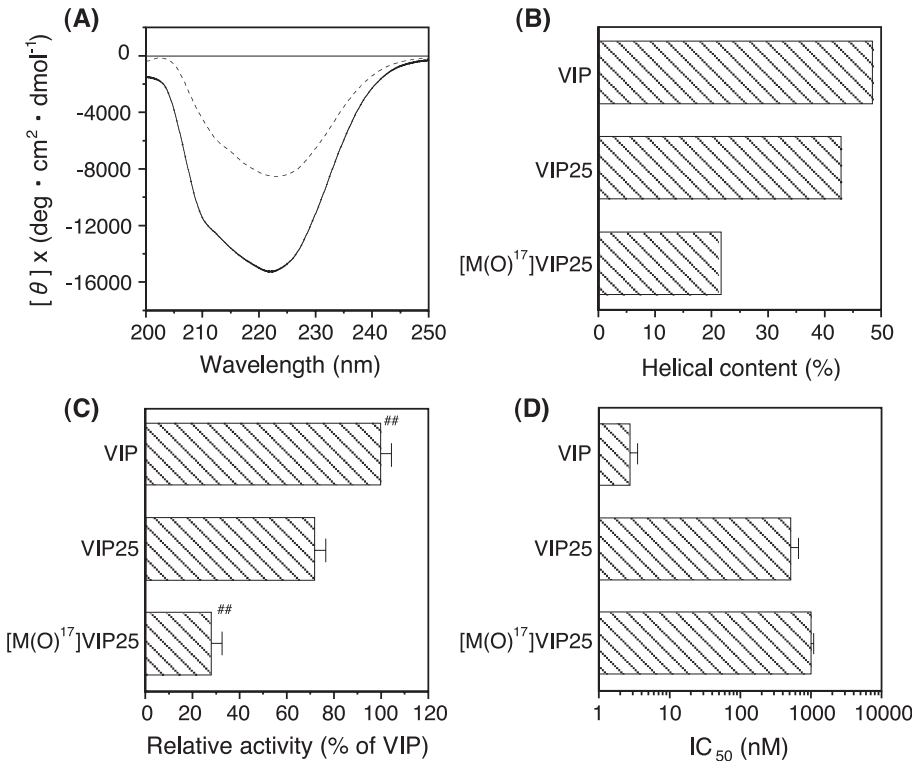


Fig. 2. Conformational and biological effects of the mid-chain modification in the α-helical region of VIP. (A) CD spectra of VIP-(1–25) (solid line) and its oxidized derivative [Met(O)^{17}]VIP-(1–25) (dashed) were measured in 50% methanol/20 mM Tris–HCl buffer (pH 7.4). (B) The α-helical contents of VIP-related compounds estimated by CD spectral analysis in 50% methanol/20 mM Tris–HCl buffer (pH 7.4). (C) The maximal relaxant effects of VIP-related compounds (10^{-6} M). The relaxant effects are represented as a percentage of VIP (10^{-6} M). The data on the relaxant activity represent the average of four determinations as the mean ± SD. The statistical analysis was performed with ANOVA followed by the Fisher's LSD test. $^{\#\#}P<0.01$ compared with VIP-(1–25). (D) Inhibitory effects (IC_{50} values) of VIP, VIP-(1–25) and [Met(O)^{17}]VIP-(1–25) on specific [^{125}I]VIP binding to the rat lung. Rat lung homogenate was incubated with [^{125}I]VIP (0.3 nM) and various concentrations of unlabeled peptides at 4 °C for 3 h, and their IC_{50} values were measured from the displacement curves of specific [^{125}I]VIP binding. Data represents the mean ± SE for seven (VIP) and four (its analogues) determinations. VIP-(1–25) and [Met(O)^{17}]VIP-(1–25) are designated as VIP25 and [M(O)^{17}]VIP25, respectively.

Furthermore, the IC_{50} value of [Met(O)^{17}]VIP-(1–25) against [^{125}I]VIP binding in rat lung membrane was 1028 nM, which was two-fold larger than VIP-(1–25) (Fig. 2D). These findings indicated that the oxidation of Met^{17} caused the disruption of a long α-helical structure formed in VIP-(1–25) as well as a significant reduction of its biological activity.

3.5. Chemical modification of VIP in the N-terminus of the α-helix

To confirm the role of the N-terminal sequence in the region of the α-helical structure of VIP, we modified VIP-(1–23), which still showed about 60% relaxant effect at the concentration of 10^{-6} M (Fig. 1A), to synthesize [Leu^{17}]VIP-(1–23) and [des-Thr^{11}, Leu^{17}]VIP-(1–23) (Table 1) and investigated their conformations and biological activities. [Leu^{17}]VIP-(1–23), in which Met at position 17 was substituted by Leu, showed no significant changes on both the α-helical content and biological activity compared with VIP-(1–23) (Fig. 3B and C), indicating that this substitution could exclude undesired disturbance caused by the oxidation of Met^{17} and be useful for the accurate evaluation of the effect of modification in the N-terminal α-helical region. CD spectra of [Leu^{17}]VIP-(1–23) and [des-Thr^{11}, Leu^{17}]VIP-(1–23), in which Thr at position 11 was deleted, in 50% methanol/20 mM Tris–HCl (pH7.4) showed distinct differences in the ellipticity among the measured range (200–400 nm) (Fig. 3A). Although both analogues exhibited an abundance of α-helix and a few β-turn structures, a decrease in the α-helical content was evident in [des-Thr^{11}, Leu^{17}]VIP-(1–23) compared to [Leu^{17}]VIP-(1–23) (Fig. 3B), indicating that the long α-helix in [Leu^{17}]VIP-(1–23) was disturbed due to the deletion of Thr^{11} at the N-terminal end of α-helix. This modification also led to a reduced biological activity of [Leu^{17}]VIP-(1–23); namely, [des-Thr^{11}, Leu^{17}]VIP-(1–23) (10^{-6} M) showed a 20-fold lower isometric relaxation against the carbachol-induced contraction of murine stomach. In addition, the deletion of Thr^{11} had a dramatic effect on the binding activity to rat lung membrane, resulting in at least 40-fold less affinity than the case of [Leu^{17}]VIP-(1–23) (Fig. 3D). These results suggested that, in addition to the C-terminus and middle-part, the N-terminus of the

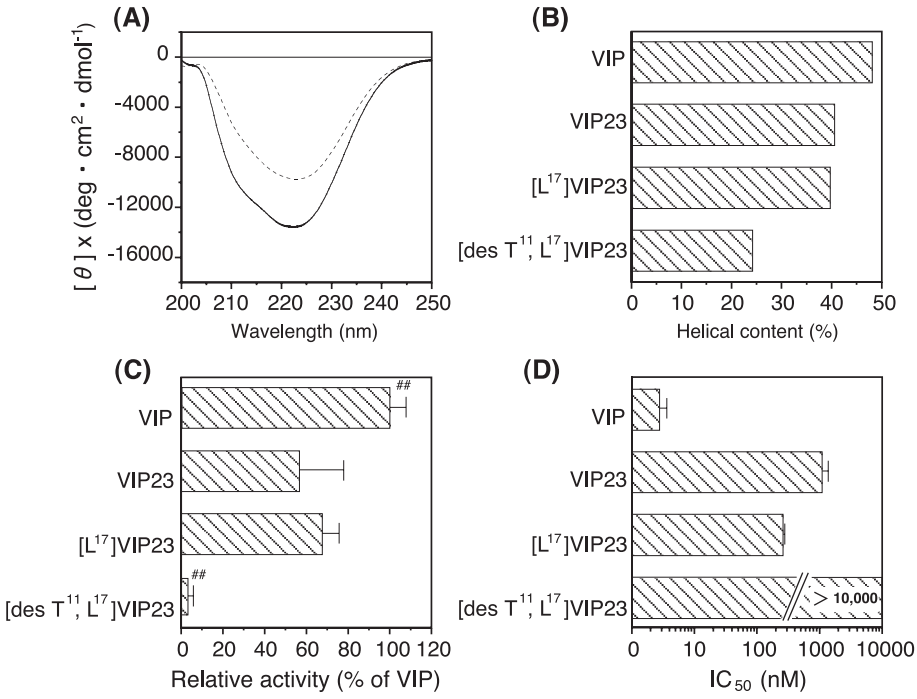


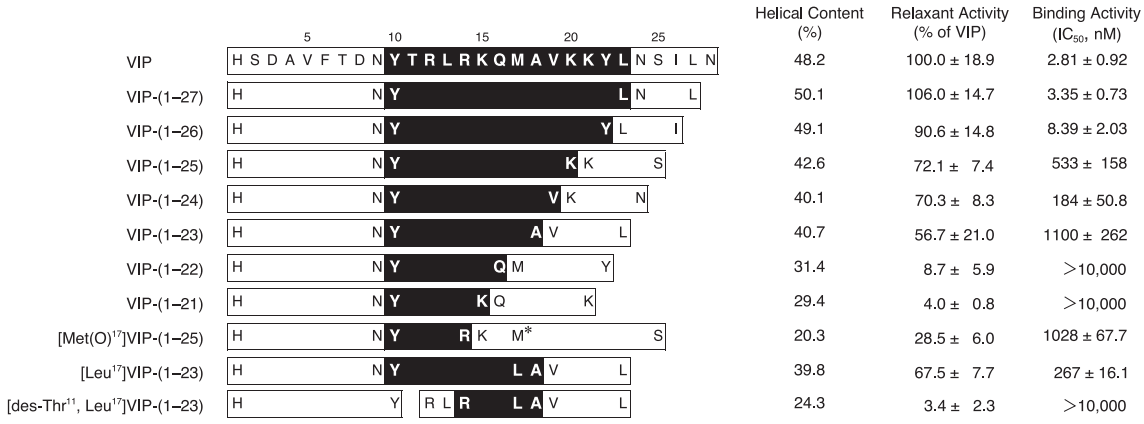
Fig. 3. Conformational and biological effects of the N-terminal modification in the α-helical structure of VIP. (A) CD spectra of [Leu^{17}]VIP-(1–23) (solid line) and [des-Thr^{11}, Leu^{17}]VIP-(1–23) (dashed line) were measured in 50% methanol/20 mM Tris–HCl buffer (pH 7.4). (B) The α-helical contents of VIP-related compounds estimated by CD spectral analysis in 50% methanol/20 mM Tris–HCl buffer (pH 7.4). (C) The maximal relaxant effects of VIP-related compounds (10^{-6} M). The relaxant effects are represented as a percentage of VIP (10^{-6} M). The data on the relaxant activity represent the average of four determinations as the mean ± SD. The statistical analysis was performed with ANOVA followed by the Fisher's LSD test. $^{\#\#}P<0.01$ compared with [Leu^{17}]VIP-(1–23). (D) Inhibitory effects (IC_{50} values) of VIP, VIP-(1–23) and its analogues on specific [^{125}I]VIP binding to the rat lung. Rat lung homogenate was incubated with [^{125}I]VIP (0.3 nM) and various concentrations of unlabeled peptides at 4 °C for 3 h, and their IC_{50} values were measured from the displacement curves of specific [^{125}I]VIP binding. Data represents the mean ± SE for seven (VIP) and four (its analogues) determinations. VIP-(1–23) is designated as VIP23. The names of the amino acids are designated as one-letter abbreviations.

α-helical structure in VIP also plays an important role in its biological functions.

4. Discussion

Numerous conformational studies including CD spectra and computational analyses have characterized that VIP possesses two segments of secondary structures; there is a random coil structure in the N-terminal region containing 10 amino acid residues between positions 1 and 10 and a long α-helical structure in the C-terminal region stretching from position 10 to its C-terminus (Bodanszky and Bodanszky, 1986; Bodanszky et al., 1974; Fournier et al., 1988; Haghjoo et al., 1996; Robinson et al., 1982). Here, we conducted three types of site-specific chemical modifications in the C-terminus, mid-chain, and N-terminus of the α-helical region of VIP and evaluated their biological implications. As summarized in Fig. 4, we showed that the α-helical contents of the VIP-related analogues calculated from CD spectra in 50% methanol/20 mM Tris–HCl (pH 7.4) displayed a tight correlation with their relaxant effects against the carbachol-induced contraction of murine stomach and their binding activities to rat lung membrane. Given the following findings, we demonstrated that the entire α-helical region consisting of residues stretching between positions 10 and 23 is essential to the biological functions of VIP and this α-helical formation is influenced by the C-terminal amino acid residues between positions 24 and 27. First, the C-terminal truncation experiments clarified the requirement of 14 amino acid residues between positions 10 and 23 for the α-helical formation as well as the biological activities of VIP, and VIP-(1–27) showed almost equal characteristics to VIP. Second, the mid-chain modified analogue, [Met(O)^{17}]-VIP-(1–25), exhibited a 2–2.5-fold reduction in relaxant and binding effects and a 50% reduction in α-helical content compared to VIP(1–25). Third, the N-terminal modified analogue, [des-Thr^{11}, Leu^{17}]VIP-(1–23), resulted in a short α-helical stretch consisting of only five amino acid residues and a 20-fold decrease in the relaxant activity compared to [Leu^{17}]VIP-(1–23) in accordance with the binding activity.

In addition to VIP, a similar structure–function relationship has been observed for PACAP27 (Kitada et al., 1991) and helodermin (Li et al., 1993). A 27 amino acid peptide, PACAP27, is a short peptide form of PACAP containing 38 amino acid residues (Miyata et al., 1989; Miyata et al., 1990), and helodermin is a 35 amino acid peptide (Hoshino et al., 1984). Both peptides show a high degree of sequence homology with peptides of the glucagon/secretin/VIP family. In the C-terminal shortening experiments on PACAP27,



	Helical Content (%)	Relaxant Activity (% of VIP)	Binding Activity (IC_{50}, nM)
VIP	48.2	100.0 ± 18.9	2.81 ± 0.92
VIP-(1–27)	50.1	106.0 ± 14.7	3.35 ± 0.73
VIP-(1–26)	49.1	90.6 ± 14.8	8.39 ± 2.03
VIP-(1–25)	42.6	72.1 ± 7.4	533 ± 158
VIP-(1–24)	40.1	70.3 ± 8.3	184 ± 50.8
VIP-(1–23)	40.7	56.7 ± 21.0	1100 ± 262
VIP-(1–22)	31.4	8.7 ± 5.9	>10,000
VIP-(1–21)	29.4	4.0 ± 0.8	>10,000
[Met(O)17]VIP-(1–25)	20.3	28.5 ± 6.0	1028 ± 67.7
[Leu17]VIP-(1–23)	39.8	67.5 ± 7.7	267 ± 16.1
[des-Thr11, Leu17]VIP-(1–23)	24.3	3.4 ± 2.3	>10,000

Fig. 4. Schematic representation of the primary structures, the contents of the α-helical structure, and the biological activities on VIP and its related analogues used in this study. Deduced α-helical regions are shown in black. The oxidized methionine at position 17 in [Met(O)^{17}]VIP-(1–25) is designated by an asterisk. The relaxant activities are represented as the relative values as a percentage of VIP (10^{-6} M).

the potent cAMP-producing activity was lost when the peptide length reached 22 from the N-terminal end, indicating that the 23 amino acid residues from the N-terminus in PACAP are required for the biological activity. As for helodermin, CD and NMR spectral analyses revealed that there is a random coil structure in the N-terminus and an α-helical structure in the C-terminal region containing amino acid residues between positions 9 and 23 (Blankenfeldt et al., 1996). Truncating helodermin from the C-terminus resulted in a decrease in its biological activity; in particular, helodermin-(1–28), in which the C-terminal 7 amino acid residues were truncated, was less potent than helodermin in binding activity (10-fold lower) and activation of adenylate cyclase (>10^2-fold lower) (Li et al., 1993). In this investigation, we demonstrated that VIP requires at least 23 amino acid residues from the N-terminus for the relaxant effect in the murine stomach as well as the binding effect to the rat lung membrane. The amino acid sequence from positions 10 to 23 in VIP, corresponding to the C-terminal α-helical-forming region, has 86% and 43% homology with the corresponding regions of PACAP27 and helodermin, respectively. Because of the high conformational and biological similarities among VIP, PACAP27 and helodermin, it is conceivable that the biological activities of PACAP27 and helodermin are associated with the formation of α-helical structures in their C-terminuses as observed in VIP.

Since VIP acts through the interaction with two subclasses of G protein-coupled receptors, VPAC1 and VPAC2, it is necessary to provide an artificial hydrophobic environment that mimics the vicinity of the peptide/receptor interface when performing conformational studies on VIP. α-Helical contents estimated by CD spectra vary depending on the calculation methods and solvent systems such as pH and polarity. The solvent system of water/alcohol, including methanol and trifluoroethanol, has often been used as a membrane-mimetic medium (Kobayashi et al., 2000; Minn et al., 1998; Park et al., 2000; Park et al., 1998), and secondary structures existing in methanol/water reflect those in the lipidic milieu. Our present CD spectral analyses on VIP-related analogues were conducted using 50% methanol/Tris–HCl buffer (pH 7.4). Helodermin is one of the VPAC receptor agonists and its previous NMR spectral analysis was performed in 50% trifluoroethanol solution (Blankenfeldt et al., 1996). Thus, our results provide further circumstantial evidence that the α-helical conformation in these peptide ligands could be formed on the surface of membrane where the VPAC receptors are expressed for their interactions.

We previously reported that chemical modifications in the N-terminal random coil regions of both VIP and PACAP resulted in significant changes in their biological activities including receptor-binding activity, activation of adenylate cyclase and neurite outgrowth (Onoue et al., 2001). When replacing the N-terminal region of VIP with that of PACAP, the VIP/PACAP chimeric peptide, PACAP-(1–11)-VIP-(12–28), showed PACAP-like activity instead of VIP-like activity. VIP regulates bronchodilation; it inhibits the histamine-stimulated contraction of bronchial smooth muscle (O'Donnell et al., 1994; Yoshihara et al., 1998). The binding activities of our C-terminally truncated VIP analogues against lysates from rat lungs that express VPAC receptors were reduced in accordance with the decrease in α-helical contents. Previous studies also indicated that the presence of the C-terminal end of VIP is important for its interaction with the VPAC1 receptor (Gourlet et al., 1996). Hence, we conclude that the N-terminal regions forming random coil structures in both VIP and PACAP may play a crucial role in the selective recognition of VPAC receptors or a PACAP-specific receptor, PAC1, and that the formation of the α-helical structure in the C-terminus of VIP is critical to its binding to VPAC receptors.

Determining secondary structures in peptide ligands that bind to their receptors provides useful information on the development of agonists and antagonists that lead to potent drug compounds. We propose that such agonists and antagonists for VPAC receptors could be designed on the basis of

the α-helix-forming sequence between positions 10 and 23 in VIP for the biological activity and receptor binding, and the N-terminal random coil-forming sequence between positions 1 and 11 in VIP for the selective recognition of VPAC receptors. In addition, our present results showed that the oxidation of Met residue at position 17 in VIP yielded the inactive form of VIP, suggesting that there may be a serious problem in the clinical application of VIP since oxidation at Met residues easily occurs. Therefore, in developing an effective VIP-related compound for medication, the Met residue at position 17 should be replaced by a different amino acid including Leu that possesses high conformational stability as well as potent biological activity, which can maintain the biological activity regardless of exposure to an oxidized condition. VIP is a potential pharmaceutical agent for clinical treatments of asthma, diabetes, impotence, inflammation, and dementia. Our present findings obtained by chemical modification experiments provide a novel structure–activity relationship of VIP, and it will be useful in the development of VIP-related drug compounds for the above diseases.

Acknowledgements

We wish to express our gratitude to Dr. Noboru Yanaihara, Professor Emeritus of University of Shizuoka, Japan, and President of Yanaihara Institute (Shizuoka, Japan), for valuable suggestions and discussions throughout this work. We are also grateful to Dr. Shigeo Kubota, DendriMolecular (San Carlos, CA, USA), for his technical advice about the determination of secondary structure. K. Ohshima is awardee of research resident fellowship from the foundation for promotion of cancer research in Japan.

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