Biochemical Pharmacology, Vol. 54, pp. 509–515, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00207-4

Vasoactive Intestinal Peptide (VIP) and Pituitary Adenylate Cyclase-Activating Peptide (PACAP-27, but not PACAP-38) Degradation by the Neutral Endopeptidase EC 3.4.24.11

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ABSTRACT. VIP (vasoactive intestinal polypeptide) and PACAP (pituitary adenylate cyclase-activating polypeptide), which are potent relaxing agents in the airways, were submitted to *in vitro* degradation by the neutral endopeptidase EC 3.4.24.11 (NEP), one of the most active peptidase in the lung, to test their relative resistance to proteolysis. Both VIP and PACAP(1–27) were cleaved by NEP, but PACAP(1–38) was not. The main fragments produced were VIP(1–22) and VIP(1–25), and PACAP(1–22) and PACAP(1–25), respectively. The degradation of VIP(1–27), PACAP(6–27), and PACAP(13–27) was also hindered by extending their C-terminal ends with the (28–38) sequence of PACAP(1–38). The sensitivity to enzyme degradation was gradually reduced when the C-terminal extension was increased from PACAP(1–27) to PACAP(1–29), PACAP(1–32) and PACAP(1–38). The biological activities of the degradation products were evaluated on the three classes of PACAP/VIP receptors, with VIP(1–25) and PACAP(1–25) retaining an important part of their activities on the VIP_1 receptor. Thus, the degradation of VIP and PACAP(1–27) by the neutral endopeptidase 24.11 might produce a VIP_1 receptor-selective active metabolite, provided that very high VIP or PACAP(1–27) concentrations are achieved in the receptor vicinity. BIOCHEM PHARMACOL 54;4:509–515, 1997.

KEY WORDS. VIP; PACAP; degradation; neutral endopeptidase; metabolite activity

Pituitary adenylate cyclase-activating polypeptide (PACAP) belongs to the vasoactive intestinal peptide (VIP)§ family of peptides [1]. PACAP occurs in a 38- and a 27-amino acid form with an identical (1–27) sequence that exhibits 68% similarity with VIP. PACAP-38 represents 80 to 90% of the total PACAP content in all the tissues studied.

PACAP, like VIP, is expressed in the central nervous system and in nerve fibres of the gut, lung, and endocrine and exocrine glands [2, 3]. PACAP acts through interaction with receptor sites identified in a large number of tissues or cells [4] and classified as PACAP-I (or PACAP-selective) and PACAP-II (or nonselective VIP/PACAP) receptors. The PACAP-II receptors, previously designated VIP receptors, were further subdivided into VIP_1 and VIP_2 receptors [5, 6]. VIP_1 receptors were identified in lung, liver, pancreas, intestinal cells, and discrete brain areas, while VIP_2 receptors were found in brain, endocrine glands, immunocompetent cells, and testis [7–9].

The respective physiological roles of VIP and PACAP in a given tissue expressing VIP receptors depend on the relative amount of VIP and PACAP secreted in the vicinity of the receptor and on the relative stability of the peptides. It has recently been shown that PACAP is a potent relaxing agent on several tracheal or bronchial preparations, as efficient as VIP. Moreover, in some preparations, PACAP exerted a more sustained effect [10–12]. It was suggested that the prolonged action of PACAP-38 [13] might result from its poor degradation by neutral endopeptidase 24.11 (NEP), an endopeptidase present on lung epithelial cells and involved in the inactivation of peptidic hormones [14]. To test this hypothesis, we compared the degradation of VIP, PACAP-27 and PACAP-38 by purified neutral endopeptidase prepared from human kidney.

MATERIALS AND METHODS

Peptide Synthesis

All peptides were synthesized as C-terminal amides by solid phase methodology using the Fmoc (9-fluorenyl-methoxy

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§ *Abbreviations:* VIP, vasoactive intestinal polypeptide; PACAP, pituitary adenylate cyclase-activating polypeptide; NEP, neutral endopeptidase EC 3.4.24.11; CHO, chinese hamster ovary cells; IC_{50}, 50% inhibition of tracer binding; EC_{50}, 50% "over basal" adenylate cyclase stimulation.

Received 8 November 1996; accepted 11 March 1997.

carbonyl) strategy with an Applied Biosystems apparatus 431A (Foster City, CA). The peptides were cleaved for 4 hr with a cleavage mixture containing 82% trifluoroacetic acid, 4% phenol, 2% ethanedithiol, 4% thioanisole, 4% H_2O, and 4% triisopropyl silane. After partial trifluoroacetic acid evaporation, the peptides were precipitated with 10 vol of cold ether. They were purified by reverse phase chromatography on Jordi-Gel DVB 300Å (Alltech, Laarne, Belgium) (10× 1 cm) and by ion exchange chromatography on Mono S HR 5/5 (Pharmacia, Uppsala, Sweden). The peptide purity was assessed (>95%) by capillary electrophoresis, and the sequence conformity was verified by sequencing and electrospray mass spectrometry.

Enzyme Purification

Neutral endopeptidase was purified from human kidney as described previously [15]. The purity of the enzyme (90%) was assessed by SDS-PAGE.

Degradation of the Peptides: Quantification and Identification of the Hydrolysis Products

In the standard assay, 2 μg/mL of neutral endopeptidase was incubated at 37°C in a 50 mM Tris-HCl pH 7.6 buffer containing 0.1% Triton X-100 and 50 μM of the peptide. The assay volume was 200 μL. The reaction was started by the addition of the peptide and stopped with 1 mL 0.1% TFA, 3% CH_3CN. The sample was immediately chromatographied on a Vydac 218TP104 column (The Separations Group, Hisperia, CA) equilibrated with 0.1% TFA, 3% CH_3CN, and eluted with a linear gradient of CH_3CN (from 3 to 80% in 100 min). The optical density of the eluent was recorded at 226 nm, and the amounts of the hydrolysis products were calculated from the peak areas measured with a Shimadzu CR5A integrator (Shimadzu Benelux, 's-Hertogenbosch, The Netherlands) and referred to the areas given by standard amounts of substrates chromatographied under the same conditions. As the absorbance at 226 nm is little affected by aromatic amino acids and approximately proportional to the number of peptide bonds, we used the same average area/μg ratio to quantify each hydrolysis peptide. The main peaks observed after enzyme action were identified by electrospray mass spectrometry and N-terminal sequencing using the Edman reaction performed on the Applied Biosystems sequencer.

Evaluation of the Biological Activity of the Main Metabolites

The major metabolites [VIP(1–22)OH, VIP(1–25)OH, PACAP(1–22)OH, and PACAP(1–25)OH] resulting from VIP and PACAP degradation were further purified and tested for their capacity to bind and to stimulate adenylate cyclase activity of membranes from chinese hamster ovary (CHO) cells expressing the recombinant PACAP-I [16], PACAP-II VIP_1 [17], and PACAP-II VIP_2 receptors [18], using previously detailed methods [16–18]. [^{125}I]-Ac-His^1-PACAP(1–27) binding to membranes from CHO cells expressing either the "normal" rat PACAP-I receptor (clone 2–10), rat PACAP-II VIP_1 receptor or human PACAP-II VIP_2 receptor was measured at 37°C, by a filtration method, in the absence or presence of unlabeled peptides (two concentrations per logarithm) [18]. Nonspecific binding was defined in the presence of 1 μM PACAP(1–27). Adenylate cyclase stimulation was measured by the method of Salomon *et al.*, as described in [18]. The peptide concentrations necessary for 50% inhibition of tracer binding (IC_{50}) and 50% "over basal" adenylate cyclase stimulation (EC_{50}) were determined in two experiments by a computer-assisted nonlinear curve fitting procedure. The IC_{50} and EC_{50} values were always within 30% of each other.

RESULTS

Degradation of VIP, PACAP-27, PACAP-38, and Fragments in the Presence of Neutral Endopeptidase

VIP and PACAP-27, but not PACAP-38, were degraded by neutral endopeptidase (Fig. 1 and Table 1). The degradation was entirely blocked by 1 μM thiorphan (data not shown). As shown in Table 1, the addition of the Gly-Lys or the Gly-Lys-Arg-Tyr-Lys sequence to the C-terminal end of PACAP-27, giving rise to PACAP(1–29) and PACAP(1–32), respectively, reduced peptide degradation. The amino terminal-deleted peptides, PACAP(6–27) and PACAP(13–27), were efficiently hydrolyzed by neutral endopeptidase; their degradation, like that of VIP, was markedly reduced by extending their carboxyl terminus with the (28–38) sequence of PACAP-38 (Table 1).

Identification of the Degradation Products

As shown in Fig. 2A, several VIP fragments were observed after VIP hydrolysis by the neutral endopeptidase. The major metabolites identified at any incubation time (15, 30, 60, 120, and 180 min) were VIP(1–22)OH and VIP(1–25)OH. As shown in Fig. 2B, the main degradation products of PACAP-27 were, as for VIP, PACAP(1–22)OH and PACAP(1–25)OH. PACAP(6–27) was degraded mainly in PACAP(6–22)OH (Fig. 2C), and PACAP(13–27) in PACAP(13–22)OH (Fig. 2D). Thus, the two main peptidic bonds cleaved were between amino acids 22–23 and 25–26. After 1 hr hydrolysis, the remaining intact VIP or PACAP plus the two main fragments (1–22) and (1–25) represented at least 90% of the starting material (Fig. 1), indicating that cleavage at other sites was negligible during that incubation period. Furthermore, the rate of appearance of VIP(1–22)OH and PACAP(1–22)OH suggested that they derived from a direct hydrolysis of VIP and PACAP and not from a secondary degradation of VIP(1–25)OH and PACAP(1–25)OH, at least during the first 60-min incubation period.

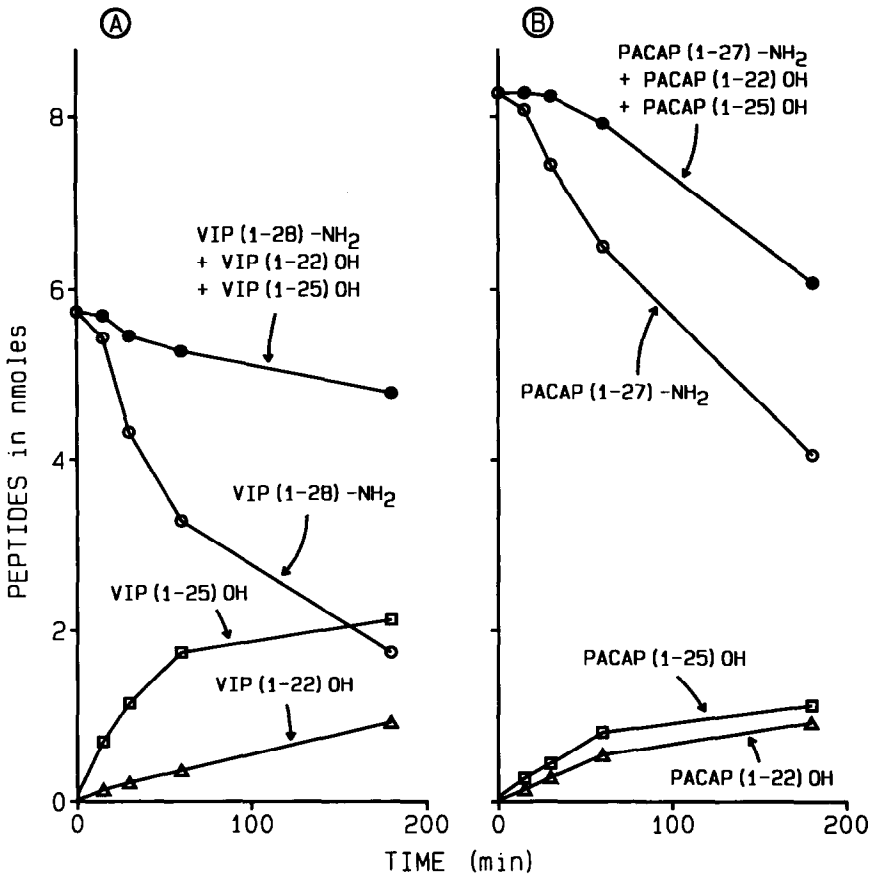

FIG. 1. Time course of VIP (left panel) and PACAP (right panel) degradation, and appearance of the two major metabolites. The amounts of peptides were estimated by integration of the peaks identified after HPLC chromatography (see Fig. 2), as indicated in Materials and Methods. The full circles indicate the mass of the undegraded peptide plus the two metabolites. The results are the means of three experiments.

Determination of the Apparent K_m et V_{max} for the Two Cleavage Points

The kinetic constants for VIP and PACAP-27 degradation were determined by incubating the enzyme in the presence of increasing concentrations of peptide. The amounts of fragments (1–25)OH and (1–22)OH generated were determined after a 45-min incubation period under the conditions described. The Lineweaver-Burk plots are shown in Fig. 3. Nonlinear curve fitting yielded apparent Km values close to 100–200 μM for the cleavage between amino acids 25–26 of both VIP and PACAP-27, and for the cleavage between amino acids 22–23 of PACAP. The cleavage between amino acids 22–23 of VIP occurred with a significantly lower apparent K_m of 15 μM. The maximal production rates of VIP(1–25)OH, VIP(1–22)OH, PACAP(1–25)OH, and PACAP(1–22)OH by 0.4 μg neutral endopeptidase are summarized in Table 2.

TABLE 1.

Peptide tested (50 μM)	Residual intact peptide (in % of control)
VIP(1–28)-NH_2	19 ± 3
VIP(1–27)/PACAP(28–38)-NH_2	100 ± 2
PACAP(1–27)-NH_2	49 ± 5
PACAP(1–38)-NH_2	100 ± 2
PACAP(1–29)-NH_2	79 ± 4
PACAP(1–32)-NH_2	91 ± 5
PACAP(6–27)-NH_2	21 ± 4
PACAP(6–32)-NH_2	90 ± 3
PACAP(6–38)-NH_2	100 ± 2
PACAP(13–27)-NH_2	7 ± 3
PACAP(13–38)-NH_2	98 ± 2

The amount of peptide remaining after a 3-hr incubation at 37°C with 2 μg/mL neutral endopeptidase is expressed in % of the peptide remaining after incubation in the absence of enzyme. The results are the means ± SEM of three determinations.

Biological Properties of the VIP and PACAP Fragments (1–22) and (1–25)

The capacity of fragments (1–22)OH and (1–25)OH to occupy the three classes of PACAP/VIP receptors was evaluated by competition curves performed on cell membranes expressing a single receptor subtype. The IC_{50} values (concentrations required for half maximal binding inhibition) are given in Table 3. The capacity of the peptides to stimulate adenylate cyclase activity was also evaluated on the same cell line membranes. The EC_{50} values (concentrations required for half maximal enzyme activation) are also given in Table 3. All the fragments tested were agonists, but in most cases, the maximal value was not obtained at 10 μM, the highest concentration tested. The observation of lower EC_{50} than IC_{50} values for VIP_1 and PACAP-I receptors reflected the presence of spare receptors [16, 17].

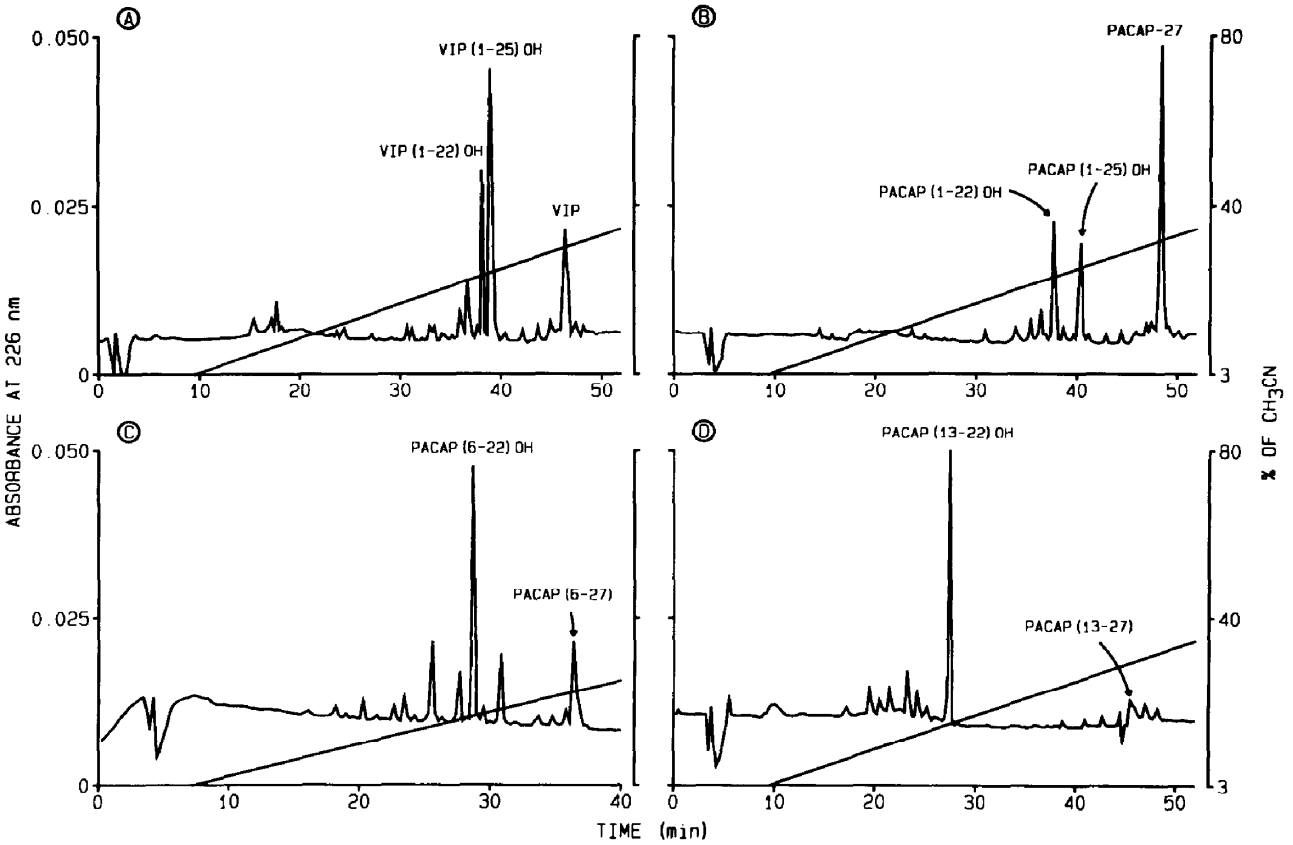

FIG. 2. Chromatographic HPLC profiles observed after incubation of VIP (panel A), PACAP-27 (panel B), PACAP (6–27) (panel C), and PACAP (13–27) (panel D) for 3 hr at 37°C in the presence of neutral endopeptidase. Details on incubation and HPLC procedures are given in Materials and Methods. The absorbance was recorded at 226 nm. The slope of the gradient is also indicated. The minor peaks were not identified.

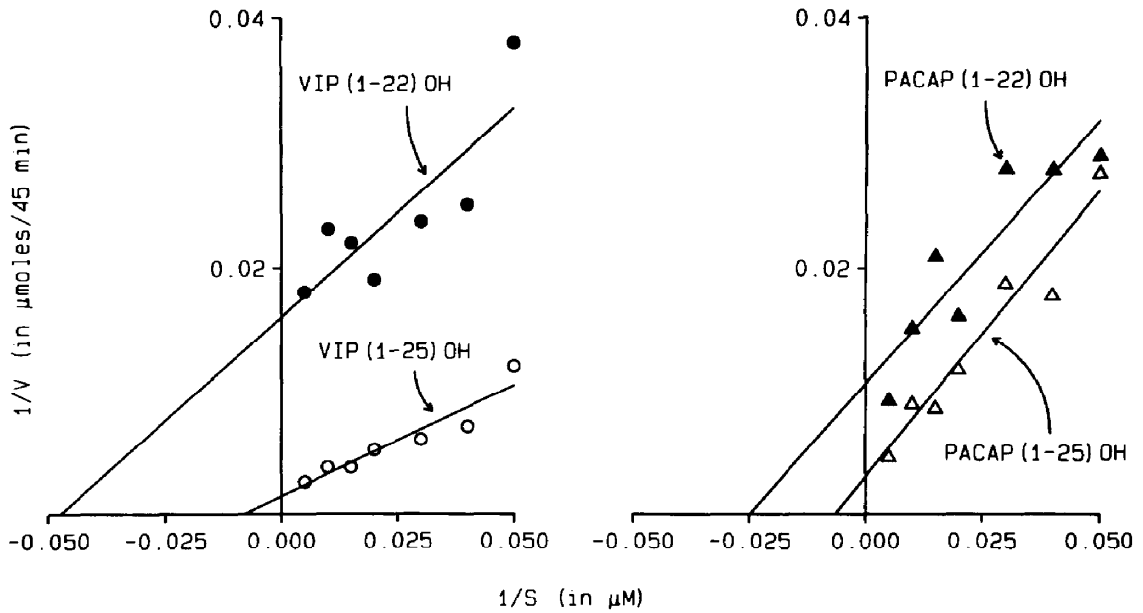

FIG. 3. Double reciprocal representation of appearance of VIP(1–25)OH (○, left panel), VIP(1–22)OH (●, left panel), PACAP(1–25)OH (△, right panel), and PACAP(1–22)OH (▲, right panel) after incubation of increasing concentrations of VIP (left panel) and PACAP-27 (right panel) for 45 min at 37°C in the presence of neutral endopeptidase (see Materials and Methods). The amounts of peptides were evaluated by integration of the peaks after HPLC chromatography, as indicated in Materials and Methods.

TABLE 2.

Substrate	Product	K_m (μM)	V_m (nmol/min)
VIP(1–28)NH_2	→VIP(1–22)OH	15 ± 6	1.3 ± 0.1
VIP(1–28)NH_2	→VIP(1–25)OH	95 ± 21	13.4 ± 1.5
PACAP(1–27)NH_2	→PACAP(1–22)OH	107 ± 46	3.6 ± 0.8
PACAP(1–27)NH_2	→PACAP(1–25)OH	240 ± 97	10.6 ± 2.8

The apparent K_m and V_m (± standard error) for hydrolysis of VIP and PACAP(1–27) in positions 22–23 and 25–26, respectively, by 0.4 μg of neutral endopeptidase were determined by nonlinear regression of the experimental data to the Michaelis-Menten equation.

DISCUSSION

Although it is not the sole degradation system for VIP and probably not for PACAP as well, neutral endopeptidase is of importance in the respiratory tract, central nervous system, and kidney. *In vitro*, tryptase and chymase from lung mast cells also hydrolyze VIP efficiently: tryptase at positions 12, 14, 20, and 21 [19, 20], and chymase between amino acids 22–23 [21], as with neutral endopeptidase in the present study. When VIP was incubated in the presence of bronchial alveolus lavages, a first cleavage was identified between amino acid residues 25–26 and a second between positions 7 and 8 [22]. Finally, VIP could be cleared in the lung through endocytosis and lysosomal degradation [23].

In enterocytes, the major degradation occurs through an amastatin- and bestatin-sensitive aminopeptidase that rapidly removes the N-terminal histidine [24]. Crude membrane fractions of gastric smooth muscle degraded VIP in fragments generated by cleavage between amino acids 2–3, 4–5, 9–10, 20–21, 21–22, 23–24, and 25–26; the hydrolysis was not markedly influenced by neutral endopeptidase inhibitors [25]. These data suggest that enzymatic activities found in lung (chymase, tryptase, and neutral endopeptidase) mainly degrade the C-terminal moiety of VIP, but that gut aminopeptidases rapidly degrade the N-terminal end.

Recent *in vivo* data [26] support the hypothesis that neutral endopeptidase plays a physiological role in VIP inactivation in the hamster cheek pouch vessels. In this model, VIP-induced vasodilatation was modulated by NEP inhibitors. The wide distribution of NEP in peripheral circulation as well as in airways [27], including the nasal mucosa [28], underlines the importance of developing tissue-specific and NEP-resistant VIP-PACAP agonists or antagonists.

The present data demonstrated that VIP and PACAP-27 could be degraded by human neutral endopeptidase. As shown in Tables 2 and 4, the two main degradation products [VIP(1–22)OH and VIP(1–25)OH; PACAP(1–22)OH and PACAP(1–25)OH] obtained from VIP and PACAP-27 are compatible with the known specificity of the enzyme that cleaves at the amino side of hydrophobic residues (Leu^{23} and Ile^{26} or Val^{26} for VIP and PACAP, respectively). This pattern of VIP degradation differs from that reported by Goetzl *et al.* [29] using human recombinant enkephalinase: these authors identified peptide bonds (3–4) and (4–5) as the main targets for the enzyme, the (21–22) and (25–26) bonds representing minor cleavage sites. It must be noted, however, that the cleavages in the N-terminal moiety of VIP, in their study, were not completely inhibited by phosphoramidon (a specific inhibitor of NEP).

Although both VIP and PACAP-27 were degraded by the neutral endopeptidase at the same positions, the kinetic parameters of hydrolysis were different. The K_m and V_m values for cleavage of the (25–26) peptide bonds were identical for PACAP (Ala-Val) and VIP (Ser-Ile), but the Vm for cleavage of the (22–23) peptide bond (Tyr-Leu) was significantly lower ($P > 0.95$). The rates of hydrolysis of the Tyr-Leu peptide bonds of PACAP(1–27)NH_2 and VIP(1–28)NH_2 were similar, but the K_m values were significantly different ($P > 0.95$). This supported the hypothesis that the cleavage site environment modulates the rate of peptide hydrolysis.

Our results also demonstrated that PACAP-38 was resistant to hydrolysis by the neutral endopeptidase. This result might account for the long-lasting effect of PACAP-38 *in vitro* on bronchial relaxation [13], as NEP is

TABLE 3. IC_{50} values (in nM) of binding and EC_{50} values (in nM) of adenylate cyclase activation of VIP, PACAP, and fragments on PACAP-I, VIP_1, and VIP_2 receptors

	PACAP I receptors		VIP_1 receptors		VIP_2 receptors	
Peptide tested	IC_{50}	EC_{50}	IC_{50}	EC_{50}	IC_{50}	EC_{50}
VIP	1000	100	1	0.1	1	3
VIP(1–25)OH	n.d.	n.d.	200	10	3000	3000
VIP(1–22)OH	n.d.	n.d.	10000	1000	10000	30000
PACAP(1–27)	3	0.1	1	0.1	3	3
PACAP(1–25)OH	800	10	100	10	2000	10000
PACAP(1–22)OH	>30000	10000	10000	1000	>30000	>30000

The IC_{50} values were established from inhibition curves of tracer binding ([^{125}I]Ac-His^1-PACAP-27 for PACAP I receptor and [^{125}I]VIP for VIP_1 and VIP_2, receptors) by increasing concentrations of unlabeled peptides. The EC_{50} values were those provoking half maximal adenylate cyclase stimulation. Means of two determinations in duplicate.

TABLE 4. Sequences of the natural peptides VIP, PACAP(1–27), and PACAP(1–38)

	1 5 10 15 20 25 30 35
VIP–28	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-NH_2
PACAP(1–27)	H S D G I F T D S Y S R Y R K Q M A V K K Y↑L A A↑V L-NH_2
PACAP(1–38)	H S D G I F T D S Y S R Y R K Q M A V K K Y L A A V L G K R Y K Q R V K N K-NH_2

The major sites of VIP and PACAP(1–27) cleavage by the neutral endopeptidase, observed in this work, are indicated by arrows.

considered as the main enzyme responsible for peptide degradation by bronchial epithelial cells [14]. The resistance of PACAP-38 to neutral endopeptidase hydrolysis did not require the presence of the whole (28–38) sequence: PACAP(1–29) and PACAP(1–32) amide were more stable than PACAP(1–27). A similar observation was made for the N-terminally truncated peptide (6–32). The fragment (13–38) was also protected from enzymatic hydrolysis. We have not yet established whether the nature of the amino acids is of importance, but the "natural extension" of PACAP-27 includes a majority of basic residues. It is tempting to speculate that the presence of positive charges on these substrates is responsible for unfavourable ionic interaction with the Arg^{102} and Arg^{747} residues of the binding site of the enzyme [30]. It is of interest in this respect that the lizard peptide helodermin, a parent peptide of VIP and PACAP with 35 amino acids, also exhibited a long-acting bronchorelaxant effect [31].

We found that VIP(1–22)OH and PACAP(1–22)OH were at least 10,000-fold less potent than VIP and PACAP, respectively, confirming that the C-terminal end of the peptides is required for binding of the ligand to the receptor [32, 33]. VIP(1–25)OH, however, was only 100- to 200- and 1000- to 3000-fold less potent than VIP on the VIP_1 and VIP_2 receptors, respectively. PACAP(1–25)OH was 100- to 300-, 100-, and 800- to 3000-fold less potent than PACAP(1–27) on the PACAP-I, VIP_1 and VIP_2 receptors, respectively. This indicated that the C-terminally truncated peptides had a higher affinity for the VIP_1—than for the VIP_2—or the PACAP receptors. A similar observation was made recently with synthetic C-terminally shortened PACAP with an amidated carboxyl terminal residue [34]. Thus, the degradation of VIP and PACAP by neutral endopeptidase may generate an active metabolite selective for the VIP_1 receptor subtype. The physiological relevance of this metabolite is unclear, because it is markedly less potent than the parent peptide.

All these observations could be useful for the design of new VIP/PACAP analogs with a prolonged biological effect.

This work was supported by Grant 3.4502.95 from the Fonds de la Recherche Scientifique Médicale, Belgium, and by an Action Concertée de Recherche from the Communauté Française de Belgique.

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