Role of endopeptidase 3.4.24.16 in the catabolism of neurotensin, *in vivo*, in the vascularly perfused dog ileum

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- 1 The degradation of tritiated and unlabelled neurotensin (NT) following close intra-arterial infusion of the peptides in ileal segments of anaesthetized dogs was examined.
- 2 Intact NT and its catabolites recovered in the venous effluents were purified by chromatography on Sep-Pak columns followed by reverse-phase h.p.l.c. and identified by their retention times or by radioimmunoassay.
- 3 The half-life of neurotensin was estimated to be between 2 and 6 min. Four labelled catabolites, corresponding to free tyrosine, neurotensin (1–8), neurotensin (1–10) and neurotensin (1–11), were detected.
- 4 Neurotensin (1–11) was mainly generated by a phosphoramidon-sensitive cleavage, probably elicited by endopeptidase 24–11.
- 5 Two endopeptidase 3.4.24.16 inhibitors, phosphodiethyl 03 and the dipeptide Pro-Ile, dose-dependently potentiated the recovery of intact neurotensin. Furthermore, both agents inhibited the formation of neurotensin (1–10), the product that results from the hydrolysis of neurotensin by purified endopeptidase 3.4.24.16. In contrast, the endopeptidase 3.4.24.15 inhibitor Cpp-AAY-pAB neither protected neurotensin from degradation nor modified the production of neurotensin (1–10).
- 6 Our study is the first evidence to indicate that endopeptidase 3.4.24.16 contributes to the catabolism of neurotensin, *in vivo*, in the dog intestine.

Keywords: Neurotensin; degradation; dog ileum; endopeptidase 24.15; endopeptidase 24.16; peptidases; phosphodiethyl 03; Pro-Ile

Introduction

Neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) functions as a paracrine regulator of intestinal motility. This statement is supported by the presence of neurotensin-like immunoreactivity (NT-LI) in mucosal N cells (Orci *et al.*, 1976) from which the peptide can be released upon various humoral or luminal stimuli (Mashford *et al.*, 1978; Hammer *et al.*, 1982). Furthermore, neurotensin receptor sites have been described in smooth muscle tissues and in enteric nerve synaptosomes of intestine from various species (Kitabgi & Freychet, 1979; Goedert *et al.*, 1984; Ahmad *et al.*, 1987; Ahmad & Daniel, 1991). These binding sites probably modulate the contractility of several gastrointestinal tissue preparations *in vitro* and *in vivo* (for review see Kitabgi, 1982). Thus, in an *ex vivo* model of vascularly perfused ileum of the dog, Sakai *et al.* (1984) demonstrated that intra-arterial administration of neurotensin inhibited the field-stimulated, atropine-sensitive contractile response of the ileum by both neural and myogenic actions.

We have established the distribution of various exo- and endopeptidases in purified membranes from muscular, nervous and mucosal origin (Checler *et al.*, 1987; Barelli *et al.*, 1989; 1993a), prepared from dog ileum. In agreement with the concept of a local regulation that implies a short term action, we have demonstrated that the various layers of the intestinal wall contain peptidases that efficiently catabolize neurotensin. In particular, our *in vitro* studies clearly demonstrated that endopeptidase 3.4.24.16 mainly contributed to neurotensin degradation in these muscular and nervous layers

(Checler *et al.*, 1987; Barelli *et al.*, 1989), as previously shown in membrane preparations and cells from central origin (Checler *et al.*, 1988a). However, the assessment of the involvement of endopeptidase 24.16 in the physiological inactivation of neurotensin has awaited the development of selective inhibitors of the enzyme. We have previously described a dipeptide, Pro-Ile (Dauch *et al.*, 1991) and more recently a phosphoramidon peptide (Barelli *et al.*, 1992) that behave as potent and selective inhibitors of purified endopeptidase 3.4.24.16. In the present study, we have used these novel tools to examine the contribution of endopeptidase 3.4.24.16 to the inactivation of neurotensin, *in vivo*, in the dog ileum.

Methods

Canine isolated perfused ileum

All procedures were approved by the McMaster Animal Care Committee. Ileal segments were prepared as previously described (Manaka *et al.*, 1989). Briefly, fasted mongrel dogs of either sex (weighing 15–30 kg) were anaesthetized with α -chloralose (50 mg kg⁻¹; Sigma) and urethane (500 mg kg⁻¹; Sigma). Ventilation was set up at approximately 15 strokes min⁻¹ with a Harvard animal ventilator pump. The femoral artery and vein were cannulated for monitoring blood pressure and for administration of sodium pentobarbitone (25–50 mg) in order to maintain anaesthesia throughout the experiment. The abdominal cavity was opened with a midline incision and an ileal segment (about 15–20 cm long and 15–35 g weight) was prepared. The largest vein and artery

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irrigating a single ileal segment were cannulated and the venous cannula left open to drain. The arterial cannula was perfused at a flow rate of 12 ml min^{-1} with a peristaltic pump (Harvard apparatus) with Krebs-Ringer bicarbonate solution (warmed to 37°C and gassed with 95% O_2 , 5% CO_2) containing (mM): NaCl 137.4, MgSO_4 1.2, NaHPO_4 1.2, NaHCO_3 21.1, CaCl_2 2.5 and glucose 5.5. The ileal segment was then ligated and the collateral circulation tied off. Five to seven segments, separated from each other by at least one complete vascular arcade, were sequentially prepared for each dog.

Experimental procedure

An equilibration period preceded the start of each experiment until the venous effluent became free of haematis by inspection. Dog ileum segments were pretreated for 10 min by perfusion of Krebs-Ringer bicarbonate buffer either without (control) or with freshly prepared solutions of peptidase inhibitors before neurotensin injection. At zero time, a mixture of tritiated (10^6 c.p.m. , 107 Ci mmol^{-1}) and unlabelled (10 nmol) neurotensin was injected in 1 ml. After 20 s, the peristaltic pump was stopped for 2 min in order to facilitate neurotensin diffusion inside ileal tissues, then the perfusion was continued for a period of 8 min (from injection). Perfusates corresponding to 2 min time intervals were collected.

Venous effluents were passed through ODS-Silica cartridges (C18 Sep-pak, Millipore) that had been previously activated by several washes with acetonitrile ($2 \times 5 \text{ ml}$) then with $4 \times 5 \text{ ml}$ of 0.1% (v/v) trifluoroacetic acid (TFA), 0.05% (v/v) triethylamine (TEA) in water. After loading of samples, resin was washed with 0.1% (v/v) TFA, 0.05% (v/v) TEA in water, then peptides were eluted with 4 ml of 80% acetonitrile containing 0.1% (v/v) TFA, 0.05% (v/v) TEA. More than 90% of the radioactivity applied was recovered after the various step-elutions. Eluates were lyophilized and reconstituted in 2 ml of water containing 0.02% bovine serum albumen. Aliquots containing about 6000 c.p.m. were submitted to reverse-phase h.p.l.c.

Reverse-phase h.p.l.c. analysis

The h.p.l.c. procedure has been described previously (Checler *et al.*, 1988a). Briefly, aliquots of reconstituted samples were loaded onto a RP18 lichrosorb column (Merck, France) and eluted at room temperature. Elutions were performed at a flow rate of 1 ml min^{-1} and absorbing material was detected at 230 nm. Intact neurotensin and its catabolites were separated by means of a 42 min linear gradient of 0.1% TFA, 0.05% TEA in water/0.1% TFA, 0.05% TEA in acetonitrile from 90:10 (v/v) to 60:40 (v/v) and 500 μl fractions were collected. Radioactive products were identified by their retention times as compared with synthetic standards run in the same conditions and neurotensin-like immunoreactivity contents were estimated by radioimmunoassay.

Radioimmunoassays

Neurotensin-like immunoreactivity (NT-LI) was determined with an antiserum directed towards the C-terminal end of NT (antiserum 29G) (Cuber *et al.*, 1990). This polyclonal antiserum does not cross-react with neurotensin (1–12) or neurotensin (9–13) ($<0.1\%$) and displays a detection limit and an EC_{50} value of 0.5 and 5 fmol of neurotensin/tube, respectively. Radioimmunoassays were carried out at 4°C in a final volume of 500 μl in phosphate-buffered saline (50 mM Na_2HPO_4 and 140 mM NaCl , pH 7.5) containing 0.1% gelatin, 6000–7000 c.p.m. of ^{125}I -labelled neurotensin (Sadoul *et al.*, 1984) and various concentrations of unlabelled competitor (synthetic neurotensin) or diluted samples (De Nadai *et al.*, 1989). Incubations with a final dilution of antiserum 1:50,000 were allowed to reach equilibrium for 24 h, then

separation of bound and free peptide was classically performed by charcoal precipitation.

Peptides and drugs

Synthetic NT was purchased from Neosystem (Strasbourg, France). Tritiated neurotensin [$3,11$ -tyrosyl- $3\text{-}^3\text{H}(\text{N})$] (107 Ci mmol^{-1}) was obtained from New England Nuclear (France). Bestatin ((2s,3r)-3 amino 2-hydroxy-4-phenyl-butanoil leucine), phosphoramidon (N-(α -rhamno-pyranosyloxy-hydroxyphosphinyl)-leucyl-tryptophan) and prolyl-isoleucine were from Sigma Chemicals. Captopril (2-D-methyl-3-mercaptopropanoyl-L-proline) was from the Squibb Institute (Princeton, U.S.A.). CPP-AAY-pAB (N-(1(R,S)-carboxyl-3-phenyl-propyl)-alanyl-alanyl-tyrosyl-pAB) was kindly given by Dr M. Orlowski (Mount Sinai School of Medicine, New York, U.S.A.). Phosphodiethyl 03 (N-(phenylethylphosphonyl)-glycyl-prolyl-hexanoic acid) was synthesized as described by Dive *et al.* (1990).

Results

Data presented in Table 1 indicated that $83.6 \pm 10.1\%$ of the infused radioactivity was recovered under control conditions. The distribution of the label within the three time-intervals indicated that most of the radioactivity ($84.4 \pm 1\%$ of total radioactivity recovered) appeared in the 0–4 min interval while the radioactivity recovered in the two following periods (4–6 min and 6–8 min) represented 9.3 ± 1.3 and 6.3 ± 0.4 of total radioactivity, respectively (Table 1). It was noteworthy that pretreatment of ileal segments with various specific peptidase inhibitors neither influence the recovery of radioactivity nor affected the distribution of the label within the sequential time intervals (Table 1).

Degradation rate of neurotensin in dog ileum

The perfusion of tritiated and unlabelled neurotensin in dog ileum led to a linear decrease in the recovery of intact peptide until the 4–6 min time interval. Then, the percentage of native neurotensin remained constant, corresponding to about 30% of intact tritiated peptide (Figure 1). The same values were obtained when recovery of unlabelled neurotensin was estimated by radioimmunoassay (not shown). This plateau value probably reflected a steady state between the rate of peptide diffusion within the ileal tissues and the degradation process taking place inside these tissues. Taking into account these considerations, it was estimated that the half-life of neurotensin in this *ex vivo* model was between 2 and 6 min.

Catabolites of neurotensin in dog ileum

We examined the nature of the tritiated catabolites recovered in the 4–6 min time interval. H.p.l.c. analysis of the fraction corresponding to the Sep-Pak washout (see Methods) indicated that all the radioactivity eluted with the retention time of free tritiated tyrosine (not shown). Accordingly, this fraction did not display any neurotensin-like immunoreactivity. Radioactivity recovered by elution of the Sep-Pak columns with acetonitrile indicated five major radiolabelled products following h.p.l.c. analysis (Figure 2a). Three of these eluted with the retention times of neurotensin(1–8), neurotensin(1–10) and neurotensin(1–11). The main radioactive product exhibited the retention time of standard neurotensin and probably accounted for the remaining intact peptide since it corresponded to immunoreactive material (Figure 2b). In contrast, other peaks did not display neurotensin-like immunoreactivity, thereby confirming that the neurotensin antiserum used in the present study did not cross-react with any of the neurotensin catabolites (Figure 2b). Finally, the small

Table 1 Effect of various peptidase inhibitors on the recovery and distribution of radioactivity during dog ileum perfusion with tritiated neurotensin

Pretreatment	Recovery	Distribution of radioactivity recovered		
		0–4 min	4–6 min	6–8 min
Control	83.6 ± 10.1	84.4 ± 1.0	9.3 ± 1.3	6.3 ± 0.4
Bestatin	90.3 ± 9.7	86.7 ± 1.8	8.2 ± 0.7	5.2 ± 1.1
Captopril	86.2 ± 2.0	84.6 ± 3.0	9.9 ± 2.0	5.5 ± 1.0
Phosphoramidon	96.3 ± 0.9	89.7 ± 0.3	7.5 ± 0.6	2.8 ± 0.9
Pro-Ile	91.4 ± 0.4	86.0 ± 2.7	8.9 ± 2.5	5.0 ± 0.2
Cpp-AAY-pAB	86.2 ± 1.3	82.0 ± 0.6	11.5 ± 0.1	6.4 ± 0.6
P03	87.1 ± 0.7	88.3 ± 4.8	8.1 ± 3.6	3.6 ± 1.0

Perfused dog ileum segments were pretreated for 10 min in the absence (control) or in the presence of the indicated peptidase inhibitor. At zero time, tritiated NT (10^6 c.p.m., 107 Ci mmol^{-1}) was injected and perfusion was continued for a total period of 8 min. Samples collected during the 0–4, 4–6 and 6–8 min time-intervals were counted. Recovery corresponds to the total radioactivity recovered expressed as the percentage of initial radioactivity infused. The distribution of radioactivity between the various time-intervals is expressed as the percentage of total radioactivity recovered. Values are the mean $\pm$ s.e.mean of three to six independent experiments carried out with three independent dogs. Peptidase inhibitors were perfused at the following final concentrations: bestatin, $10 \mu\text{M}$; captopril, $1 \mu\text{M}$; phosphoramidon, $1 \mu\text{M}$; Pro-Ile, 10 mM ; Cpp-AAY-pAB, $2 \mu\text{M}$; P03 (phosphodiethyl 03) $0.5 \mu\text{M}$.

Figure 1 Kinetics of disappearance of tritiated neurotensin (NT) after infusion in dog ileum. Dog ileum segments were pretreated for 10 min by perfusion with Krebs-Ringer-bicarbonate buffer. Tritiated neurotensin (10^6 c.p.m., 107 Ci mmol^{-1}) was then infused in a 1 ml bolus. Labelled catabolites and intact tritiated neurotensin recovered in the venous effluent during the three time-intervals were loaded on Sep-Pak cartridges. Samples corresponding to the acetonitrile step elution were lyophilised, reconstituted in water and an aliquot (about 6000 c.p.m.) was applied to reverse-phase h.p.l.c. as described in Methods. Labelled peptides were identified by comparison of their retention times with those of synthetic standards run in the same conditions. Values represented the radioactivity corresponding to intact tritiated NT recovered after h.p.l.c. analysis and are expressed as the percentage of total radioactivity recovered in the two Sep-Pak elution steps. Values are the mean $\pm$ s.e.mean of four separate experiments carried out with four dogs.

amount of radioactivity that behaved as free labelled tyrosine could reflect a small proportion of tyrosine that would have resisted the washing step of the Sep-Pak procedure.

It was noteworthy that the nature of the catabolites generated during the various time intervals appeared identical although they were recovered in lesser amounts (Table 2) in the 0–4 min interval. By contrast, a quantitatively similar production of the different catabolites was observed between 4–6 and 6–8 min. This indicated that the plateau value observed for intact neurotensin recovery (Figure 1) was accompanied by a steady-state level of the catabolites formation (Table 2).

Effect of peptidase inhibitors on the recovery of intact neurotensin and on the neurotensin (1–10) formation

We recently described a potent phosphonamide peptide inhibitor, phosphodiethyl 03, that displayed a K_i value of about 1 nM towards endopeptidase 3.4.24.16 (Barelli *et al.*, 1992). This agent elicited a dose-dependent increase of the neurotensin-like immunoreactivity recovered in the venous effluent (Figure 3). This protecting effect of phosphodiethyl 03 on neurotensin was accompanied by a concomitant decrease of the production of tritiated neurotensin (1–10) estimated after h.p.l.c. (Figure 3). This inhibitor appeared to be without effect on the formation of the other neurotensin catabolites (not shown).

It was crucial to examine the effect of the endopeptidase 3.4.24.15 inhibitor, Cpp-AAY-pAB since we have reported that phosphodiethyl 03 not only blocks endopeptidase 3.4.24.16, but also inhibits endopeptidase 3.4.24.15 although with a lower potency ($K_i = 7.5 \text{ nM}$) (Barelli *et al.*, 1992). Figure 3 clearly shows that Cpp-AAY-pAB neither modified neurotensin recovery nor affected neurotensin (1–10) production. Altogether, these data strengthen the hypothesis that the protective effect of phosphodiethyl 03 on neurotensin catabolism is mediated via the inhibition of endopeptidase 3.4.24.16. Such a conclusion was further confirmed by the examination of the effect of Pro-Ile, a dipeptide which we have previously reported is a selective blocker of endopeptidase 3.4.24.16 (Dauch *et al.*, 1991). Figure 4 indicates that Pro-Ile elicited a drastic increase in the recovery of NT-LI, together with an important decrease in neurotensin (1–10) formation. These data confirm the involvement of endopeptidase 24.16 in the catabolism of neurotensin, *in vivo*, in dog ileum. It should be noted that $10 \mu\text{M}$ phosphoramidon, a potent and specific inhibitor of endopeptidase 24.11 (Suda *et al.*, 1973), also induced a protection of NT-LI and slowed down the formation of neurotensin(1–10) (Figure 4) and neurotensin(1–11) (data not shown). This was in keeping with our previous data showing that thiorphan (another endopeptidase 24.11 inhibitor, Roques *et al.*, 1980) led to the partial protection of neurotensin in dog ileum (Checler *et al.*, 1988b). Finally, the aminopeptidase M inhibitor, bestatin (Umezawa *et al.*, 1976) slightly affected neurotensin recovery and neurotensin(1–10) formation (Figure 4). This effect could probably be explained by the slight inhibition (12% at $10 \mu\text{M}$) elicited by this inhibitor on purified endopeptidase 3.4.24.16 (Barelli *et al.*, 1988b).

Finally, the angiotensin-converting enzyme inhibitor captopril ($10 \mu\text{M}$) did not modify neurotensin recovery and neurotensin(1–10) production (Figure 4). This is in agreement with our previous work showing that angiotensin-converting enzyme does not contribute to the primary catabolism of neurotensin, in dog ileum (Checler *et al.*, 1988b).

Discussion

In this study, we have shown that neurotensin is rapidly catabolized in a vascularly perfused isolated ileal segment of anaesthetized dog with an estimated half-life between 2 and 6 min. This agrees with the very short-life of the peptide

(about 1 min) after intravenous bolus administration in rat and man (for review see Checler, 1991). It is noteworthy that the experimental procedure that included the flushing of the intestinal segment prior to administration of neurotensin precluded the possibility that plasma or blood cell factors could contribute to peptide degradation.

The major N-terminal degradation products of neurotensin generated during the transit of the peptide across intestinal tissues corresponded to neurotensin(1–8), neurotensin(1–10) and neurotensin(1–11). The formation of these products increased and reached an apparent plateau value at the 4–6 min and 6–8 min time intervals. This probably reflected a resistance of these products to secondary cleavages. This was not the case for their C-terminal counterparts neurotensin(9–13) and neurotensin(11–13) that were recovered in much lower amounts. This explained the recovery of free tyrosine that probably corresponded to the production of the tyrosine 11 resulting from the proteolytic subsequent breakdown of neurotensin(11–13) and neurotensin(9–13).

Figure 2 High performance liquid chromatography (h.p.l.c.) analysis of neurotensin catabolites recovered in 4–6 min time-interval of the venous effluent. Dog ileum segments were pretreated for 10 min by perfusion with Krebs-Ringer bicarbonate buffer. Tritiated neurotensin (10^6 c.p.m., 107 Ci mmol^{-1}) and unlabelled neurotensin (10 nmol) were then infused in a 1 ml bolus. Peptides recovered in the venous effluent were extracted on Sep-Pak cartridges, lyophilized and reconstituted in 2 ml of water as described in Methods. An aliquot (about 6000 c.p.m.) of the sample corresponding to the acetonitrile elution step was applied to reverse-phase h.p.l.c. and eluted in the conditions described in Methods. Fractions corresponding to 0.5 min ($500\ \mu\text{l}$) were collected, counted and tested for their content in neurotensin-LI as described in Methods. Labelled peptides (a) were identified by comparison of their retention times with those of synthetic standards run under the same conditions. The radioactivity (a) and NT-LI (b) contents are expressed as the percentage of total radioactivity or total NT-LI recovered after h.p.l.c. analysis.

Figure 3 Effect of phosphodiethylpyl 03 and Cpp-AAY-pAB on neurotensin recovery and neurotensin (1–10) formation. Dog ileum segments were pretreated for 10 min by perfusion with Krebs-Ringer-bicarbonate buffer in the absence (A) or in the presence of phosphodiethylpyl 03 $0.05\ \mu\text{M}$ (B) or $0.5\ \mu\text{M}$ (C) or with Cpp-AAY-pAB $0.5\ \mu\text{M}$ (D) or $2\ \mu\text{M}$ (E). At zero time, tritiated neurotensin (10^6 c.p.m., 107 Ci mmol^{-1}) and unlabelled neurotensin (10 nmol) were injected in 1 ml, then the perfusion was continued for a total period (from injection) of 8 min and 2 min-samples were collected. Samples were loaded on Sep-Pak cartridges and the peptides eluted in the acetonitrile step were lyophilized, reconstituted in 2 ml of water and an aliquot was h.p.l.c. analyzed as described in Methods. All collected fractions were counted and tested for their content of neurotensin-LI. Values are expressed as pmol of neurotensin-like immunoreactivity (a) or correspond to tritiated neurotensin (1–10) formation (b) expressed as the percentage of neurotensin(1–10) recovered in absence of inhibitor (control). Values are the means $\pm$ s.e.mean of ten determinations derived from three independent experiments carried out on three dogs. Asterisks indicate the statistical significance determined by Student's unpaired t test of inhibitory versus control conditions: $*P < 0.0001$. NS = non-significant.

Table 2 Identification and quantification of the tritiated catabolites recovered after perfusion of tritiated neurotensin in dog ileal segments

Time interval (min)	Radioactivity recovered in the Sep-Pak washout		Radioactivity recovered in the Sep-Pak acetonitrile step				
	Tyr	NT(1-8)	NT(1-10)	NT(1-11)	NT(11-13)	NT(9-13)	NT
0-4	18.0 ± 6.2	2.7 ± 0.3	2.2 ± 0.3	2.7 ± 0.7	2.3 ± 0.5	2.1 ± 0.4	70.0 ± 1.7
4-6	37.4 ± 6.5	8.6 ± 1.6	7.2 ± 1.5	7.4 ± 2.0	1.6 ± 0.4	2.5 ± 0.8	35.3 ± 3.3
6-8	37.3 ± 4.3	11.0 ± 1.8	7.4 ± 1.2	8.9 ± 1.3	1.3 ± 0.6	1.9 ± 0.1	32.2 ± 1.8

Dog ileum segments were pretreated for 10 min with Krebs-Ringer Bicarbonate buffer. Tritiated NT (10^6 c.p.m., 107 Ci mmol^{-1}) was infused as a 1 ml bolus and the labelled products recovered in the various time-intervals were submitted to the Sep-Pak extraction procedure described in the Methods. Samples recovered in the washout and acetonitrile steps were lyophilized, reconstituted in water and aliquots were h.p.l.c. analyzed as described. Neurotensin catabolites were identified by comparison of their retention times with those of synthetic standards run in the same conditions. The content of radioactivity under each peak was expressed as the percentage of total radioactivity recovered by the Sep-Pak extraction procedure. Values are the means $\pm$ s.e.mean of four independent experiments performed with four dogs.

Figure 4 Effect of specific peptidase inhibitors on the degradation of neurotensin in perfused ileum segment of dog. Dog ileum segments were pretreated for 10 min by perfusion with Krebs-Ringer-bicarbonate buffer in the absence (control conditions) or in the presence of various peptidase inhibitors. At zero time, tritiated neurotensin (10^6 c.p.m., 107 Ci mmol $^{-1}$) and unlabelled neurotensin (10 nmol) were injected in a 1 ml bolus then the perfusion was continued for a total period (from injection) of 8 min and 2 min-samples were collected. Samples were loaded on Sep-Pak cartridges, and the peptides recovered in the acetonitrile step were lyophilized, reconstituted in 2 ml of water and an aliquot was h.p.l.c. analyzed as described in Methods. Labelled peptides were identified by their retention time as compared with those of synthetic standards run under the same conditions. Values are the mean $\pm$ s.e.mean of six determinations (three independent experiments carried out on three dogs). Asterisks indicate the statistical significance determined by Student's unpaired t test of inhibitory versus control conditions: * $P < 0.01$, ** $P < 0.005$, *** $P < 0.001$. Peptidase inhibitors were used at the following final concentrations in the perfusion buffer: control without inhibitor (A); bestatin, $10 \mu\text{M}$ (B); captopril, $1 \mu\text{M}$ (C); phosphoramidon, $1 \mu\text{M}$ (D); Pro-Ile, 10 mM (E).

We previously showed that endopeptidase 24.11 is responsible for neurotensin(1–11) formation and also contributes to neurotensin(1–10) production (Checler *et al.*, 1988b) in dog ileum. In agreement with these studies, the present data indicate that the endopeptidase 24.11 inhibitor phosphoramidon fully blocked neurotensin(1–11) formation and partly inhibited neurotensin(1–10) production. However, there is an important phosphoramidon-insensitive generation of neurotensin(1–10). A good candidate for mediating the formation of this peptide could be endopeptidase 3.4.24.16. Thus, endopeptidase 3.4.24.16 was first detected and later purified from central (Checler *et al.*, 1986; Barelli *et al.*, 1988a) and peripheral (Barelli *et al.*, 1988b; 1993a,b) tissue sources on the basis of its ability to degrade neurotensin, leading to neurotensin(1–10) formation. This activity remains the major candidate for the catabolism of neurotensin since we have established that endopeptidase 3.4.24.16 is the only proteolytic activity that was shown to participate ubiquitously in the inactivation of neurotensin in various tissue sources, cells or membrane preparations from central or peripheral origin (Checler *et al.*, 1988a).

We recently described various inhibitors which allowed us to examine the putative contribution of endopeptidase 3.4.24.16 in the formation of neurotensin(1–10). Phosphodipepryl 03 was reported to block endopeptidase 3.4.24.16 with a K_i value of about 1 nM (Barelli *et al.*, 1992). This inhibitor clearly protected neurotensin from degradation in dog ileum and induced a selective inhibition of the expected catabolite i.e. neurotensin(1–10) (Figure 3). Although phosphodipepryl 03 (at a 1000 fold concentration above its K_i value for endopeptidase 24.16) did not inhibit purified endopeptidase 3.4.24.11, angiotensin-converting enzyme, leucine aminopeptidase and carboxypeptidase A, we established that this inhibitor also inhibited endopeptidase 24.15 ($K_i \sim 7.5 \text{ nM}$)

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Figure 5 Model of neurotensin catabolism in vascularly perfused ileum segments of dog. Full arrows indicate primary cleavages taking place on the neurotensin molecule (present study) and dashed arrows indicate secondary cleavages occurring on the neurotensin catabolites (our previous study Checler *et al.*, 1988b). Neurotensin (NT): pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu. E:24.11, endopeptidase 3.4.24.11; E:24.16, endopeptidase 3.4.24.16; ACE, angiotensin-converting enzyme; AP, aminopeptidases.

(Barelli *et al.*, 1992). We therefore examined the effect of the endopeptidase 24.15 inhibitor, Cpp-AAY-pAB. We demonstrated (Figure 3) that this agent was unable to protect neurotensin from degradation in dog ileum and did not modify neurotensin(1–10) formation. These data indicated that the effect of phosphodipepryl 03 on neurotensin catabolism was clearly mediated via the inhibition of endopeptidase 24.16. This conclusion was further reinforced by the fact that the dipeptide, Pro-Ile, elicited an effect similar to that of phosphodipepryl 03 on neurotensin recovery and neurotensin(1–10) formation (Figure 4). Thus, we previously showed that Pro-Ile, which mimics the Pro-Tyr bond of neurotensin targeted by endopeptidase 24.16, inhibited endopeptidase 24.16 ($K_i = 90 \mu\text{M}$). Furthermore, the inhibitory spectrum of this dipeptide towards other exo- and endopeptidases indicated that it behaved as a fully selective endopeptidase 24.16 inhibitor (Dauch *et al.*, 1991). Altogether, these observations clearly established the participation of endopeptidase 24.16 in the metabolism of neurotensin and represented the first demonstration that neurotensin could behave as a physiological substrate of endopeptidase 24.16. A model that summarizes the proteolytic events taking place on neurotensin, *in vivo*, in dog ileum is presented in the Figure 5.

Phosphodipepryl 03, which displays high potency and solubility, can be regarded as a novel tool with which to assess whether endopeptidase 24.16 is involved in the central mechanisms of neurotensin inactivation. Furthermore, on account of the large specificity of endopeptidase 24.16, which can also hydrolyse a series of other neuropeptides besides neurotensin, phosphodipepryl 03 could prove useful in examining the contribution of endopeptidase 24.16 in the physiological termination of the action of other biologically active peptides.

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