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Phosphorus-containing peptides as mixed inhibitors of endopeptidase 3.4.24.15 and 3.4.24.16: effect on neurotensin degradation *in vitro* and *in vivo*

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1 We have examined several phosphorus-containing peptides as potential mixed inhibitors of two neurotensin-degrading zinc metallopeptidases, endopeptidase 3.4.24.15 and endopeptidase 3.4.24.16.

2 Among a series of 13 phosphonamide peptides, N-(2-(2-naphtyl)ethylphosphonyl-glycyl-prolyl-norleucine (phosphodiepryl 08) was found to inhibit potently the hydrolysis of neurotensin by purified endopeptidase 3.4.24.15 and 3.4.24.16 with an identical K_i value of 0.4 nM.

3 Phosphodiepryl 08 displayed a strong selectivity towards the two peptidases since it failed to inhibit several other zinc-containing peptidases such as endopeptidase 3.4.24.11, angiotensin-converting enzyme, aminopeptidase M, leucine aminopeptidase and carboxypeptidases A and B.

4 The protective effect of phosphodiepryl 08 on neurotensin degradation was examined *in vitro* and *in vivo* in central and peripheral bioassays.

5 Phosphodiepryl 08 virtually abolished neurotensin degradation by 4-day-old plated pure cultured neurones from mouse embryos and greatly potentiated neurotensin-induced antinociception in the mouse hot plate test.

6 In the periphery, phosphodiepryl 08 inhibited neurotensin degradation by membranes prepared from isolated longitudinal smooth muscle of guinea-pig ileum and greatly potentiated the neurotensin-induced contraction of the same longitudinal smooth muscle preparation.

7 Our study indicates that phosphodiepryl 08 behaves as a potent and selective mixed inhibitor of endopeptidase 3.4.24.15 and 3.4.24.16 and can be used as a powerful agent to prevent neurotensin degradation, *in vitro* and *in vivo*, in central and peripheral assays.

Keywords: Neurotensin; degradation; peptidases; endopeptidase 3.4.24.15; endopeptidase 3.4.24.16; phosphorus peptides; inhibitors; ileum contraction; cultured neurones; analgesia

Introduction

We previously examined the catabolic fate of the tridecapeptide neurotensin by various membrane preparations and cells of central and peripheral origin (Checler *et al.*, 1988). It appeared consistently that neurotensin was hydrolysed at its Arg^8-Arg^9 and Pro^{10}-Tyr^{11} peptidyl bonds, leading to the biologically inactive products, neurotensin (1-8) and neurotensin (1-10) (Checler *et al.*, 1988). By means of specific inhibitors, we demonstrated that endopeptidase 3.4.24.15 was responsible for the formation of neurotensin (1-8) (Checler *et al.*, 1985) while neurotensin (1-10) mainly resulted from cleavage by an unidentified peptidase (Checler *et al.*, 1983; 1984; 1985) later purified in our laboratory (Checler *et al.*, 1986b) and now called endopeptidase 3.4.24.16 (or neurolysin, Checler *et al.*, 1994). Therefore, the development of mixed inhibitors able to block potently endopeptidase 3.4.24.15 and endopeptidase 3.4.24.16 would be of considerable use for *in vitro* and *in vivo* work with neurotensin by preventing the degradation of the peptide.

Phosphonamide peptides have been shown to behave as very potent inhibitors of metallopeptidases that contain a zinc atom at their active site (Barlett & Marlowe, 1987), a physicochemical feature that is shared by both endopeptidases 3.4.24.15 and 3.4.24.16 (Pierotti *et al.*, 1990; Checler *et al.*, 1986b). Here we examine a series of phosphorus-containing peptides as potential blockers of endopeptidases 3.4.24.15 and 3.4.24.16. One of them, N-[2-(2-naphtyl)ethylphosphonyl]-Gly-Pro-norleucine (referred to as phosphodiepryl 08), inhibits both enzymes equally (K_I=0.4 nM) and displays high selectivity towards these peptidases compared to other zinc metalloexo- and endo-peptidases. In addition, we report on the ability of this agent to reduce greatly neurotensin degradation by pure cultured neurones from mouse embryos and by membranes prepared from isolated longitudinal smooth muscle of guinea-pig ileum. Finally, we examine the effect of phosphodiepryl 08 on the neurotensin-induced nociceptive response in mice after i.c.v. administration as well as on the neurotensin-induced contraction of isolated longitudinal smooth muscle from guinea-pig ileum.

Methods

Purification of endopeptidase 3.4.24.16 and endopeptidase 3.4.24.15

Rat brain endopeptidase 3.4.24.16 and endopeptidase 3.4.24.15 were purified according to procedures described previously by Barelli *et al.* (1988, 1991).

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Preparation of membrane homogenates from rat brain and guinea-pig ileal longitudinal smooth muscle

Rats were decapitated, the brain immediately removed and homogenized by polytron in 5 mM Tris/HCl, pH 7.5. Protein concentrations were adjusted to 10 mg ml^{-1} in the same buffer. Longitudinal smooth muscle strips (including myenteric plexus) of about 2 cm were dissected out from the ileum of two- to three-month old guinea-pigs. Homogenization was carried out as above and protein concentrations were adjusted to 3.5 mg ml^{-1}.

Primary cultured neurones

Primary cultures of neurones were prepared from hemispheres of 14-day-old mouse embryos as previously described (Chabry *et al.*, 1990). At the beginning of the third day, cytosine arabinofuranoside (5 μM) was added to the medium for 24 h to prevent glial cell proliferation. We previously established that, in our experimental conditions, neuronal cultures appeared totally devoid of glial fibrillary acidic protein immunoreactivity, a specific astrocytic marker (Vincent *et al.*, 1994).

Degradation experiments and h.p.l.c. analysis

H.p.l.c. analysis Samples were applied on a reverse-phase C_{18} lichrosorb column (Merck) and eluted as previously described (Checler *et al.*, 1988). Absorbance was monitored at 230 nm with a detector setting of 0,05 full scale.

Neurotensin degradation by endopeptidase 3.4.24.16 and endopeptidase 3.4.24.15 Neurotensin (2 nmol, 20 μM) was incubated at 37°C with 10 μl of purified endopeptidase 3.4.24.16 or endopeptidase 3.4.24.15 in a final volume of 100 μl of 5 mM Tris/HCl pH 7.5, in the absence or in the presence of increasing concentrations of inhibitors. Incubations were acidified (10 μl of 1 M HCl) and analysed by h.p.l.c.

Neurotensin degradation by plated pure cultured neurones Monolayers of four-day-old plated neurones were washed twice with PBS buffer, pH 7.4 (mM: NaCl 140, Na_2HPO_4 8.5, KCl 2.7, KH_2PO_4 1.5). After aspiration of the buffer, neurotensin (10 nmol, 10 μM) was incubated with cells for 4 h at 37°C in a final volume of 1 ml of PBS, pH 7.4, in the absence or in the presence of various concentrations of peptidase inhibitors. At the end of the incubations, supernatants were removed, acidified (with 100 μl of 1 M HCl) and analysed by h.p.l.c. as above.

Neurotensin degradation by membranes from guinea-pig ileum longitudinal smooth muscle Neurotensin (2 nmol, 20 μM) was incubated for various times at 37°C with membrane homogenates (35 μg of protein) in a final volume of 100 μl of 5 mM Tris/HCl, pH 7.5, in the absence or in the presence of peptidase inhibitors. Incubations were then stopped by acidification (10 μl, 1 M HCl) and analysed by h.p.l.c. as above.

Carboxypeptidases A and B assays Hip-Phe and Hip-Lys (3 nmol, 30 μM) were incubated for 15 min at 37°C with 0.4 μg or 0.5 μg of purified carboxypeptidases A or B respectively, in a final volume of 100 μl of 5 mM Tris/HCl pH 7.5, in the absence or in the presence of inhibitor. Incubations were stopped and analysed by h.p.l.c. as above.

Fluorimetric assays of purified peptidases

Endopeptidase 3.4.24.16 and endopeptidase 3.4.24.15 Endopeptidase 3.4.24.16 (10 μl) was incubated with 50 μM of QFS for 1 h at 37°C, in a final volume of 100 μl of 5 mM Tris/HCl pH 7.5, in the absence or in the presence of increasing concentrations of phosphodiepryl 08. Incubations were stopped and enzymatic activities were fluorimetrically recorded as previously described (Dauch *et al.*, 1991a). Endopeptidase 3.4.24.15 was monitored with QFS as above or with 0.5 mM of Bz-Gly-Ala-Ala-Phe-pAB as the substrate (Checler, 1993).

Endopeptidase 3.4.24.11 Suc-Ala-Ala-Phe-7AMC (0.5 mM) was incubated with 25 ng of purified endopeptidase 3.4.24.11 for 10 min at 37°C in a final volume of 100 μl of 5 mM Tris/HCl pH 7.5. The 7AMC group was released from the Phe-7AMC degradation product upon exogenous aminopeptidase attack (5 μg of leucine aminopeptidase for 1 h at 37°C as described by Checler, 1993). Inhibition experiments were performed by addition of 1 μM phosphoramidon or various concentrations of phosphodiepryl 08.

Angiotensin-converting enzyme Hippuryl-His-Leu (1 mM) was incubated with 0.6 μg of purified angiotensin-converting enzyme for 10 min at 37°C in a final volume of 100 μl of 5 mM Tris/HCl pH 7.5 containing 0.3 M NaCl. Inhibition of angiotensin-converting enzyme was performed by addition of 1 μM captopril or various concentrations of phosphodiepryl 08.

Aminopeptidase M and leucine aminopeptidase Leu-7AMC (0.5 mM) was incubated for 6 min with 0.2 μg of leucine aminopeptidase or 0.5 μg of aminopeptidase M in 100 μl of 5 mM Tris/HCl pH 7.5. Inhibition studies were performed by prior incubations of the enzymes with 50 μM bestatin or increasing concentrations of phosphodiepryl 08.

Fluorimetric substrate-hydrolysing activities in whole rat brain homogenate Incubations were performed for various times at 37°C in 5 mM Tris/HCl pH 7.5 with a final concentration of 1 mg ml^{-1} of brain homogenate protein and activities were quantified as previously described (Checler, 1993). Briefly, proline endopeptidase activity was monitored by following the hydrolysis of 0.1 mM Z-Gly-Pro-7AMC and inhibition of the activity was obtained with 1 μM Z-Pro-Prolinal. Post-proline dipeptidyl aminopeptidase (DAP IV) was detected with 1 mM Gly-Pro-7AMC and inhibited by 100 μM of diprotin A. Aminopeptidase B was assayed with 0.2 mM Arg-7AMC and inhibited by 0.5 μM arphamenine B. All other activities were detected in the conditions described for assaying purified peptidases.

Hot plate test Male mice weighing 18–22 g were injected i.c.v. with 10 μl of sterile saline buffer (0.9% NaCl) or neurotensin (in saline) in the presence or in the absence of various quantities of phosphodiepryl 08. The hot plate test was derived from that described by Eddy & Leimbach (1953). The plate was maintained at a temperature of 55±0.5°C with a regulated water circulating pump. The antinociception test was assessed 15 min after i.c.v. injection and the reaction times (latency of both fore-paw licking with a cut-off time of 30 s) of mice placed in a glass cylinder (20 cm high, 16 cm diameter) surrounding the plate were determined as described (Schmidt *et al.*, 1991).

Guinea-pig ileal longitudinal smooth muscle contraction assays Female guinea-pigs (2–3 months) were used in all studies. The longitudinal smooth muscle (including the myenteric plexus) was dissected out from whole ileum according to Paton & Zar (1968) and strips 1.5–2 cm in length were set up for isometric tension recording in 3 ml organ baths containing modified Tyrode solution (mM: NaCl 136.8, KCl 2.7, $MgSO_4$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, $CaCl_2$ 2.5, glucose 5.5) to which was added 100 nM neostygmine methylsulphate. The strips were allowed to equilibrate for 45 min in modified Tyrode solution (maintained at 37°C under 95% O_2 and 5% CO_2) and washed every 10 min. Peptidase inhibitors were included in organ baths before the addition of various concentrations of neurotensin.

Protein concentrations Protein concentrations were determined by the Bradford method with white egg lysozyme as the standard.

Materials

Mcc-Pro-Leu-Gly-Pro-DLys-Dnp (QFS) and diprotin A were from Novabiochem. Di-isopropylfluorophosphate-treated carboxypeptidase A, leucine aminopeptidase (microsomal, type IV), angiotensin-converting enzyme, bestatin and all the chromogenic and fluorimetric substrates were from Sigma chemicals. Carboxypeptidase B and aminopeptidase M were from Boehringer Mannheim. Neurotensin was from Neosystem. Endopeptidase 3.4.24.11 was purified and kindly provided by Dr Patrick Auberger (INSERM U 364, Faculté de Médecine, Nice, France). Arphamenine B was from Interchim. Benzyloxycarbonyl-prolyl-prolinal (Z-Pro-Prolinal) and Bz-Gly-Ala-Ala-Phe-pAB were kindly donated by Drs S. Wilk and M. Orlowski (Mount Sinai, School of Medicine, New York, U.S.A.). The synthesis of phosphorus-containing peptides has been described previously (Dive *et al.*, 1990; Yiotakis *et al.*, 1994).

Results

Effect of phosphorus-containing peptides on endopeptidases 3.4.24.15 and 3.4.24.16

Table 1 summarizes the affinities of various phosphorus-containing peptides for endopeptidase 3.4.24.15 and endopeptidase 3.4.24.16. This was assessed by means of neurotensin that was previously shown to behave as a good substrate of both peptidases. The most striking feature concerns the parallel observed between the K_i values obtained for endopeptidases 3.4.24.15 and 3.4.24.16. Indeed, there exists only a threefold factor between the K_i values determined for the inhibitors (compounds 05 and 11) that discriminate the most between the two enzymes.

As compared to phosphodiepryl 03 (compound 03), the low potency of compound 01 underlines the positive contribution of an aromatic substituent to the binding process. Interestingly, there is an optimal length (corresponding to 2 or 3 methylene groups) between this aromatic group and the phosphonamide moiety that leads to favourable interactions (compounds 03 and 04). Introduction of a substituent in the *para* position of the phenyl group (compound 05) slightly improves the K_i values for both enzymes. The fact that the addition of a naphtyl group further increases the potency of the inhibitor (compare compounds 03 and 08) probably indicates that the S_1 subsite can accomodate hydrophobic groups with distinct sizes. Compound 06 reveals that the hindrance introduced by an unsaturated double bond, drastically affects the inhibitory potency of the agent.

Replacement of the glycyl residue in the P'_1 position by an alanyl residue in the L configuration slightly improves the affinity of the compound for both peptidases (compare 03 and 07). By contrast, a D-alanyl residue in this P'_1 site strongly diminishes the affinity of the inhibitor (compare compounds 07 and 09) for both enzymes. This emphasizes the strict stereochemical requirements of the S'_1 site of these two peptidases. Introduction of an alanyl residue in P'_2 (compound 10) leads to a 24 fold reduction in K_i while such replacement in the P'_3 position (compound 11) does not modify the inhibitory potency of the molecule for endopeptidase 3.4.24.16 and only slightly increases K_i against endopeptidase 3.4.24.15.

The activity of phosphonamide and phosphinic peptides containing a pseudo amino-acid on both sides of the phosphorus moiety was also examined. The rather good potency displayed by compound 13 confirms the preference of the S_1 subsite of these peptidases for aromatic groups. Interestingly, the substitution of phosphonamide by a phosphinic group reduces the affinity of the inhibitor only by a factor of 4 and 7 for endopeptidase 3.4.24.15 and endopeptidase 3.4.24.16, respectively (compare compounds 13 and 14). This reduction is relatively slight when compared with the introduction of a phosphonate group that induces a drastic loss of affinity (compare compounds 03 and 16).

One of the best inhibitors (compound 08, referred to as phosphodiepryl 08) was thoroughly examined to establish its inhibitory potency against hydrolysis of neurotensin by purified endopeptidases 3.4.24.15 and 3.4.24.16, its selectivity towards other zinc metalloproteases and its ability to interfere with neurotensin degradation, *in vitro* and *in vivo*, in central and peripheral assays.

Effect of phosphodiepryl 08 on purified endopeptidases 3.4.24.15 and 3.4.24.16

As previously described purified endopeptidase 3.4.24.16 generates the two catabolites neurotensin (1 – 10) and neurotensin (11 – 13) (Figure 1a). The formation of both degradation products appears to be inhibited dose-dependently by phosphodiepryl 08 and was virtually abolished at a concentration of 3×10^{-8} M (Figure 1). Similarly, phosphodiepryl 08 prevents

Table 1 Inhibition of endopeptidase 3.4.24.15 and endopeptidase 3.4.24.16 by phosphorus-containing peptides

Compound	Inhibitor	K_i (nM) Endopeptidase 3.4.24.16	Endopeptidase 3.4.24.15
01	CH_3 ψ [PO_2NH]-Gly-Pro-Nle	78	150
02	ϕ CH_2 ψ [PO_2NH]-Gly-Pro-Nle	379	553
03	ϕ CH_2-CH_2 ψ [PO_2NH]-Gly-Pro-Nle	1.6	2.6
04	ϕ CH_2-CH_2-CH_2 ψ [PO_2NH]-Gly-Pro-Nle	3.4	3.9
05	pCF_3-ϕ CH_2-CH_2 ψ [PO_2NH]-Gly-Pro-Nle	0.7	2.2
06	Cin ψ [PO_2NH]-Gly-Pro-Nle	299	518
07	ϕ CH_2-CH_2 ψ [PO_2NH]-Ala-Pro-Nle	0.4	0.4
08	2-Nap CH_2-CH_2 ψ [PO_2NH]-Gly-Pro-Nle	0.4	0.4
09	ϕ CH_2-CH_2 ψ [PO_2NH]-DAla-Pro-Nle	369	251
10	ϕ CH_2-CH_2 ψ [PO_2NH]-Gly-Ala-Nle	39	40
11	ϕ CH_2-CH_2 ψ [PO_2NH]-Gly-Pro-LAla	1.4	4.2
12	ϕ CH_2-CH_2 ψ [PO_2NH]-Gly-Pro-LTyr	4.5	9.4
13	Z-(DL)Phe [PO_2NH]-Gly-Pro-Nle	5.5	8.5
14	Z-(L)Phe ψ [PO_2CH_2]-Gly-Pro-Nle	33	39
15	Z-(D)Phe ψ [PO_2CH_2]-Gly-Pro-Nle	209	323
16	ϕ CH_2-CH_2 ψ [PO_2O]-Gly-Pro-Nle	375	377

Neurotensin (2 nmol, 20 μM) was incubated with 10 μl of purified endopeptidase 3.4.24.16 or with 10 μl purified endopeptidase 3.4.24.15 for 1 h at 37°C in the presence of increasing concentrations (0.1 nM to 10 μM) of the indicated compounds. The K_i values were derived from the IC_{50} values (see Results) and are the mean of 4 independent determinations. ϕ, phenyl; Cin, cinnamoyl; 2-Nap, 2-naphtyl; Nle, norleucine, 2-aminohexanoic acid.

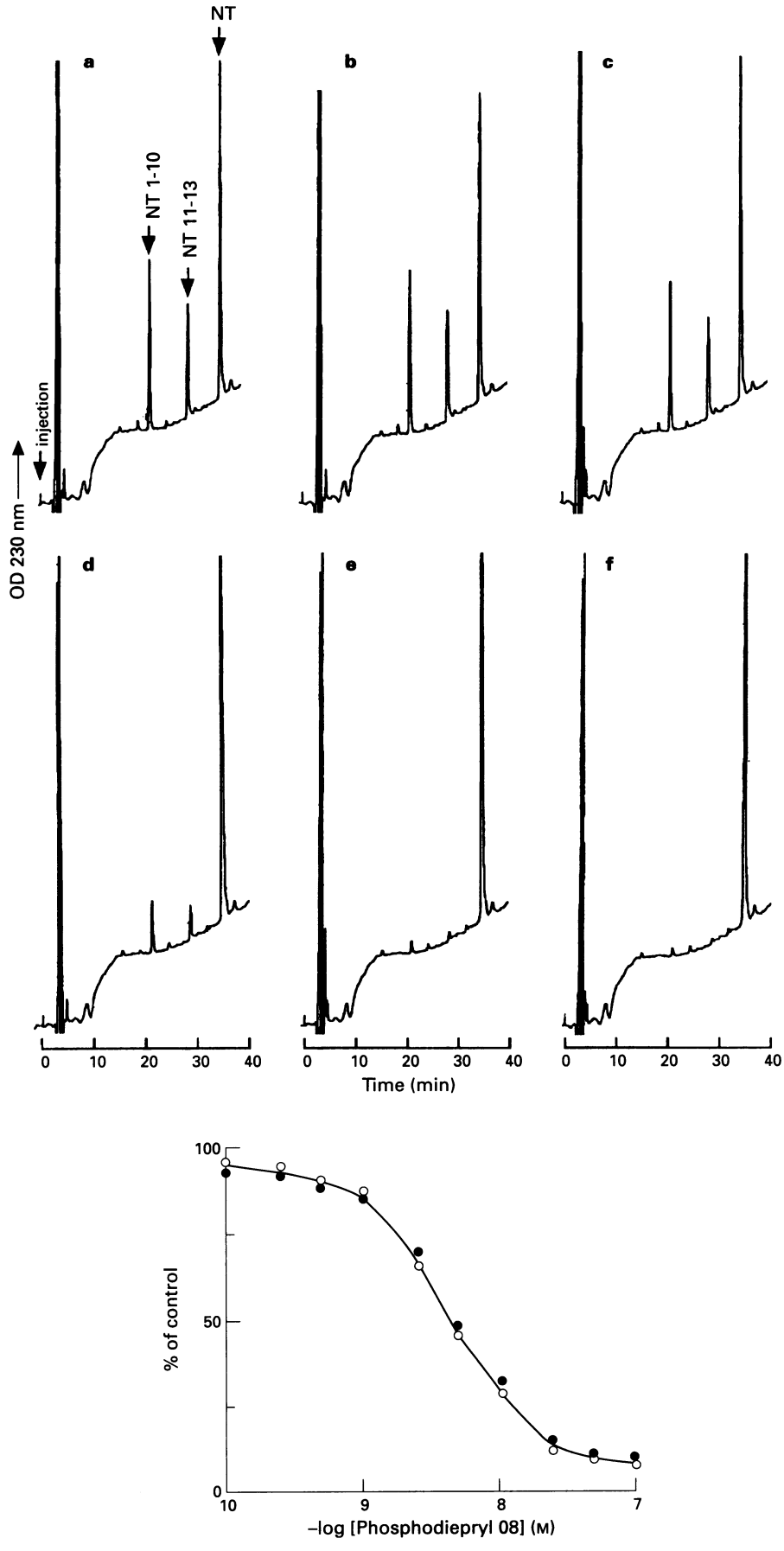

Figure 1 Effect of phosphodiepryl 08 on neurotensin hydrolysis by purified rat endopeptidase 3.4.24.16. Neurotensin (2 nmol, 20 μM) was incubated for 1 h at 37°C with 10 μl of purified endopeptidase 3.4.24.16 in 100 μl of 5 mM Tris/HCl, pH 7.5, in the absence (control, a) or in the presence of 0.1 nM (b); 1 nM (c); 10 nM (d); 100 nM (e) and 1 μM (f) phosphodiepryl 08. Incubations were acidified (10 μl, HCl 1 M) and submitted to reverse-phase h.p.l.c. as described in Methods. The curve represents the amount of neurotensin (1–10) (●) and neurotensin (11–13) (○) recovered in the presence of inhibitor and expressed as a percentage of the control value obtained in absence of phosphodiepryl 08 (a).

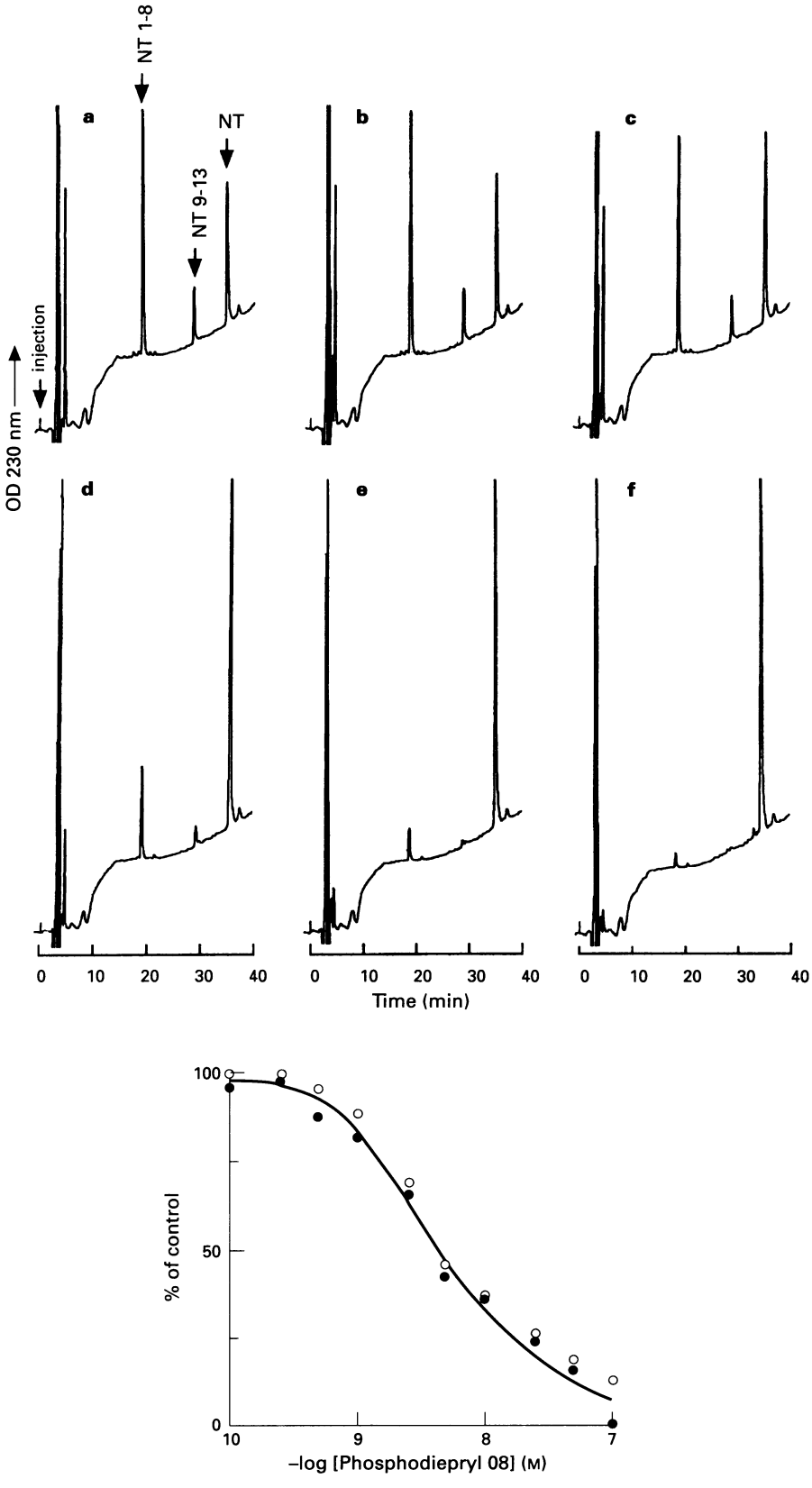

Figure 2 Effect of phosphodiepryl 08 on neurotensin hydrolysis by purified rat endopeptidase 3.4.24.15. Neurotensin (2 nmol, 20 μM) was incubated for 1 h at 37°C with 10 μl of purified endopeptidase 3.4.24.15 in 100 μl of 5 mM Tris/HCl pH 7.5 in the absence (control, a), or in the presence of 0.1 nM (b); 1 nM (c); 10 nM (d); 100 nM (e) and 1 μM (f) phosphodiepryl 08. Incubations were acidified (10 μl, HCl 1 M) and submitted to reverse-phase h.p.l.c. as described in Methods. The curve represents the amount of neurotensin (1–8) (●) and neurotensin (9–13) (○) recovered in the presence of inhibitor and expressed as the control degradation products obtained in absence of phosphodiepryl 08 (a).

the production of neurotensin (1–8) and neurotensin (9–13) (Figure 2a), the two catabolites that were generated by purified endopeptidase 3.4.24.15 (Orlowski *et al.*, 1983). The formation of these two products was diminished in parallel and was maximally inhibited at a concentration of 10^{-7} M phosphodiepryl 08 (Figure 2).

Figure 3 illustrates the effect of phosphodiepryl 08 on the hydrolysis of QFS, a quenched fluorimetric substrate of endopeptidase 3.4.24.16 (Dauch *et al.*, 1991a) and endopeptidase 3.4.24.15 (Barrett & Tisljar, 1989; Tisljar *et al.*, 1990). The curves indicate a dose-dependent and complete inhibition of the hydrolysis of the substrate. The K_i values in Table 2 were calculated according to the single relationship $IC_{50} = K_i (1 + \frac{S}{K_m})$ using K_m values of endopeptidase 3.4.24.16 for QFS and neurotensin of 25 μM and 2 μM, respectively (Dauch *et al.*, 1991a; Checler *et al.*, 1986b) and 9 μM and 2 μM for endopeptidase 24-15 (Tisljar *et al.*, 1990; Barelli *et al.*, 1991). These data indicate a K_i value of 0.4 nM for endopeptidase 3.4.24.16 whatever the substrate that was examined. The same affinity was observed for endopeptidase 3.4.24.15. Futhermore, we established that phosphodiepryl 08 inhibited the hydrolysis of Bz-Gly-Ala-Ala-Phe-pAB (a selective substrate for endopeptidase 3.4.24.15) with a virtually identical affinity (K_i = 2.2 nM, Table 2).

Selectivity of phosphodiepryl 08

We examined the putative inhibition of various purified zinc-metallopeptidases by phosphodiepryl 08. Figure 4 indicates that a concentration of 1 μM phosphodiepryl 08 did not affect endopeptidase 3.4.24.11 (neprylisin), angiotensin-converting

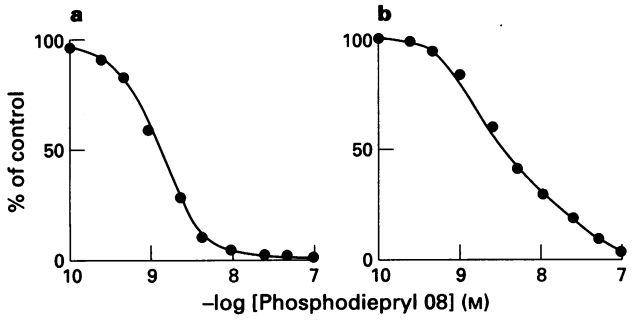

Figure 3 Effect of phosphodiepryl 08 on Mcc-Pro-Leu-Gly-Pro-DLys-Dnp (QFS) hydrolysis by purified endopeptidase 3.4.24.16 and endopeptidase 3.4.24.15. QFS (5 nmol, 50 μM) was incubated for 1 h at 37°C with 10 μl of purified endopeptidase 3.4.24.16 (a) or purified endopeptidase 3.4.24.15 (b) in 100 μl of 5 mM Tris/HCl pH 7.5 in the presence of various concentrations of phosphodiepryl 08. Incubations were stopped and fluorimetrically analysed as described in Methods. Values represent the mean of 3 independent determinations and are expressed as the percentage of activity obtained in absence of phosphodiepryl 08.

Table 2 K_i values of the inhibition of purified endopeptidase 3.4.24.15 and endopeptidase 3.4.24.16 by phosphodiepryl 08

		K_i (nM)	
Endopeptidase	*Neurotensin*	*QFS*	*Bz-Gly-Ala-Ala-Phe-pAB*
3.4.24.15	0.4	0.6	2.2
3.4.24.16	0.4	0.4	Not hydrolyzed

QFS and Bz-Gly-Ala-Ala-Phe-pAB hydrolyses were determined as described in Methods and neurotensin degradation was monitored by h.p.l.c. analysis as described in Methods. Values represent the mean of 5–8 independent determinations.

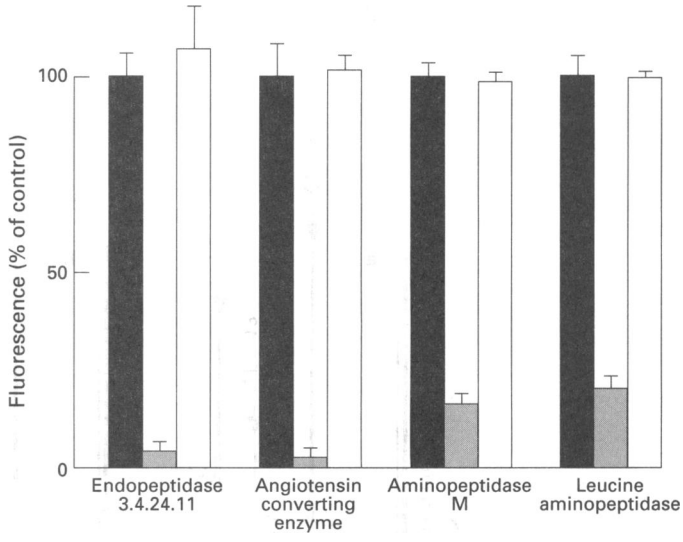

Figure 4 Effect of phosphodiepryl 08 and specific inhibitors on various purified peptidases. Activities were measured by incubating 0.5 mM Suc-Ala-Ala-Phe-7AMC (endopeptidase 3.4.24.11), 1 mM Hippuryl-His-Leu (angiotensin-converting enzyme) and 0.5 mM Leu-7AMC (aminopeptidase M and leucine aminopeptidase) under the conditions described in Methods, in the absence (solid columns) or in the presence of specific inhibitors (hatched columns) or 1 μM phosphodiepryl 08 (open columns). Incubations were stopped and activities were recorded as described in the Methods. Concentrations of inhibitors were as follows: endopeptidase 3.4.24.11, phosphoramidon 1 μM; angiotensin converting enzyme, captopril 1 μM; aminopeptidase M and leucine aminopeptidase, bestatin 50 μM. Values represent the mean ± s.e.mean of 4 independent determinations.

enzyme, leucine aminopeptidase and aminopeptidase M, the identities of which were confirmed by the sensitivity to their specific inhibitors (Figure 4). Furthermore, phosphodiepryl 08 was unable to modify the hydrolysis of Hip-Phe and Hip-Lys by carboxypeptidase A and carboxypeptidase B, respectively (Figure 5). Finally, the usefulness of phosphodiepryl 08 when employed with a crude membrane preparation from rat brain was assessed. Table 3 summarizes the effect of several specific inhibitors and phosphodiepryl 08 on the cleavage of various fluorimetric substrates by rat brain homogenates. Some of these substrates could not be considered as exclusive substrates for their pseudospecific peptidase. Indeed, the specific inhibitors of DAP IV and aminopeptidase B (i.e. diprotin A and arphamenine B, respectively) elicited only a faint or very partial inhibition of their respective peptidase. This indicated that Gly-Pro-7AMC and Arg-7AMC also behaved as substrates of additional peptidases. However, Table 3 clearly shows that these other peptidases were also unaffected by a concentration of 1 μM phosphodiepryl 08.

Effect of phosphodiepryl 08 on neurotensin degradation in the central nervous system

Two of the main neurotensin catabolites generated by plated 4-day-old pure cultured neurones are neurotensin (1–8) and neurotensin (1–10) (Figure 6a) consistent with our previous data (Checler *et al.*, 1986a; Vincent *et al.*, 1994). Phosphodiepryl 08 exerts a potent and dose-dependent inhibition of the formation of both products (Figure 6b) and potently increases the recovery of intact neurotensin. The IC_{50} observed (0.5 nM) led to the estimation of a K_i value of about 0.1 nM, in agreement with those observed with purified endopeptidases 3.4.24.15 and 3.4.24.16 (Table 2). This clearly suggests that these two peptidases mainly contribute to the inactivation of neurotensin by pure cultured neurones. In order to assess whether such conclusions also apply *in vivo* in the CNS, we examined the effect of phosphodiepryl 08 on the neurotensin-

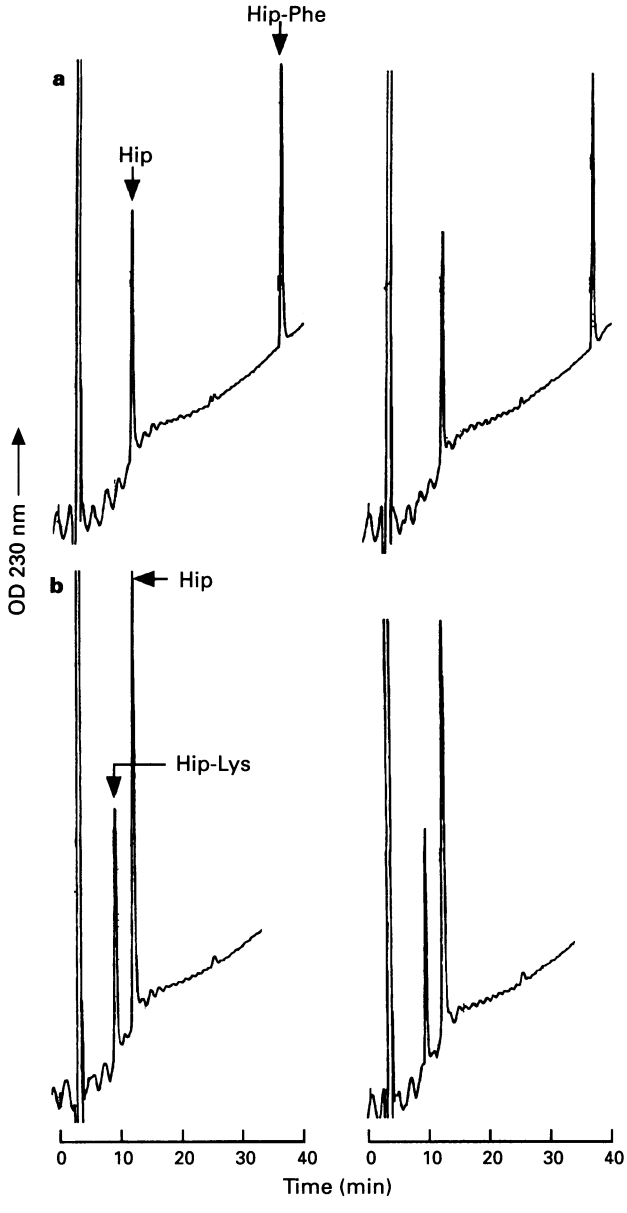

Figure 5 Effect of phosphodiepryl 08 on purified carboxypeptidase A and carboxypeptidase B. Carboxypeptidase A (0.4 μg) (a) and carboxypeptidase B (0.5 μg) (b) were incubated with Hip-Phe (3 nmol, 30 μM) and Hip-Lys (3 nmol, 30 μM), respectively, for 15 min at 37°C in a final volume of 5 mM Tris/HCl pH 7.5 in the absence (control, left panels) or in the presence of 1 μM phosphodiepryl 08 (right panels). Incubations were stopped by acidification and analysed by h.p.l.c. as described in Methods.

induced analgesic effect in mice. As shown in Figure 7, i.c.v. administration of neurotensin induced a dose-dependent antinociceptive response in the hot-plate tested mice. The administration of phosphodiepryl 08 with ineffective or submaximal doses of neurotensin greatly potentiated the antinociceptive effect of the peptide (Table 4).

Effect of phosphodiepryl 08 on peripheral neurotensin degradation

It has been shown previously that neurotensin contracts the isolated longitudinal smooth muscle from guinea-pig ileum (Kitabgi, 1982). This prejunctional effect appears to be mediated through cholinergic nerves located in the myenteric plexus that adheres to the longitudinal smooth muscle. Figure 8a illustrates the dose-dependent neurotensin-induced contraction of the longitudinal smooth muscle strip. Phosphodiepryl 08 greatly enhanced the contractile response elicited by a submaximal concentration (3 nM) of neurotensin (Figure 8b). Altogether, this seemed to indicate that endopeptidases 3.4.24.15 and 3.4.24.16 could participate in neurotensin catabolism in the gastrointestinal tract. In order to test this hypothesis further we examined the metabolic fate of neurotensin after exposure to homogenate membranes prepared from isolated longitudinal smooth muscle. Phosphodiepryl 08 slowed down the kinetics of neurotensin hydrolysis (Figure 9a) (half-lives of 13 and 47 min, in absence or in the presence of inhibitor, respectively) and virtually abolished the formation of neurotensin (1–8) and neurotensin (1–10) that corresponded to the only cleavage products identified after h.p.l.c. (Figure 9b).

Discussion

A classical strategy for the development of zinc-metalloprotease inhibitors is based on the use of the N- or C-terminal portion of the substrate to which a zinc chelating group (thiol, hydroxamate, carboalkyls) is attached in a suitable position. This approach has allowed the development of powerful and convenient molecules designed as mixed inhibitors of various metalloenzymes. Fournié-Zaluski *et al.* (1984) successfully designed a highly potent inhibitor of several enkephalin-degrading peptidases, kelatorphan, that fully prevents the degradation of exogenous enkephalins (Waksman *et al.*, 1985). Later, the same group developed the first systemically active inhibitor of endopeptidase 3.4.24.11 and aminopeptidase N, the two major peptidases that metabolize endogenous enkephalins (Noble *et al.*, 1992). This agent, designed as the prodrug RB101, produced a potent analgesic response in murine species through the elevation of extracellular levels of enkephalins (Noble *et al.*, 1992). French *et al.* (1994) also

Table 3 Effect of specific inhibitors and phosphodiepryl 08 on peptidase activities present in rat brain homogenates

		Enzyme activity	
Enzyme	*Substrate/Inhibitor*	*Specific inhibitor*	*Phospho-diepryl 08*
EC3.4.24.11	Suc-Ala-Ala-Phe-7AMC/phosphoramidon	22.2 ± 5	100 ± 4
ACE	Hip-His-Leu/Captopril	5.8 ± 1.5	93 ± 1
LAP/APM	Leu-7AMC/Bestatin	28.1 ± 2.4	87 ± 10
PE	Z-Gly-Pro-7AMC/Z-Pro-Prolinal	6.2 ± 0.8	100 ± 5
DAP IV	Gly-Pro-7AMC/Diprotin A	87 ± 0.4	100 ± 2
APB	Arg-7AMC/Arphamenine B	49 ± 3.6	96 ± 3

Activities were monitored by incubation of rat brain homogenate with the indicated substrates in the presence of phosphodiepryl 08 (1 μM) or saturating concentrations of the indicated specific inhibitors (for concentrations, see Methods). Values are expressed as a percentage of control activity obtained in absence of inhibitor and represent the mean ± s.e.mean of 4 independent determinations.

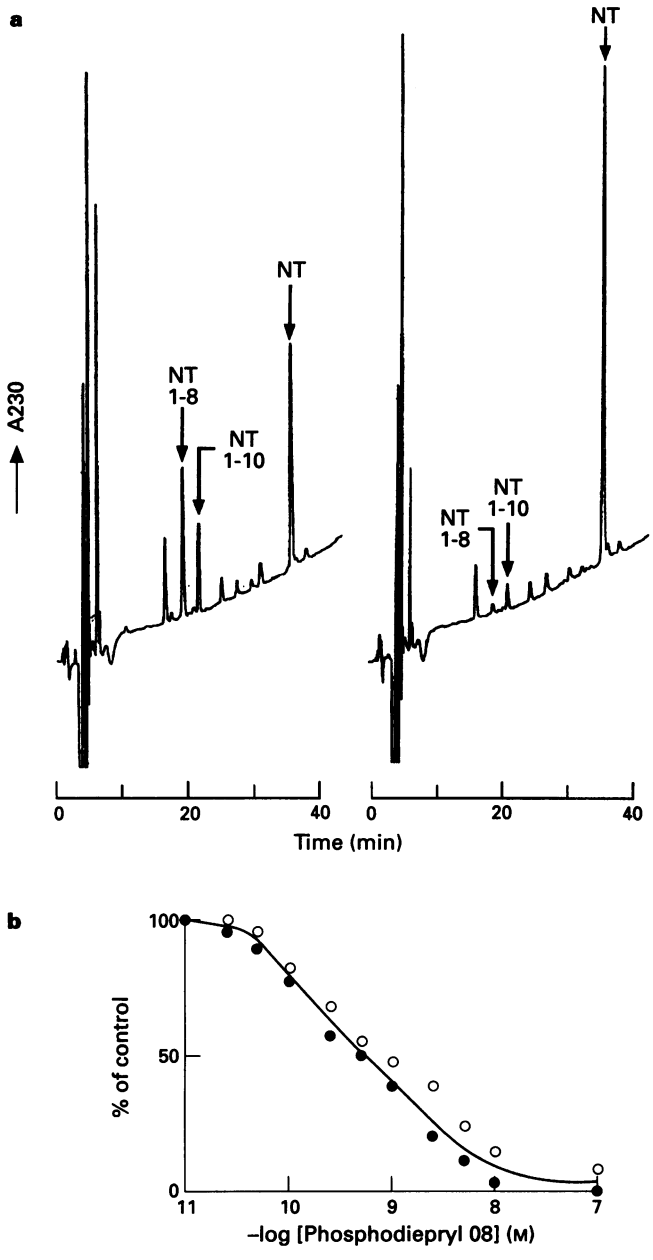

Figure 6 Effect of phosphodiepryl 08 on neurotensin hydrolysis by primary-cultured neurones. Neurotensin (10 nmol, 10 μM) was incubated with 4-day-old plated primary cultured neurones, for 4 h at 37°C, in 1 ml of PBS pH 7.4, in the absence (a, left panel), or in the presence of 10 nM phosphodiepryl 08 (a, right panel). Supernatants were then removed, acidified, (100 μl of 1 M HCl), and submitted to h.p.l.c. as described in Methods. Complete dose-response curves were established by incubating neurotensin (10 nmol, 10 μM) with the indicated increasing concentrations of phosphodiepryl 08 (b). Values are expressed as a percentage of control NT (1–8) (●) or NT (1–10) (○) recovered in absence of inhibitor and represented the mean of 4 independent determinations.

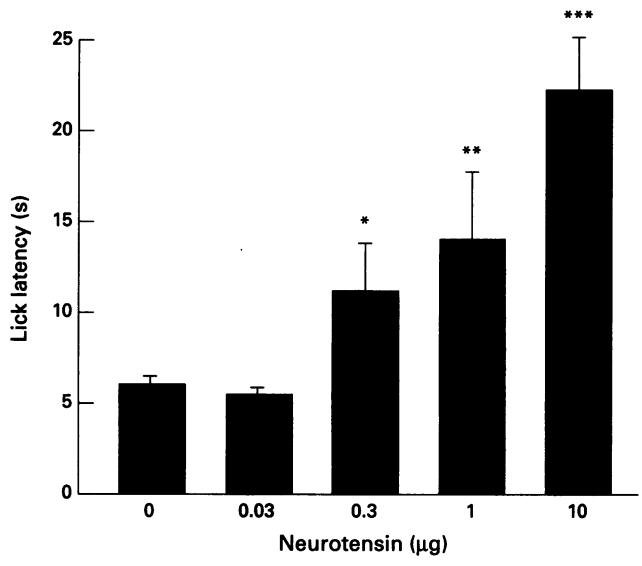

Figure 7 Neurotensin-induced antinociceptive effect on hot-plate tested mice. Fifteen min after the i.c.v. administration of saline buffer or the indicated dose of neurotensin, mice were placed on the hot plate (55°C) and the latency for the appearance of forepaw licking behaviour was determined. Columns represent the mean ± s.e.mean of results from 8 mice. $*P<0.1$ compared to the control (saline); $**P<0.05$; $***P<0.01$.

Table 4 Effect of phosphodiepryl 08 on neurotensin-induced antinociception in mice tested on the hot plate

Treatment	*Dose* (μg)	*Latency* (s)
Saline	–	5.99 ± 0.35
Phosphodiepryl 08	1	6.67 ± 1.25
Phosphodiepryl 08	3	7.50 ± 1.23
Neurotensin	0.03	5.40 ± 0.76
+ Phosphodiepryl 08	1	16.75 ± 3.34*
+ Phosphodiepryl 08	3	15.56 ± 3.71*
Neurotensin	0.3	11.27 ± 2.54
+ Phosphodiepryl 08	1	12.75 ± 2.63
+ Phosphodiepryl 08	3	18.65 ± 3.10*

Neurotensin was administered i.c.v. at the doses indicated in the absence or in the presence of various quantities of phosphodiepryl 08. Latency of appearance of forepaw licking behaviour was recorded 15 min after peptide and/or inhibitor administration. Results are given as means ± s.e.mean of 8 independent animals per group. $*P<0.05$ as compared to the same dose of neurotensin without inhibitor.

characterized a dual blocker of angiotensin-converting enzyme and endopeptidase 3.4.24.11. This agent increased the hypotensive, diuretic and natriuretic responses mediated by atrial natriuretic peptide and inhibited the hypertensive response thought to be dependent upon the conversion of angiotensin I into angiotensin II (French *et al.*, 1994).

From our previous studies, it appeared clear that potent inhibitors of the physiological degradation of neurotensin should target endopeptidases 3.4.24.16 and 3.4.24.15. Thus, we showed previously that endopeptidase 3.4.24.16 was the only peptidase that consistently participated in the metabolism of neurotensin by membrane preparations and cell cultures of central and peripheral origin (Checler *et al.*, 1988). Furthermore, we recently established that endopeptidase 3.4.24.16 was involved in the degradation of neurotensin, *in vivo*, in a model of vascularly perfused dog ileum (Barelli *et al.*, 1994). The present study indicates that endopeptidase 3.4.24.15, as well as endopeptidase 3.4.24.16, also contributed to neurotensin hydrolysis in plated pure cultured neurones. Furthermore, these two proteolytic activities appeared to contribute fully to neurotensin breakdown *in vitro* in membranes prepared from longitudinal smooth muscle of guinea-pig ileum. Therefore, the ubiquitous participation of the two enzymes in the central and peripheral degradation of neurotensin emphasizes the potential interest of developing mixed inhibitors of both activities to serve as blockers of physiological neurotensin proteolysis.

Our approach consisted in the synthesis of phosphorus-containing analogues of peptide substrates. To develop such transition-state analogues, the use of the phosphonamide

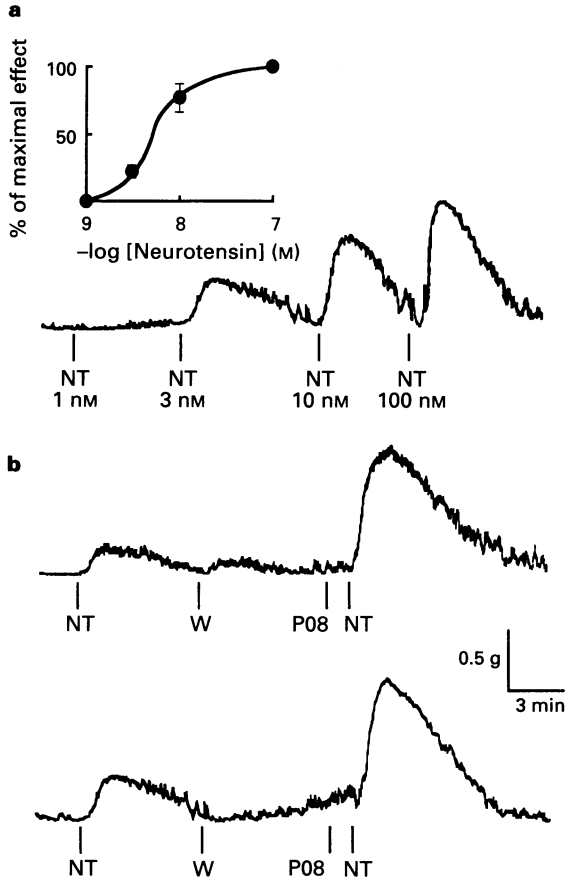

Figure 8 Effect of phosphodiepryl 08 on the neurotensin-induced contraction of isolated longitudinal smooth muscle strips from dog ileum. Preparations were allowed to equilibrate at 37°C under 95% O_2, 5% CO_2 for 45 min, in a modified Tyrode solution containing 100 nM neostigmine methylsulphate. (a) The isometric tension of the preparation was recorded in response to the indicated concentrations of neurotensin (NT). Note that between each neurotensin application, the preparation was washed out and subsequent peptide application occurred when the preparation returned to baseline level. Inset, dose-response curve of neurotensin-induced contraction. Values are expressed as a percentage of maximal contraction and represent the mean ± s.e.mean of 4 independent preparations. (b) Effect of 10 nM phosphodiepryl 08 (P08) on the contractile response elicited by 3 nM neurotensin in two separate ileal preparations.

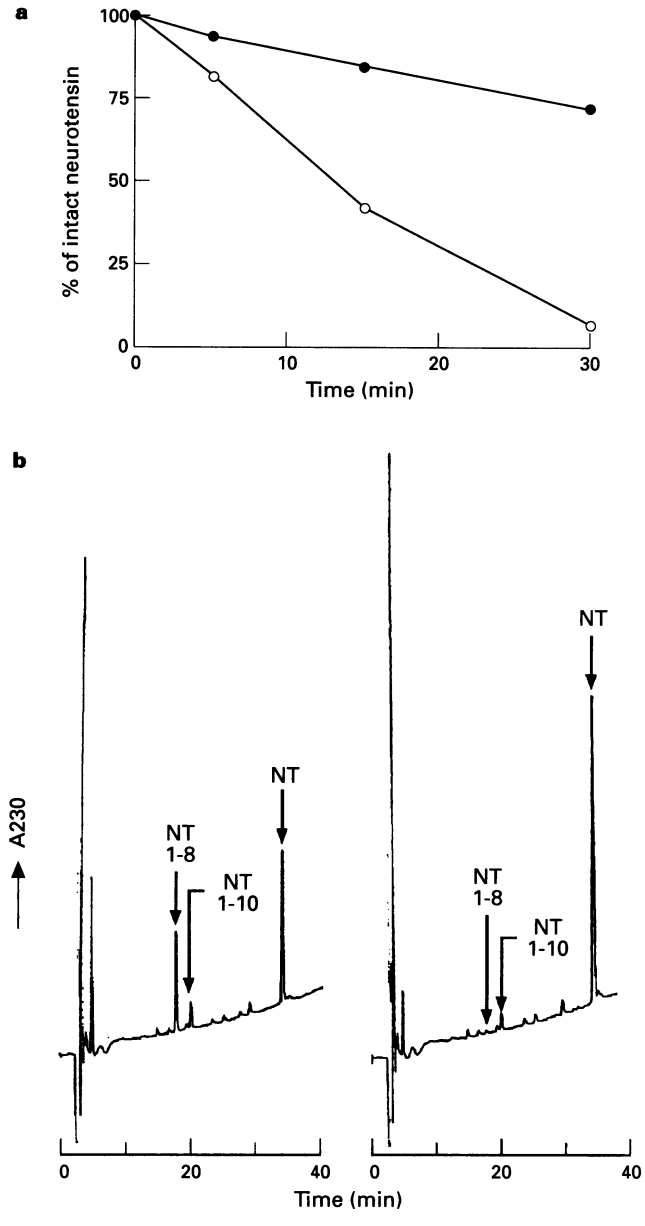

Figure 9 Effect of phosphodiepryl 08 on neurotensin hydrolysis by homogenates of guinea-pig ileal longitudinal smooth muscle. The kinetics of neurotensin hydrolysis by membrane homogenates of guinea-pig ileal longitudinal smooth muscle (a) were obtained by incubating neurotensin (2 nmol, 20 μM) at 37°C as described in Methods for various times in the absence (○) or in the presence of 10 nM phosphodiepryl 08 (●). Incubations were acidified, centrifuged (5 min, 13.00 r.p.m.), and supernatants were submitted to h.p.l.c. as described in the Methods. Intact neurotensin was quantified by comparing the remaining absorbing material with the standard amount of neurotensin incubated with preacidified membranes. Values represent the mean of 3 independent determinations. (b) Illustrates the h.p.l.c. analysis of neurotensin hydrolysis by membrane homogenates from guinea-pig ileum longitudinal smooth muscle. Neurotensin (2 nmol, 20 μM) was incubated for 15 min at 37°C with 35 μg of proteins in 100 μl of 5 mM Tris/HCl pH 7.5 in the absence (control, a) or in the presence of 10 nM phosphodiepryl 08 (b). Incubations were stopped by acidification (10 μl, 1 M HCl) and h.p.l.c. analysed as described in the Methods section.

group as a surrogate of the scissile peptide bond appeared to be the most suitable choice. Among a series of phosphorus-containing peptides, phosphodiepryl 08 behaved as a very potent mixed inhibitor ($K_i = 0.4$ nM) of endopeptidases 3.4.24.15 and 3.4.24.16. This inhibitor appears so far to be the most potent blocking agent of these two enzymes since it displays about 100 fold higher affinity than that of JMV 390-1 ($K_i = 31$ nM and 58 nM for endopeptidases 3.4.24.15 and 3.4.24.16 respectively) (Doulut *et al.*, 1993). Furthermore, JMV 390-1 also inhibits the enkephalin-degrading enzymes, endopeptidase 3.4.24.11 and aminopeptidase M *in vitro* ($K_i = 40$ and 52 nM, respectively) and *in vivo* as illustrated by the enhanced naltroxone-sensitive analgesic effect elicited by JMV 390-1 after i.c.v. administration (Doulut *et al.*, 1993). Finally, phosphodiepryl 08 is 225,000 fold more potent than the selective endopeptidase 3.4.24.16 dipeptide blocker, Pro-Ile (Dauch *et al.*, 1991b) and exhibits a 45 fold higher affinity for endopeptidase 3.4.24.15 than that observed with Cpp-Ala-Ala-Tyr-pAB, a rather selective inhibitor of this peptidase (Orlowski *et al.*, 1988).

It is important to underline the fact that phosphodiepryl 08 displays high selectivity towards endopeptidases 3.4.24.15 and 3.4.24.16. Thus, a 1 μM concentration of inhibitor (2500 fold higher than the K_i values for the two peptidases) appears unable to affect various zinc metallopeptidases including endopeptidase 24-11, angiotensin-converting enzyme, aminopeptidase M, leucine aminopeptidase and carboxypeptidases A and B.

The powerful and selective pharmacological spectrum of phosphodiepryl 08, *in vitro*, prompted us to examine whether

this agent could prevent neurotensin degradation in a peripheral bioassay as well as *in vivo*, in the central nervous system. Phosphodiepryl 08 potentiated the neurotensin-induced contraction of an isolated longitudinal smooth muscle preparation from guinea-pig ileum. Furthermore, this inhibitor increases the nociceptive response elicited by neurotensin after intracerebroventricular administration in mice. Therefore, these studies clearly established that endopeptidases 3.4.24.15 and 3.4.24.16 participate in the central and peripheral inactivation of neurotensin and suggested that phosphodiepryl 08 might be a useful tool that could ultimately permit a better understanding of the physiological role of this peptide.

It has been shown that phosphonamide bonds can easily be cleaved depending on the pH of the medium. This problem of lability can be overcome by the replacement of the phosphonamide by a phosphonate or a phosphinic group. Our study shows that the replacement by a phosphonate led to a 937 fold reduced affinity for both peptidases (compare compounds 03 and 16 in Table 1) while the introduction of a phosphinic group only diminished the potency towards the two peptidases by a 5 to 6 fold factor (compare compounds 13 and 14). These data are of importance in the context of the development of highly potent and specific inhibitors of endopeptidases 3.4.24.16 and 3.4.24.15. This should be achieved by the screening of peptide libraries containing a phosphinic moiety as a peptide bond surrogate. This work is in progress in our laboratories.

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