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Potent inhibition of endopeptidase 24.16 and endopeptidase 24.15 by the phosphonamide peptide *N*-(phenylethylphosphonyl)-Gly-L-Pro-L-aminohexanoic acid

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A phosphonamide peptide, *N*-(phenylethylphosphonyl)-Gly-L-Pro-L-aminohexanoic acid, previously shown to block *Clostridium histolyticum* collagenases, was examined as a putative inhibitor of endopeptidase 24.16 and endopeptidase 24.15. Hydrolysis of two endopeptidase 24.16 substrates, i.e. 3-carboxy-7-methoxycoumarin (Mcc)-Pro-Leu-Gly-Pro-D-Lys-dinitrophenyl (Dnp) and neurotensin, were completely and dose-dependently inhibited by the phosphonamide inhibitor with K_I values of 0.3 and 0.9 nM respectively. In addition, the phosphonamide peptide inhibited the hydrolysis of benzoyl (Bz)-Gly-Ala-Ala-Phe-(pAB) *p*-aminobenzoate and neurotensin by endopeptidase 24.15 with about a 10-fold lower potency (K_I values of 5 and 7.5 nM respectively). The selectivity of this inhibitor towards several exo- and endopeptidases belonging to the zinc-containing metallopeptidase family established that a 1 μM concentration of this inhibitor was unable to affect leucine aminopeptidase, carboxypeptidase A, angiotensin-converting enzyme and endopeptidase 24.11. The present paper therefore reports on the first hydrophilic highly potent endopeptidase 24.16 inhibitor and describes the most potent inhibitory agent directed towards endopeptidase 24.15 developed to date. These tools should allow one to assess the contribution of endopeptidase 24.16 and endopeptidase 24.15 to the physiological inactivation of neurotensin as well as other neuropeptides.

INTRODUCTION

We previously isolated a novel endopeptidase from rat brain (Checler *et al.*, 1986) and intestine (Barelli *et al.*, 1988) on the basis of its ability to inactivate the tridecapeptide neurotensin. Interestingly, this peptidase [now called endopeptidase 3.4.24.16, neurotensin endopeptidase or neurolysin (Webb, 1984)] was shown to be ubiquitously involved in the mechanisms of neurotensin inactivation in membrane and cell preparations that bear the specific receptors that mediate the neurotensinergic message (Checler *et al.*, 1988). This feature, together with the observation that endopeptidase 24.16 was co-located with neurotensin receptors on a discrete population of primary cultured neurons of cerebral hemispheres from mice embryos (Chabry *et al.*, 1990), suggested the enzyme as a putative candidate for the physiological inactivation of neurotensin. However, such a hypothesis is not antagonistic to the possibility that endopeptidase 24.16 participates in the metabolism of other neuropeptides. In agreement with this fact was the observation that several peptides, including angiotensin I, substance P, bradykinin and dynorphin 1–8, behaved as substrates of endopeptidase 24.16 *in vitro* (Checler *et al.*, 1986; Yoshikawa *et al.*, 1988).

Endopeptidase 24.16 was reported to belong to the metallopeptidase family with respect to its potent inhibition by several metal chelators such as *o*-phenanthroline and EDTA (Checler *et al.*, 1986; Barelli *et al.*, 1988), and its sensitivity to various bivalent cations, including Zn^{2+}, Cu^{2+} and Co^{2+} (Barelli *et al.*, 1988). The fact that Zn^{2+} seemed to be the most powerful agent capable of restoring the activity of the apoenzyme (Barelli *et al.*, 1988) suggested that endopeptidase 24.16 could be a novel member of the growing family of the zinc-containing metallopeptidases. A crucial step in the development of specific inhibitors of such a class of proteolytic activities consists in the co-ordination of the zinc atom. This can be achieved by several metal co-ordinating groups that include carboxyalkyls, thiols, hydroxamates and phospho groups. Concerning the latter, it is well known that the natural phospho-group-containing substance phosphoramidon behaves as an excellent inhibitor of two metallopeptidases, namely thermolysin and endopeptidase 24-11 (Suda *et al.*, 1973; Mumford *et al.*, 1981). More recently it was reported that synthetic substrate analogues in which the scissile bond was replace by a tetrahedral phosphonamide moiety could act as potent thermolysin and carboxypeptidase A inhibitors (Barlett & Marlowe, 1987; Hanson *et al.*, 1989), most likely by mimicking the transition state of the catalysis reaction. Indeed, the phosphonamide moiety is similar to the tetrahedral intermediate that arises from the addition of the zinc–water complex to the carbonyl side of the cleaved amide bond. Phosphonamide peptides were also successfully designed as potent inhibitors of bacterial collagenases from *Clostridium histolyticum* (Dive *et al.*, 1990). Interestingly, the structure of one of these inhibitors, *N*-(phenylethylphosphonyl)-Gly-L-Pro-L-aminohexanoic acid (referred to hereafter as phosphodiepryl 03) resembled the Leu-Gly-Pro sequence feature of a fluorimetric substrate 3-carboxy-7-methoxycoumarin (Mcc)-Pro-Leu-Gly-Pro-DLys-dinitrophenyl (Dnp) that was recently designed as a probe to monitor Pz peptidase [endopeptidase 24.15; Thimet oligopeptidase (Tisljar & Barrett, 1990)] and that was shown to undergo an identical proteolytic cleavage at the Leu–Gly bond by endopeptidase 24.16 (Dauch *et al.*, 1991*a*). Therefore, according to the above statements, phosphodiepryl 03 could be

Abbreviations used: Mcc, 3-carboxy-7-methoxycoumarin; Dnp, dinitrophenyl; Bz, benzoyl; pAB, *p*-aminobenzoate; phosphodiepryl 03, *N*-(phenylethylphosphonyl)-Gly-L-Pro-L-aminohexanoic acid; DFP, di-isopropyl fluorophosphate; IC_{50}, concentration causing 50% of maximum inhibition; CPP, *N*-[(1*R*,1*S*)-carboxy-3-phenylpropyl].

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considered as a putative blocker of these two enzymes. The present paper reports on the ability of this phosphonamide peptide to inhibit endopeptidase 24.16 and endopeptidase 24.15 and examines its selectivity towards other zinc-containing exo- and endo-metallopeptidases such as leucine aminopeptidase, carboxypeptidase A, angiotensin-converting enzyme and endopeptidase 24.11.

MATERIALS AND METHODS

Materials

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp was from Novabiochem. Di-isopropyl fluorophosphate (DFP)-treated carboxypeptidase A, leucine aminopeptidase, angiotensin-converting enzyme, bestatin and phosphoramidon were purchased from Sigma Chemical Co. Endopeptidase 24.11 was purified and kindly provided by Dr. P. Crine and Dr. G. Boileau (Département de Biochimie, Université de Montréal, Montréal, Canada). Benzoyl (Bz)-Gly-Ala-Ala-Phe-*p*-aminobenzoate (pAB) was generously provided by Dr. M. Orlowski (Mount Sinaï School of Medicine, New York, NY, U.S.A.). Arphamenine B was from Interchim (Paris, France). Neurotensin was obtained from Neosystem (Strasbourg, France). The phosphonamide inhibitor phosphodiepryl 03 was prepared as previously described (Dive *et al.*, 1990).

H.p.l.c. and fluorimetric assay of Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by endopeptidase 24.16

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (50 μM) was incubated for 1 h at 37 °C with 10 μl of purified endopeptidase 24.16 (Checler *et al.*, 1986) in a final volume of 100 μl of 50 mM-Tris/HCl, pH 7.5, in the absence (control) or in the presence of 10^{-2} M-prolylisoleucine or increasing concentrations of phosphodiepryl 03. Incubations were stopped and fluorimetrically recorded as previously described (Dauch *et al.*, 1991*a*) or h.p.l.c.-analysed by means of a 42 min linear gradient of 0.1% trifluoroacetic acid/0.05% triethylamine in acetonitrile from 3:1 (v/v) to 1:4 (v/v) as previously described (Checler *et al.*, 1988; Checler, 1992).

H.p.l.c. analysis of neurotensin and Bz-Gly-Ala-Ala-Phe-pAB hydrolysis

Neurotensin (2 nmol) or Bz-Gly-Ala-Ala-Phe-pAB (5 nmol) were incubated for various times at 37 °C with 10 μl of post-hydroxyapatite fractions of endopeptidase 24.15 (neurotensin and Bz-Gly-Ala-Ala-Phe-pAB) or endopeptidase 24.16 (neurotensin) in the absence or in the presence of increasing concentrations of inhibitor. Incubations were acidified and reverse-phase-h.p.l.c.-analysed by means of 42 min [from 9:1 (v/v) to 3:2 (v/v); neurotensin incubations] or 63 min [from 9:1 (v/v) to 11:9 (v/v); Bz-Gly-Ala-Ala-Phe-pAB incubations] linear gradients in the above solvent/buffer system.

Purification of endopeptidases 24.16 and 24.15

Endopeptidase 24.16 was purified as previously described (Checler *et al.*, 1986), and endopeptidase 24.15 was isolated as recently reported (Barelli *et al.*, 1991; Dauch *et al.*, 1991*a*).

RESULTS

Effect of phosphodiepryl 03 on endopeptidase 24.16

We previously reported on the hydrolysis of the fluorimetric substrate Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp by purified endopeptidase 24.16 (sp. activity 215 nmol/h per mg of protein; Dauch *et al.*, 1991*a*). As expected, h.p.l.c. analysis indicated that a single proteolytic cleavage occurred at the Leu–Gly peptide bond (Fig. 1*a*). The formation of these products was abolished by prior incubation of endopeptidase 24.16 with the dipeptide prolylisoleucine, which was previously shown to selectively block endopeptidase 24.16 (Fig. 1*b*) (Dauch *et al.*, 1991*b*). Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by endopeptidase 24.16 could be inhibited in a dose-dependent manner and completely by phosphodiepryl 03, with a half-maximal effect obtained at a concentration of 1 nM (Fig. 1*c*).

The effect of the inhibitor was confirmed by monitoring the ability of endopeptidase 24.16 to cleave neurotensin. As previously reported, endopeptidase 24.16 hydrolysed neurotensin at the Pro^{10}–Tyr^{11} bond, yielding the biologically inert fragments neurotensin-(1–10)-peptide and neurotensin-(11–13)-peptide (Checler *et al.*, 1986; Barelli *et al.*, 1988) (Fig. 2*a*). The total lack of neurotensin-(1–8)-peptide and neurotensin-(9–13)-peptide formation, together with the inability of endopeptidase 24.16 to cleave the endopeptidase 24.15 chromogenic substrate Bz-Gly-Ala-Ala-Phe-pAB, clearly indicated that endopeptidase 24.16 was not contaminated by endopeptidase 24.15, as previously discussed (Barelli *et al.*, 1991). Neurotensin hydrolysis appeared completely abolished by 1 μM-phosphodiepryl 03 (Fig. 2*a*), with an IC_{50} (concentration causing 50% of maximal inhibition) of 10 nM (Fig. 2*c*). Taking into account the concentrations of Mcc-

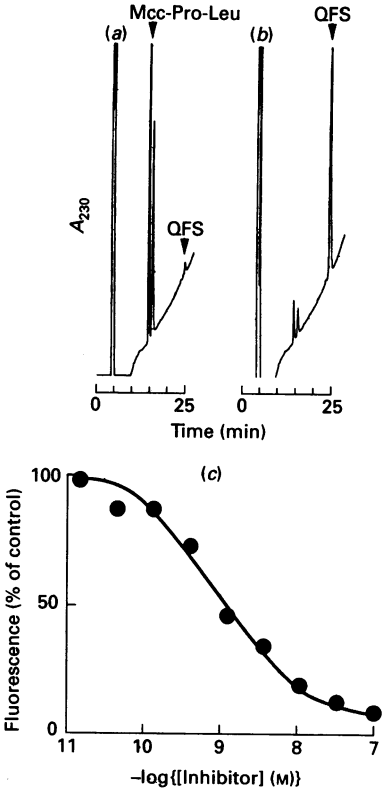

Fig. 1. Effect of prolylisoleucine and phosphodiepryl 03 on Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp hydrolysis by endopeptidase 24.16

Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (5 nmol, 50 μM) was incubated for 1 h at 37 °C with 8 μg of post-hydroxyapatite endopeptidase 24.16 in a final volume of 100 μl of 50 mM-Tris/HCl, pH 7.5, in the absence (*a*) or in the presence (*b*) of 10^{-2} M-Pro-Ile or increasing concentrations of phosphodiepryl 03 (*c*). Incubations were either h.p.l.c.-analysed (*a* and *b*) or fluorimetrically monitored (*c*) as described in the Materials and methods section. Arrows indicate the elution time of intact Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp (QFS) and Mcc-Pro-Leu. Values in curve (*c*) correspond to fluorescence and are expressed as the percentage of control fluorescence recovered in absence of inhibitor.

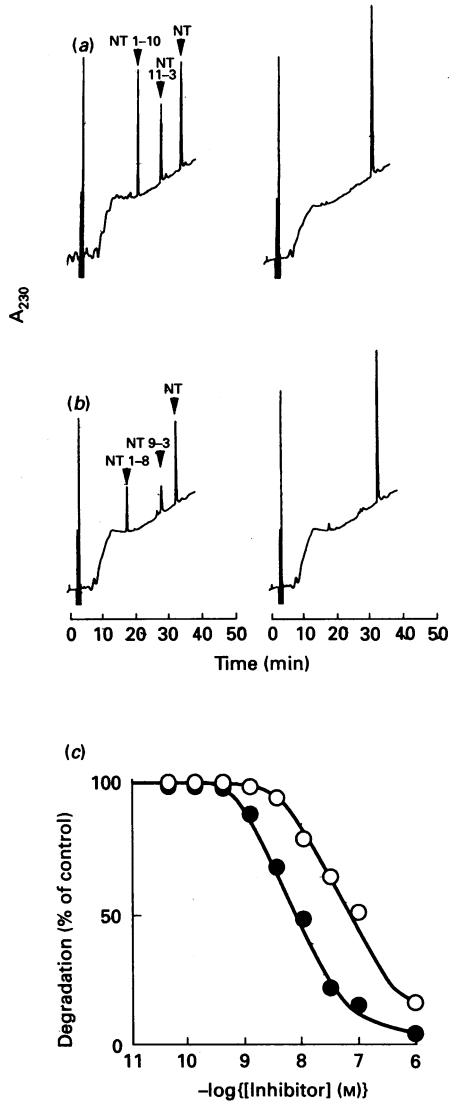

Fig. 2. Effect of phosphodiepryl 03 on neurotensin hydrolysis by endopeptidase 24.16 and endopeptidase 24.15

Neurotensin (NT; 2 nmol, 20 μM) was incubated at 37 °C with 8 μg of endopeptidase 24.16 (*a*) or 7 μg of endopeptidase 24.15 (*b*) in a final volume of 100 μl of 50 mM-Tris/HCl, pH 7.5, in the absence (left panels) or in the presence (right panels) of 1 μM-phosphodiepryl 03. Incubations were stopped by acidification and analysed by h.p.l.c. as described in the Materials and methods section. Complete dose–response curves (*c*) were established by incubating neurotensin (2 nmol, 20 μM) at 37 °C with the above quantities of endopeptidases 24.16 (●) or 24.15 (○) in the absence (control) or in the presence of increasing concentrations of phosphodiepryl 03. Values correspond to percentage of neurotensin degradation after h.p.l.c. analysis and are expressed as the percentage of degradation (control taken as 100) obtained in absence of phosphodiepryl 03. Abbreviations: NT 1–8 (etc.), neurotensin-(1–8)-peptide (etc.).

Pro-Leu-Gly-Pro-D-Lys-Dnp (50 μM) and neurotensin (20 μM), together with the K_m of endopeptidase 24.16 for these two substrates (25 μM and 2 μM respectively) (Checler *et al.*, 1986; Barelli *et al.*, 1988; Dauch *et al.*, 1991*a*), according to the simple relationship:

$$IC_{50} = K_I\left(1+\frac{[S]}{K_m}\right)$$

it was possible to derive K_I values. As shown in Table 1, these K_I values were virtually identical for the two endopeptidase 24.16 substrates.

Effect of phosphodiepryl 03 on endopeptidase 24.15

When neurotensin was used as the substrate of endopeptidase 24.15, as previously reported (Orlowski *et al.*, 1983; Barelli *et al.*, 1991), we observed (Fig. 2*b*) the formation of equimolar amounts of neurotensin-(1–8)-peptide and neurotensin-(9–13)-peptide deriving from a single breakdown at the Arg^8–Arg^9 peptidyl bond of neurotensin that also appeared totally prevented by 1 μM-CPP-Ala-Ala-Tyr-pAB (results not shown). Here again, a 1 μM-concentration of the phosphonamide inhibitor induced a complete inhibitory effect of endopeptidase 24.15 (Fig. 2*b*) with a half-maximal effect occurring at about 100 nM (Fig. 2*c*).

The endopeptidase 24.15 chromogenic substrate Bz-Gly-Ala-Ala-Phe-pAB underwent a single proteolytic cleavage by endopeptidase 24.15, leading to Bz-Gly and its complementary sequence Ala-Ala-Phe-pAB (Fig. 3*a*). This cleavage appeared fully abolished by 1 μM-*N*-(1*R*,1*S*)-carboxy-3-phenylpropyl-(CPP)-Ala-Ala-Tyr-pAB (results not shown), a compound that was described as a potent ($K_I \sim 16$ nM) inhibitor of endopeptidase 24.15 (Orlowski *et al.*, 1988). Phosphodiepryl 03 at 1 μM prevented the formation of the two degradation products (Fig. 3*b*), with a half-maximal effect elicited at 5 nM (Fig. 3*c*).

The K_I values of phosphodiepryl 03 on the hydrolysis of Bz-Gly-Ala-Ala-Phe-pAB and neurotensin derived from the above experiments appeared in close agreement (5–7.5 nM; Table 1).

Effect of phosphodiepryl 03 on other zinc-containing exo- and endo-metallopeptidases

Leucine aminopeptidase, carboxypeptidase A, angiotensin-converting enzyme and endopeptidase 24.11 activities were h.p.l.c.-monitored by measuring the hydrolysis of leucyl 7-amido-4-methylcoumarylamide, Hippuryl-Phe, Hippuryl-His-Leu and neurotensin respectively as previously described (Dauch *et al.*, 1991*b*). The chromatograms showed that the various substrates were cleaved at the expected peptide sites (Checler, 1992) and were fully abolished by pretreatment with saturating concentrations of their specific inhibitors [bestatin (leucine aminopeptidase), arphamenine B (carboxypeptidase A), captopril (angiotensin-converting enzyme) and phosphoramidon (endopeptidase 24.11) (results not shown)], indicating that the observed hydrolyses were indeed due to the expected proteolytic activities and not attributable to contaminating peptidases present in the commercial batches. All these metallopeptidases

Table 1. IC_{50} and K_I values of phosphonamide inhibition of endopeptidases 24.15 and 24.16

	Neurotensin		Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp		Bz-Gly-Ala-Ala-Phe-pAB	
Endopeptidase	IC_{50} (nM)	K_I (nM)	IC_{50} (nM)	K_I (nM)	IC_{50} (nM)	K_I (nM)
24.15	100	7.5	–	–	5	5
24.16	10	0.9	1	0.3	–	–

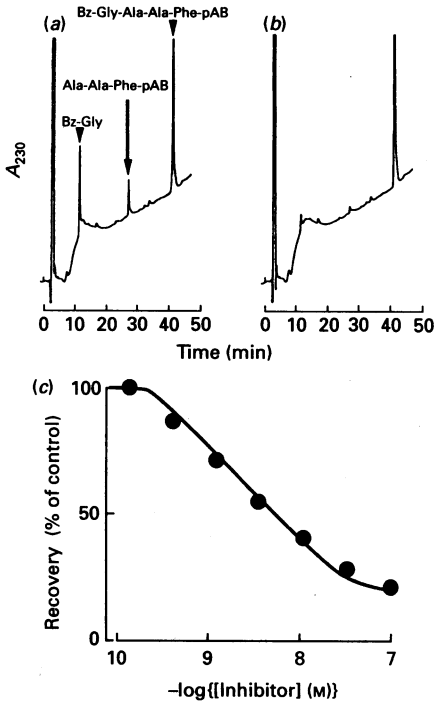

Fig. 3. Effect of phosphodiepryl 03 on Bz-Gly-Ala-Ala-Phe-pAB hydrolysis by endopeptidase 24.15

Bz-Gly-Ala-Ala-Phe-pAB (5 nmol, 50 μM) was incubated as 37 °C with 0.7 μg of post-hydroxyapatite endopeptidase 24.15 in a final volume of 100 μl of 50 mM-Tris/HCl, pH 7.5, in the absence (*a*) or in the presence (*b*) of 0.1 μM-phosphodiepryl 03. Values in curve (*c*) represent the amount of Bz-Gly recovered as a function of phosphodiepryl 03 concentration and are expressed as the percentage recovered (taken as 100) in the absence of phosphodiepryl 03.

remained totally insensitive to 1 μM-phosphodiepryl 03 (results not shown).

DISCUSSION

The possibility of assessing the contribution of any peptidase in the inactivation of neuropeptides is dependent on the development of potent, selective and bioavailable inhibitors. This prompted us to search for inhibitory agents of endopeptidase 24.16. We recently demonstrated that several dipeptides mimicking the Pro–Tyr bond of neurotensin hydrolysed by endopeptidase 24.16 could be used as selective blockers of this enzyme (Dauch *et al.*, 1991*b*). Among them, prolylisoleucine was shown to inhibit endopeptidase 24.16 with a K_I value of 90 μM, whereas a 5 mM concentration of this agent had no effect on endopeptidases 24.11 and 24.15, proline endopeptidase, angiotensin-converting enzyme, leucine aminopeptidase, carboxypeptidases A and B and trypsin (Dauch *et al.*, 1991*b*). However, the relatively low potency, as well as its poor solubility at high concentrations, precluded the possibility of using this inhibitor in experiments *in vivo*. These drawbacks were not shared by the phosphonamide peptide inhibitor reported in the present study. Thus, phosphodiepryl 03 potently inhibited the hydrolysis of two substrates of endopeptidase 24.16, i.e. Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp and neurotensin, with IC_{50} values in the subnanomolar range (see the Results section). Such K_I values showed phosphodiepryl 03 to be one of the most potent synthetic inhibitors developed against neuropeptidases (for a review, see Checler, 1991). This tight-binding constant confirms that phosphonamide analogues can efficiently mimic the transition state of the scissile peptide bond and has already led to their development as potent inhibitors of metalloproteinases (Kam *et al.*, 1979; Galardy, 1980; Hanson *et al.*, 1989; Thorsett *et al.*, 1982).

Endopeptidase 24.15 was examined as a possible target of phosphodiepryl 03 by monitoring the hydrolysis of two of its substrates, neurotensin and Bz-Gly-Ala-Ala-Phe-pAB. The fact that their cleavage was fully abolished by pretreatment with a 1 μM concentration of the endopeptidase 24.15 inhibitor CPP-Ala-Ala-Tyr-pAB precluded the possibility that endo-oligopeptidase A participated in these cleavages, since this enzyme was reported to be unaffected by a 0.3 mM concentration of endopeptidase-24.15 inhibitor (Cicilini *et al.*, 1988).

Phosphodiepryl 03 blocked the hydrolysis of both substrates of endopeptidase 24.15 with K_I values (5–7.5 nM) that were about 10-fold higher than those obtained for endopeptidase 24.16 (see Table 1). It was not unexpected that phosphodiepryl 03 blocks endopeptidase 24.15. The two enzymes are clearly distinct, since they display different sensitivities towards various inhibitors or agents such as CPP-Ala-Ala-Tyr-pAB or dithiothreitol (Checler *et al.*, 1986; Orlowski *et al.*, 1988; Barelli *et al.*, 1991), were not immunologically related (Barelli *et al.*, 1991) and attacked distinct cleavage sites on neurotensin and other natural peptides (Checler *et al.*, 1986; Chu & Orlowski, 1985; Orlowski & Michaud, 1989; Yoshikawa *et al.*, 1988). In spite of all these differences, it was noteworthy that Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp was cleaved by both peptidases at the same Leu–Gly peptide bond (Dauch *et al.*, 1991*a*; Tisljar *et al.*, 1990), an observation that sustained the reasoning concerning the possibility that phosphodiepryl 03 could act as an endopeptidase 24.16 inhibitor (see the Introduction).

The suitability of phosphodiepryl 03 as a selective agent able to discriminate between various zinc exo- and endo-metallopeptidases was examined. A 1 μM concentration of this agent did not affect carboxypeptidase A, leucine aminopeptidase, angiotensin-converting enzyme and endopeptidase 24.11, in agreement with the fact that phosphodiepryl 03 does not exhibit the structural requirements that led to the development of potent specific inhibitors of these metallopeptidases (Ondetti *et al.*, 1979; Llorens *et al.*, 1980; Jacobsen & Bartlett, 1981; Mumford *et al.*, 1982; Malfroy & Schwartz, 1985).

In conclusion, we report here on the ability of a phosphonamide peptide inhibitor, phosphodiepryl 03, to potently block endopeptidase 24.16 activity. This is the first agent that displays an affinity for the enzyme in the subnanomolar range. Furthermore, this inhibitor exhibits a high affinity for endopeptidase 24.15, such as to make it the most potent inhibitor of endopeptidase 24.15 developed to date. Finally, phosphodiepryl 03 exerts a rather selective inhibitory profile, since a 1000-fold higher concentration than is the K_I value for endopeptidase 24.16 does not affect other zinc metallopeptidases such as leucine aminopeptidase, carboxypeptidase A, angiotensin-converting enzyme and endopeptidase 24.11. It is our hope that the described phosphonamide will be the starting point of a strategy leading to more potent and selective agents capable of blocking only endopeptidase 24.16 or 24.15, in order to assess the contribution of these enzymes in the physiological inactivation of neurotensin and other natural peptides in the central nervous system and in the periphery.

We thank Dr. M. Orlowski for generously providing the endopeptidase 24.15 substrate Bz-Gly-Ala-Ala-Phe-pAB. We are indebted to Dr. G. Boileau and Dr. P. Crine for kindly providing endopeptidase 24.11. We thank Mr. F. Aguila for artwork and Ms. V. Dalmasso for expert secretarial assistance. This work was supported by the Institut de la Santé et de la Recherche Médicale (CRS N° 886017), the Centre National de la Recherche Scientifique and the Fondation pour la Recherche Médicale.

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