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Short communication

Contribution of endopeptidase 3.4.24.15 to central neurotensin inactivation

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Abstract

The tridecapeptide, neurotensin elicits naloxone-insensitive analgesia after its intracebroventricular administration in mice. We used this central pharmacological effect to assess the putative contribution of the endopeptidase 3.4.24.15 to central inactivation of the peptide. By means of combinatorial chemistry, we previously designed the first potent endopeptidase 3.4.24.15 inhibitor. This agent, Z-(L,D)PheΨ(PO_2CH_2)(L,D)Ala–Lys–Met (phosphodiepryl 21), is shown here to behave as a fully specific endopeptidase 3.4.24.15 inhibitor, as demonstrated by the absence of effect on a series of other exo- and endopeptidases belonging to various classes of proteolytic activities present in murine brain membranes. Furthermore, central administration of phosphodiepryl 21 drastically prolongs the forepaw licking latency of mice tested on the hot plate and injected with sub-maximally active doses of neurotensin. Altogether, our results demonstrated that, in addition to endopeptidase 3.4.24.16, endopeptidase 3.4.24.15 likely contributes to the physiological termination of the neurotensinergic message in murine brain. © 1997 Elsevier Science B.V.

Keywords: Endopeptidase 3.4.24.15; Phosphinic inhibitor; Neurotensin; Analgesia; (Mouse)

1. Introduction

Neurotensin elicits naloxone-resistant analgesia after its central administration in mice (Clineschmidt et al., 1979; Coquerel et al., 1988). This lack of effect of opiate receptor antagonists suggests that neurotensin acts as an endogenous analgesic substance potentially devoid of opioids side effects. As a corollary, it was of major interest to investigate the central catabolic processes responsible for neurotensin inactivation. Thus, any putative specific blocker of peptidases involved in such a function could possibly be a highly potent analgesia enhancer.

Several lines of evidence suggest the involvement of two endopeptidases in the central termination of the neurotensinergic message. Thus, endopeptidase 3.4.24.15 and endopeptidase 3.4.24.16 mainly contribute to the degradation of the peptide in various cell lines, and in tissue slices or homogenates of central origin (Camargo et al., 1984; Checler et al., 1985, 1986, 1988, 1991; Davis et al., 1992; Mentlein and Dahms, 1994). Our search for potent, specific and bioavailable inhibitors of these two peptidases led us to design phosphonamide peptides acting as potent mixed blockers of the two proteolytic activities (Barelli et al., 1992; Vincent et al., 1995). One of them, phosphodiepryl 08, was shown to strongly potentiate the neurotensin-induced antinociception in the mouse hot plate test (Vincent et al., 1995), indicating that one peptidase or both likely contributed to the central inactivation of neurotensin. In order to delineate the respective contributions of theses enzymes to the metabolism of this peptide, we designed by combinatorial chemistry, the first highly potent and selective phosphinic peptide inhibitors of endopeptidase 3.4.24.16 (Jiràcek et al., 1996) and endopeptidase 3.4.24.15 (Jiràcek et al., 1995). In a recent study, we clearly established that phosphodiepryl 33, a potent en-

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dopeptidase 3.4.24.16 blocker, greatly enhanced the neurotensin-induced analgesia (Vincent et al., 1997), thereby confirming the involvement of this peptidase in neurotensin inactivation. We now assessed the putative contribution of endopeptidase 3.4.24.15 in neurotensin metabolism by means of phosphodiepryl 21, a specific and potent inhibitor of this enzymatic activity. We demonstrated that the central administration of this agent highly potentiates the neurotensin-induced analgesia in hot plate tested mice, thereby showing that besides endopeptidase 3.4.24.16, endopeptidase 3.4.24.15 also participates to the central inactivation of neurotensin.

2. Materials and methods

2.1. Materials

Diprotin A was from Boehringer and arphamenine B was from Interchim. All other peptidase inhibitors, chromogenic and fluorimetric substrates were from Sigma Chemicals. Neurotensin was from Neosystem. Phosphodiepryl 21 was synthetized as described previously (Jiràcek et al., 1995; Yiotakis et al., 1996).

2.2. Preparation of whole rat brain homogenates

Adult male Wistar rats were killed and their brain was rapidly excised and homogenized with a polytron in cold 5 mM Tris/HCl, pH 7.5 (buffer A). Protein concentrations were then adjusted to 10 mg/ml in buffer A and immediately tested for their fluorimetric substrate-hydrolysing activities as described below.

2.3. Fluorimetric enzymatic assays

All incubations were performed for various times at 37°C in a final volume of 100 μl of buffer A containing 100 μg of proteins. Peptidase activities were monitored by means of fluorimetric or chromogenic substrates in the absence or presence of specific peptidase inhibitors, then initial velocities measurements of the activities were monitored and quantified as previously described (Checler, 1993).

2.4. Hot plate test

Hot plate tests were mostly performed according to the procedure described by Eddy and Leimblach (1953). Briefly, male mice weighing about 20 g were injected intracerebroventricularly with either sterile saline buffer (0.9% NaCl) or various doses of neurotensin in the absence or presence of 1–3 μg of phosphodiepryl 21 then placed on a plate, the temperature of which was maintained at 55°C. The antinociception test was performed 15 min after administration of the pharmacological agents and involved the measurement of the latency of forepaw licking (with a cut-off time of 30 s) as previously described (Schmidt et al., 1991).

2.5. Protein concentrations

Protein concentrations were determined by the method of Bradford (1976) with egg lysozyme as the standard.

3. Results

We have previously reported on the design of endopeptidase 3.4.24.15 inhibitors via a combinatorial chemistry approach (Jiràcek et al., 1995). The screening of a library of several hundred phosphinic peptides led us to identify the very first inhibitor able to fully discriminate between two closely related peptidases, namely endopeptidase 3.4.24.16 and 3.4.24.15 (Dauch et al., 1995). Thus, this agent, *Z*-(L,D)PheΨ(PO_2CH_2)(L,D)Ala–Lys–Met (phosphodiepryl 21), displays a high affinity for endopeptidase 3.4.24.15 ($K_i = 0.12$ nM) and is 1920-fold less potent on endopeptidase 3.4.24.16 (Jiràcek et al., 1995).

Interestingly, a 1 μM concentration of phosphodiepryl 21 was also unable to affect various exo- and endo-metal-

Table 1
Effect of specific inhibitors and phosphodiepryl 21 on peptidase activities present in rat brain homogenates

Peptidase	Substrate/inhibitor	Enzymatic activity (% of control)	
		specific inhibitor	P21 (1 μM)
E.24.11	Suc-A-A-F-7AMC/Phosphoramidon 1 μM	4.4 ± 0.4	104.4 ± 8.9
ACE	Hip–His–Leu/Captopril 1 μM	37.4 ± 1.4	93.6 ± 4
LAP	Leu-7AMC/Bestatin 50 μM	14.5 ± 0.2	100.7 ± 1.7
APM	Leu-7AMC/Bestatin 50 μM	14.8 ± 0.3	97.9 ± 1.8
CPA	Hip–Phe/Arphamenine B 100 μM	27.2 ± 3.7	90.2 ± 2.1
CPB	Hip–Lys/	n.d.	88 ± 1.5
DAP IV	Gly–Pro-7AMC/Diprotin A 100 μM	16.5 ± 0.2	92.8 ± 1.1
APB	Arg-7AMC/Arphamenine B 0.5 μM	34.1 ± 0.9	100.9 ± 2.8
PE	Z–Gly–Pro-7AMC/Z-Pro–Prolinal 1 μM	12.6 ± 0.7	111.3 ± 5.7

Activities were measured by incubation of rat brain homogenates with the indicated substrates (for concentrations, see Section 2) in the absence or in the presence of the indicated specific inhibitors or phosphodiepryl 21 (1 μM). Values are expressed as percentages of control activity obtained in the absence of inhibitor and are the means ± SEM of 6 independent experiments.

Table 2
Effect of phosphodiepryl 21 (P21) on neurotensin-induced antinociception in mice tested on the hot-plate

Treatment	Dose (μg)	Latency (s)
Control	—	7.31 ± 0.96
P21	0.1	9.56 ± 1.40
P21	0.3	7.75 ± 0.56
P21	1	6.27 ± 0.5
P21	3	8.27 ± 1
Neurotensin (0.03 μg)		10.27 ± 1.41
+ P21	0.1	10.08 ± 1.2
+ P21	0.3	12.22 ± 1.13
+ P21	1	15.35 ± 1.37 [a]
+ P21	3	18.47 ± 1.93 [b]

Neurotensin (0.03 μg) was administered i.c.v. in the absence or in the presence of the indicated doses of phosphodiepryl 21 (P21). Latency of appearance of forepaw licking behavior was recorded 15 min after peptide and/or inhibitor administration. Values are expressed as means ± S.E.M. for 10 mice per group.
[a] $P < 0.05$ and [b] $P < 0.01$ (Newman–Keuls test) as compared to the same dose of neurotensin without inhibitor.

lopeptidases including endopeptidase 3.4.24.11, angiotensin-converting enzyme, leucine aminopeptidase/aminopeptidase M, aminopeptidase B, and carboxypeptidases A and B (Table 1). Furthermore, two serine peptidases, namely dipeptidyl aminopeptidase IV and proline endopeptidase were not sensitive to the endopeptidase 3.4.24.15 inhibitor (Table 1). Altogether, phosphodiepryl 21 can be regarded as the very first highly potent, and more important fully selective inhibitor of endopeptidase 3.4.24.15. The latter properties of this agent together with its high solubility and stability prompted us to use it to examine the putative contribution of endopeptidase 3.4.24.15 in the central inactivation of neurotensin.

As we previously showed, neurotensin elicits dose-dependent analgesia after intracerebro-ventricular administration to mice (Vincent et al., 1995). The first dose triggering a statistically significant increase in the forepaw licking latency of hot plate-tested mice was 0.3 μg of neurotensin, while a maximal effect was observed for 10 μg of this peptide (not shown).

We chose a dose of neurotensin ineffective per se (0.03 μg) to test for a possible potentiation of the neurotensin-induced analgesia by phosphodiepryl 21. The administration of this phosphinic peptide, up to a dose of 3 μg, did not significantly modify the observed latency (Table 2). When administered in combination with neurotensin, phosphodiepryl 21 increased the latency significantly ($p < 0.05$) at a dose of 1 μg and more significantly ($p < 0.01$) at a 3 μg dose (Table 2).

4. Discussion

Several studies have clearly established that endopeptidases 3.4.24.16 and 3.4.24.15 mainly contribute to the central inactivation of neurotensin, in vitro (Camargo et al., 1984; Checler et al., 1985, 1986, 1988, 1991; Davis et al., 1992; Mentlein and Dahms, 1994). The possibility that such activities also participate in the catabolism of the peptide in vivo was suggested by experiments with mixed inhibitors, the pharmacological spectrum of which includes these two peptidases. Thus, these agents were shown to drastically potentiate the recovery of neurotensin-like immunoreactivity released from hypothalamic slices (Kitabgi et al., 1992) and the neurotensin-induced analgesia elicited by intracerebrovascular injection of the peptide in mice (Vincent et al., 1995). However, evaluation of the respective contribution of each peptidase to the physiological inactivation of neurotensin clearly necessitated the design of potent, specific, bioavailable and metabolically stable inhibitors.

Several inhibitors of endopeptidase 3.4.24.15 have been described, the design of which was based on carboalkyl peptide derivatives (Chu and Orlowski, 1984; Orlowski et al., 1988; Knight and Barrett, 1991). The most potent and selective agent, *N*-[1(*R*,*S*)-carboxy-3-phenylpropyl]-Ala–Ala–Tyr-*p*-aminobenzoate, displays an apparent affinity of 16 nM towards endopeptidase 3.4.24.15 (Orlowski et al., 1988) but only 60-fold selectivity towards endopeptidase 3.4.24.16 (Checler et al., 1995). More important, several studies have clearly demonstrated that inhibitors of this family were metabolically unstable, yielding after their rapid conversion a potent angiotensin-converting enzyme inhibitor (Chappell et al., 1992; Cardozo and Orlowski, 1993; Williams et al., 1993; Lew et al., 1996).

We designed another strategy based on the screening of a library of phosphinic peptides constructed by combinatorial chemistry (Jiràcek et al., 1995). This led us to describe the first inhibitor, Z-(L,D)PheΨ(PO_2CH_2)(L,D)Ala–Lys–Met (phosphodiepryl 21) able to fully discriminate between endopeptidase 3.4.24.15 and endopeptidase 3.4.24.16 (Jiràcek et al., 1995), a structurally closely related peptidase (Dauch et al., 1995). This compound displays most of the properties expected for an inhibitor amenable to physiological studies. First, phosphodiepryl 21 is highly potent (K_i value about 0.1 nM), a feature which, combined with very good solubility in aqueous solutions, allows its use in pharmacological in vivo experiments. Second, phosphodiepryl appears to be fully specific for endopeptidase 3.4.24.15. Thus, besides its inability to block endopeptidase 3.4.24.16, we now showed that this inhibitor does not affect a series of classical exo-and endo-metallopeptidases, even when used at a concentration four orders of magnitude higher than its K_i value. Third, it is very important to note that prolonged incubations (15 h at 37°C) of phosphodiepryl 21 with whole rat brain homogenate membranes did not lead to a detectable loss of inhibitor absorbance as assessed by HPLC, indicating remarkable metabolic stability of this agent, at least in vitro (not shown). Phosphodiepryl 21 was therefore tested as a potential modulator of neurotensin-induced analgesia in mice.

As previously established (Clineschmidt et al., 1979; Coquerel et al., 1988; Al-Rhodan et al., 1991), neurotensin induced antinociception after its central administration in mice (see Fig. 1). Assuming the first statistically significant effect at a dose of 0.3 μg and a maximal analgesic effect at 10 μg, one could roughly estimate an ED_{50} value of between 1 and 3 μg (about 1 nmol), in good agreement with that described (1.5 nmol) by Al-Rhodan et al. (1991). The hot plate test was used to assess the contribution of endopeptidase 3.4.24.15 to the catabolism of neurotensin in mice. Table 2 indicates that the injection of phosphodiepryl 21 alone up to a dose of 3 μg did not modify forepaw licking latency.

Combined administration of an ineffective dose of neurotensin with low doses of phosphodiepryl 21 led to a dose-dependent potentiation of the forepaw licking latency (Table 2), indicating the likely contribution of endopeptidase 3.4.24.15 to the central mechanisms of neurotensin inactivation. Such a pharmacological study, carried out with a very recently developed phosphinic peptide inhibitor of endopeptidase 3.4.24.16 (Jiràcek et al., 1996), indicated a similar increase in latency (Vincent et al., 1997). This implies that both endopeptidases 3.4.24.15 and 3.4.24.16 participate in neurotensin metabolism, as was previously suggested by results of studies with phosphonamide peptide mixed inhibitors (Vincent et al., 1995).

In conclusion, we now showed that endopeptidase 3.4.24.15 contributes to the central inactivation of neurotensin by means of a highly potent and fully selective phosphinic peptide inhibitor of this activity. Phosphodiepryl 21 should be useful in pharmacological studies aimed at establishing the participation of endopeptidase 3.4.24.15 in the metabolism of other neuropeptides in vivo, in the central nervous system or in the periphery.

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