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Effect of a novel selective and potent phosphinic peptide inhibitor of endopeptidase 3.4.24.16 on neurotensin-induced analgesia and neuronal inactivation

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1 We have examined a series of novel phosphinic peptides as putative potent and selective inhibitors of endopeptidase 3.4.24.16.

2 The most selective inhibitor, Pro-Phe-$\Psi(PO_2CH_2)$-Leu-Pro-NH_2 displayed a K_i value of 12 nM towards endopeptidase 3.4.24.16 and was 5540 fold less potent on its related peptidase endopeptidase 3.4.24.15. Furthermore, this inhibitor was 12.5 less potent on angiotensin-converting enzyme and was unable to block endopeptidase 3.4.24.11, aminopeptidases B and M, dipeptidylaminopeptidase IV and proline endopeptidase.

3 The effect of Pro-Phe-$\Psi(PO_2CH_2)$-Leu-Pro-NH_2, *in vitro* and *in vivo*, on neurotensin metabolism in the central nervous system was examined.

4 Pro-Phe-$\Psi(PO_2CHH_2)$-Leu-Pro-NH_2 dose-dependently inhibited the formation of neurotensin 1-10 and concomittantly protected neurotensin from degradation by primary cultured neurones from mouse embryos.

5 Intracerebroventricular administration of Pro-Phe-$\Psi(PO_2CH_2)$-Leu-Pro-NH_2 significantly potentiated the neurotensin-induced antinociception of mice in the hot plate test.

6 Altogether, our study has established Pro-Phe-$\Psi(PO_2CH_2)$-Leu-Pro-NH_2 as a fully selective and highly potent inhibitor of endopeptidase 3.4.24.16 and demonstrates, for the first time, the contribution of this enzyme in the central metabolism of neurotensin.

Keywords: Phosphinic peptide; peptidase inhibitor; neurotensin; analgesia; inactivation; neurons; combinatorial; chemistry.

Introduction

Unlike other classical neurotransmitters, physiological clearance of neuropeptides is thought to be due to proteolytic enzymes (for reviews see Turner *et al*., 1985; McKelvy & Blumberg, 1986; Littlewood *et al*., 1988; Checler, 1991). Our previous studies indicated that endopeptidase 3.4.24.16, an oligopeptidase widely distributed within central and peripheral tissues (Checler *et al*., 1989), was mainly responsible for the catabolism of the tridecapeptide neurotensin, *in vitro* (Checler *et al*., 1988). Several lines of evidence suggest that this enzyme also contributes to the physiological termination of the neurotensinergic signal in the periphery. Thus, the dipeptide Pro-Ile, described as the first fully selective inhibitor of endopeptidase 3.4.24.16 (Dauch *et al*., 1991b), significantly potentiated the venous recovery of intact neurotensin after its infusion in the vascularly perfused ileum of the anaesthetized dog (Barelli *et al*., 1994). This was accompanied by a concomittant decrease in the formation of the endopeptidase 3.4.24.16-derived neurotensin degradation product, neurotensin 1-10 (Barelli *et al*., 1994). However, the low affinity of Pro-Ile for endopeptidase 3.4.24.16 precluded its use in the central nervous system, where bolus intracerebrovascular administration would have necessitied high concentrations of the dipeptide, i.e. far above its threshold of solubility. These observations have led us to search for highly soluble, bioavailable, potent and selective inhibitors of endopeptidase 3.4.24.16. Initially, we showed that phosphonamide peptides behaving as mixed inhibitors of endopeptidase 3.4.24.15 and endopeptidase 3.4.24.16 (Barelli *et al*., 1992) greatly potentiated the neurotensin-induced antinociception of mice in the hot plate test (Vincent *et al*., 1995). Later, a new systematic approach based on the chemistry of phosphinic peptides allowed us to design the first highly potent endopeptidase 3.4.24.16 inhibitor (K_i = 4 nM, Jiràcek *et al*., 1996). Interestingly, this inhibitor Pro-Phe-$\Psi(PO_2CH_2)$-Gly-Pro (P22) was 2000 times less potent on another neurotensin-cleaving activity, endopeptidase 3.4.24.15 and therefore, appeared to be the first inhibitor able to discriminate between these two related peptidases (Jiràcek *et al*., 1996). However, P22 still behaves as a potent blocker of the angiotensin-converting enzyme (ACE). Here we describe a novel phosphinic peptide, Pro-Phe-$\Psi(PO_2CH_2)$-Leu-Pro-NH_2 (P33) that appears about 500 fold less potent than P22 on ACE. This agent, that is the very first highly potent and fully selective inhibitor of endopeptidase 3.4.24.16, fully prevents the formation of neurotensin 1–10 by primary cultured neurones from mouse embryos and drastically potentiates the neurotensin-induced analgesia of the mouse tested on a hot plate.

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 as previously described (Checler *et al*., 1986b, Barelli *et al*., 1991).

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Preparation of rat brain homogenates

Rats were decapitated, the brain immediately removed and homogenized by polytron at 4°C in 5 mM TrisHCl, pH 7.5. Protein concentrations were adjusted to 10 mg ml^{-1} in the same buffer.

Primary cultures of neurones from mouse embryos

Primary cultures of neurones were prepared from the cerebral hemispheres of 14 day-old mouse embryos as previously described (Chabry *et al*., 1990). Briefly, cells were mechanically dissociated with a pipette in HAM F12 medium supplemented with 10% foetal calf serum and 0.6% glucose. Dissociated cells were then plated at a density of 3×10^6 cells in 35 mm plastic tissue culture dishes precoated with polylysine (10 μg ml^{-1}) and grown in a humidified atmosphere of 5% CO_2, 95% air. At the beginning of the third day and every two days thereafter, cytosine arabinofuranoside (5 μM) was added in the medium for 24 h to prevent glial cell proliferation. We previously established that these neuronal cultures were virtually devoid of astrocytic cells, as shown by the lack of glial fibrillary acidic protein (GFAP) immunoreactivity (Vincent *et al*., 1994).

Analysis of neurotensin degradation by plated cultured neurones with h.p.l.c.

Monolayers of 4 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) and incubated for 150 min at 37°C, with 10 nmol 10 μM) neurotensin in PBS-containing 1% glucose, in the absence and presence of 100 nM phosphodiepryl 33. At the end of the incubation, supernatants were taken out, acidified (with 100 μl of 1 M HCl) and the equivalent of 1 nmol of neurotensin was submitted to h.p.l.c. analysis as described previously (Checler *et al*., 1988). Briefly, samples were applied onto a reverse-phase C18 lichrosorb column (Merck) at room temperature. Elutions were performed at a flow rate of 1 ml min^{-1} by means of a 42 min linear gradient of 0.1% trifluoroacetic acid, 0.05% triethylamine/0.1% trifluoroacetic acid, 0.05% triethylamine in acetonitrile, from 90/10 (vol/vol) to 60/40 (vol/vol). Absorbance was monitored at 230 nm with a detector setting of 0.05 full scale.

Fluorimetric assays of purified peptidases

Endopeptidase 3.4.24.16 and endopeptidase 3.4.24.15 QFS (5 nmol) was incubated at 37°C with purified endopeptidase 3.4.24.16 or endopeptidase 3.4.24.15 in a final volume of 100 μl of 5 mM TrisHCl, pH 7.5 in the absence and presence of various concentrations of P33. Incubations were acidified with 2 ml 80 mM sodium formate, pH 3.7 and the activity was fluorimetrically recorded as previously described (Dauch *et al*., 1991a).

Angiotensin-converting enzyme (ACE) Hippuryl-His-Leu (100 nmol) was incubated with purified ACE at 37°C in a final volume of 100 μl of 5 mM TrisHCl, pH 7.5 containing 0.3 M NaCl, in the absence and presence of various concentrations of P33. His-Leu was measured by a fluorimeter within 365 and 495 nm as excitation and emission wavelengths, respectively, as previously described (Huggins & Thampi, 1968).

Fluorimetric substrate-hydrolysing activities in whole rat brain homogenate membranes

Incubations were performed for various times at 37°C in 5 mM TrisHCl, pH 7.5 with a final concentration of 1 mg ml^{-1} of membrane proteins and activities were quantified by means of fluorimetric substrates in the absence and presence of specific inhibitors, as previously described (Checler, 1993).

Hot plate test

All procedures were done under animal care and handling approved by the university. Male mice weighing 18–22 g were injected i.c.v. with 10 μl of sterile saline buffer (0.9% NaCl) or neurotensin (0.03 μg in saline) in the absence and presence of 1 or 3 μg P33. The hot plate test was derived from that described by Eddy & Leimbach (1991). The plate was maintained at 55 ± 0.5°C with a regulated water circulating pump. The antinociceptive test was begun 15 min after i.c.v. injection and the reaction times (latency of forepaw licking with a cut-off time of 30 s) of mice placed on the plate surrounded by a glass cylinder (20 cm high, 16 cm diameter) were determined as described previously (Schmidt *et al*., 1991).

Protein concentrations

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

Materials

Mcc-Pro-Leu-Gly-Pro-DLys-Dnp (QFS) and diprotin A were from Novabiochem (Meudon, France). Neurotensin was from Neosystem (Strasbourg, France). Angiotensin-converting enzyme, phosphoramidon, bestatin and all the fluorimetric and chromogenic substrates were purchased from Sigma chemicals (St Louis, MO). Arphamenine B was from Interchim. Z-prolyl prolinal was generously given by Drs S. Wilk and M. Orlowski (Mount Sinaï School of Medicine, New York, U.S.A.). All phosphinic compounds were synthetized according to the procedures previously described (Jiràcek *et al*., 1996; Yiotakis *et al*., 1996).

Results

Table 1 illustrates the potencies of a series of phosphinic peptide inhibitors on purified endopeptidases 3.4.24.15, 3.4.24.16 and ACE. All compounds displaying a free C-terminus dipeptide behaved as very potent inhibitors of ACE and were particularly poor inhibitors of endopeptidase 3.4.24.15. As previously established (Jiràcek *et al*., 1996), the introduction of a proline residue at the P2 position of the inhibitors led to analogues with higher affinity for endopeptidase 3.4.24.16 while they remained poor agonists of endopeptidase 3.4.24.15 (Table 1, compare compounds P23-P25 to P26-P31). Introduction of an additional proline residue in the P′2 position drastically enhanced the affinity for endopeptidase 3.4.24.16 (P22) without noticeable influence on the selectivity towards endopeptidase 3.4.24.15 (Table 1, compare P26-P31 to P22). However, P22 also behaved as a potent inhibitor of ACE (Table 1). Therefore, we attempted to increase the selectivity of P22-derived molecules for endopeptidase 3.4.24.16 by modifying the C-terminus of P22. Since a previous study documented the strong requirement of free C-terminus for ACE inhibition (Ondetti *et al*., 1977), we amidated the C-terminal end of P22. Table 2 shows that such a modification triggered about a 30 fold decrease in the K_i for ACE (compare P22 and P32). Interestingly, P32 not only displayed high affinity for endopeptidase 3.4.24.16 ($K_i = 12$ nM) but also appeared 6670 fold less potent on endopeptidase 3.4.24.15 (Table 2). Finally, the substitution of the glycine residue located in P′1 by a leucine achieved an additional 15 fold decrease in the apparent affinity of the compound for ACE (Table 2, compare P32 and P33). Altogether, the modifications introduced at the C-terminal dipeptide of P22 ultimately led to a 500 fold less potent inhibitor of ACE (Table 2, compare P22 and P33).

In order to establish the selectivity of P33 towards other purified or yet unknown proteolytic activities, we examined the ability of P33 to prevent the hydrolysis of various fluorimetric substrates thought to be selective for a series of

enzymes belonging to metallo- and serine proteases families (Checler, 1993) in a crude rat brain homogenate. Strikingly, discrepancy exists between the theoretical complete inhibition expected from the use of saturating concentrations of specific inhibitors (Checler, 1993) and the extent of inhibition observed for the activities present in the crude membrane homogenates (Table 3). This indicates that, in the rat brain, additional, yet unknown, proteolytic enzymes, able to cleave 'pseudo specific' chromogenic substrates, exist. However, it is clear that inhibitor-sensitive activities and additional unexpected enzymes are both totally insensitive to a 1 μM P33 (Table 3).

Therefore, as P33 was 12.5 more potent on purified endopeptidase 3.4.24.16 than on ACE and was virtually inactive on endopeptidase 3.4.24.15 (Table 2) and other activities (Table 3), it was used to examine the putative contribution of endopeptidase 3.4.24.16 on the central catabolism of neurotensin, *in vitro* and *in vivo*. We recently established that endopeptidase 3.4.24.16 is expressed at the cell surface of pure primary culture neurones (Vincent *et al*., 1996). Accordingly, one of the main neurotensin degradation products elutes with the retention time of neurotensin 1-10 after h.p.l.c. analysis (Figure 1a). Pretreatment of the cells with 100 nM P33 resulted in about a 50% decrease in the recovery of neurotensin 1-10 without any change in the production of other catabolites (Figure 1b). The dose-response curve indicates that P33 inhibits neurotensin 1-10 formation with an IC_{50} of about 100 nM (Figure 1c). By use of the equation $IC_{50} = K_i(1 + S/K_m)$, where S and K_m are 10 μM and 1.6 μM, respectively, a K_i value of 13 nM was derived.

Neurotensin elicits analgesia in mice after central administration (for review see Nemeroff *et al*., 1980). Therefore P33 was examined as a potential modulator of neurotensin-induced antinociception in the hot plate test. In our series of tested mice, the administration of P33 alone triggered an apparent algesic effect at the two doses of inhibitor that were used (Table 4). When neurotensin was administered at a low dose (0.03 μg), previously shown to be ineffective

Table 1 Inhibition of endopeptidase 3.4.24.16 (E24.16), endopeptidase 3.4.24.15 (E24.15) and angiotensin-converting enzyme (ACE) by phosphinic peptides

Compound	*Inhibitor*	K_i (nM) *E24.16*	*E24.15*	*ACE*
23	Trp-Phe (PO_2CH_2) Gly-Yaa′	600	75000	0,7
24	Val-Phe (PO_2CH_2) Gly-Yaa′	600	75000	2.5
25	Arg-Phe (PO_2CH_2) Gly-Yaa′	1200	18000	3.2
26	Pro-Phe (PO_2CH_2) Gly-Ala	20	53000	1.1
27	Pro-Phe (PO_2CH_2) Gly-Nle	40	37000	0.3
28	Pro-Phe (PO_2CH_2) Gly-Met	50	33000	0.3
29	Pro-Phe (PO_2CH_2) Gly-Ser	100	120000	16
30	Pro-Phe (PO_2CH_2) Gly-Leu	200	140000	5.4
31	Pro-Phe (PO_2CH_2) Gly-Gly	200	100000	32
22	Pro-Phe (PO_2CH_2) Gly-Pro	4	8100	0,3

Enzymatic activities were measured by incubating 50 μM QFS (E24.16 and E24.15) or 1 mM of Hip-His-Leu (ACE) with 10 μl of purified peptidases for 1 h at 37°C after overnight preincubation in the absence or presence of increasing concentrations (0.1 nM to 100 μM) of the indicated compounds. Incubations were stopped and fluorimetrically recorded as described in the Methods. K_i values were derived from the IC_{50} values according to the equation: $IC_{50} = K_i(1 + S/K_m)$ and are the mean of four independent determinations. Yaa′ represents a mix of 20 different amino acids.

Table 2 K_i values and selectivity of P22, P32 and P33 for purified endopeptidase 3.4.24.16 (E24.16), endopeptidase 3.4.24.15 (E24.15) and ACE

Inhibitor	K_i (nM) *E24.16*	*E24.15*	*ACE*	*Selectivity factor* $\frac{K_i\ 24.15}{K_i\ 24.16}$	$\frac{K_i\ ACE}{K_i\ 24.16}$
Pro-Phe (PO_2CH_2) Gly-Pro (P22)	4	8100	0.3	2025	0.075
Pro-Phe (PO_2CH_2) Gly-ProNH_2 (P32)	12	80000	8,5	6670	0.7
Pro-Phe (PO_2CH_2) Leu-ProNH_2 (P33)	12	66500	150	5540	12.5

QFS (E24.15 and E24.16) and Hip-His-Leu (ACE) hydrolysis was fluorimetrically measured as described in the Methods and the K_i values were derived from the IC_{50} according to the relation $IC_{50} = K_i\ (1 + S/K_m)$. Values are the mean of 3 to 6 independent determinations.

Table 3 Effect of specific inhibitors and P33 on peptidase activities present in rat brain homogenates

Peptidase	*Substrate/inhibitor*	*Enzymatic activity* (% of control) *Specific inhibitor*	*P33*
E24.11	Suc-A-A-F-7AMC/Phosphoramidon 1 μM	32.2 ± 0.7	96 ± 0.8
LAP/APM	Leu-7AMC/Bestatin 50 μM	31.5 ± 0.4	114.1 ± 5.7
DAP IV	Gly-Pro-7AMC/Diprotin A 100 μM	84.9 ± 0.5	99.3 ± 1.7
APB	Arg-7AMC/Arphamenine B 0,5 μM	44.5 ± 0.7	108.5 ± 4.5
PE	Z-Gly-Pro-7AMC/Z-Pro-Prolinal 1 μM	8 ± 0.4	100.4 ± 0.9

Activities were measured by incubation of rat brain homogenates with the indicated substrate as described in the Methods in the absence or presence of the indicated specific inhibitors or P33 (1 μM). Values are expressed as a percentage of control activity obtained in the absence of inhibitor and are the mean ± s.e.mean of 6 independent experiments. E24.11, endopeptidase 3.4.24.11; LAP/APM, leucine aminopeptidase/aminopeptidase M; DAPIV, dipeptidylaminopeptidase IV; APB, aminopeptidase B; PE, proline endopeptidase.

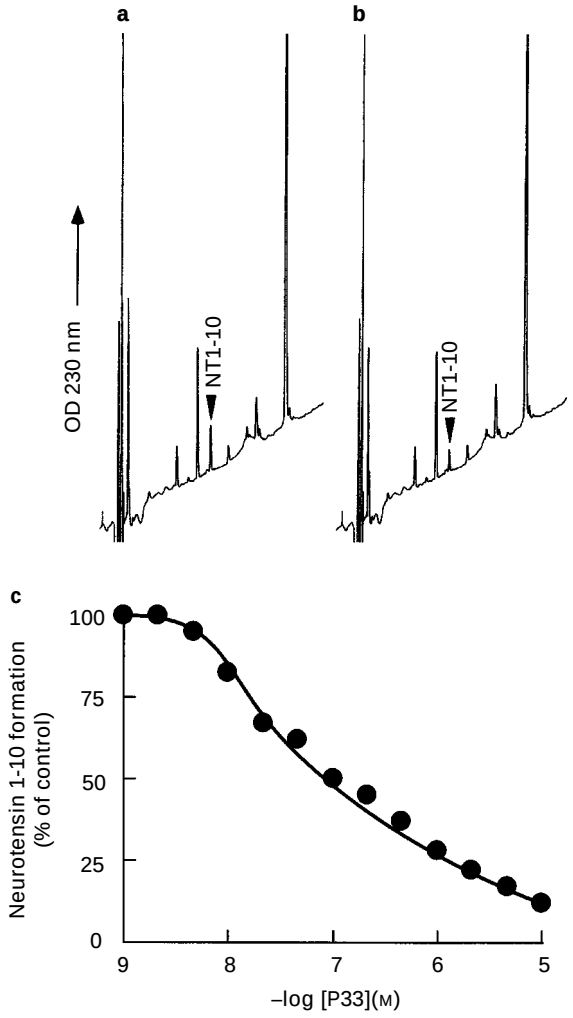

Figure 1 Effect of phosphodiepryl 33 on neurotensin hydrolysis by plated primary cultured neurones. Neurotensin (10 nmol) was incubated with 4-day-old plated primary cultured neurones for 150 min at 37°C, in 1 ml of PBS pH 7.4, in the absence (a) and presence (b) of 100 nM phosphodiepryl 33. Supernatants were then removed, acidified with 100 μl of 1 M HCl and analysed by h.p.l.c. as described in the Methods. (c) A complete dose-response curve was established by incubating neurotensin in the presence of the indicated concentrations of phosphodiepryl 33. Values are expressed as the percentage of neurotensin (1-10) (NT (1–10)) recovered in the absence of inhibitor and represent the mean of three independent experiments.

Table 4 Effect of P33 on neurotensin-induced antinociception in mice tested on the hot-plate

Treatment	*Dose* (μg)	*Latency* (s)
Saline	–	11.51 ± 1
P33	1	5.63 ± 0.18*
P33	3	5.55 ± 0.48*
Neurotensin 0.03 μg		9.76 ± 1.62
+ P33	1	19.92 ± 2.74**
+ P33	3	20.15 ± 3.73**

Neurotensin was administered i.c.v. at the doses indicated in the absence or in the presence of 1 or 3 μg of P33. Latency of appearance of forepaw licking behaviour was recorded 15 min after peptide and/or inhibitor administration. Values are expressed as means ± s.e.mean of 8 independent mice per group. *$P<0.01$ as compared to saline. **$P<0.01$ as compared to the same dose of neurotensin without inhibitor.

(Vincent *et al*, 1995), there was no modification of the forepaw licking behaviour (Table 4). However, 1 μg P33 drastically enhanced the observed latency (Table 4). This effect was not further augmented by an increased dose of P33 (Table 4).

Discussion

Elucidation of the mechanisms by which neurotensin is inactivated in the brain appears of primary importance, since this neuropeptide has been described as a neuroleptic-like agent with respect to its clear pharmacological spectrum as an endogenous dopamine antagonist (for review see Kitabgi, 1989). Furthermore, this peptide triggers a naloxone-insensitive analgesia (Clineschmidt *et al*., 1979; Coquerel *et al*., 1988) that is, therefore, not mediated by opiate receptors.

Several lines of evidence suggest that endopeptidases 3.4.24.15 and 3.4.24.16 are the main contributors of neurotensin catabolism in the central nervous system. Thus, these two peptidases were shown to participate in neurotensin inactivation by rat brain synaptic membranes (Checler *et al*., 1983; 1986b), brain or hypothalamic slices (Davis *et al*., 1992; Kitabgi *et al*., 1992; Konkoy *et al*., 1994;) and in pure cultured neurones (Checler *et al*., 1986a; Vincent *et al*., 1996) and astrocytes (Mentlein & Dahms, 1994; Vincent *et al*., 1996). These *in vitro* observations were strongly reinforced by the fact that several phosphorus-containing peptides, designed as mixed inhibitors of endopeptidase 3.4.24.15 and endopeptidase 3.4.24.16, drastically potentiated the neurotensin-induced analgesia after central administration in mice (Vincent *et al*., 1995). In order to establish the contribution of endopeptidase 3.4.24.16 in the inactivating process of neurotensin, we have designed a strategy aimed at discriminating between the two related peptidases. By a novel approach based on the chemistry of phosphinic peptides, we have obtained the first highly selective inhibitor of endopeptidase 3.4.24.16 (Pro-Phe-$\Psi(PO_2CH_2)$-Gly-Pro, P22) that was 2025 fold less potent on endopeptidase 3.4.24.15 (Jiràcek *et al*., 1996). However, P22 also behaves as a highly potent ACE inhibitor.

The further design of a fully selective inhibitor of endopeptidase 3.4.24.16 was achieved by modifying the C-terminus part of P22. In order to keep a strong selectivity towards endopeptidase 3.4.24.15, we did not modify the C-terminal dipeptide extension from the phosphinic group. Thus we showed previously that the length of this C-terminal tail was an important requirement for endopeptidase 3.4.24.15 inhibition, and that a minimal extension of three amino acids from the phosphinic group was necessary (Jiràcek *et al*., 1996). On the other hand, it appeared that ACE clearly displayed preferred affinity for inhibitors bearing a free carboxyl group (Ondetti *et al*., 1977). As expected, amidation of the C-terminus of P22 (Table 2, compound P32) elicited about a 30 fold decrease in the potency towards ACE (Table 2). A further 15 fold lowering of the potency for ACE was obtained by substitution of the glycine residue in P′1 position by an aliphatic leucine residue (Table 2, compound P33). Overall, P33 appeared 500 fold less potent than P22 on ACE without a drastic change in its inhibitory potency towards endopeptidase 3.4.24.16. Most important was the observation that P33 remained a very poor inhibitor of endopeptidase 3.4.24.15 (Table 2). Finally, we established that P33 was unable to affect a series of other peptidases belonging to the metallo and serine enzyme families (Table 3). Altogether, P33 behaves as the first highly potent and fully selective inhibitor of endopeptidase 3.4.24.16 and was therefore used to assess the contribution of endopeptidase 3.4.24.16 in the central inactivation of neurotensin, *in vitro* and *in vivo*.

As previously shown, neurotensin is rapidly inactivated by pure cultured plated neurones (Checler *et al*., 1986a; Vincent *et al*., 1996) with a noticeable production of neurotensin 1–10, the formation of which is reminiscent of that generated by purified endopeptidase 3.4.24.16 (Checler *et al*., 1986b). This formation was dose-dependently inhibited by P33, with a K_i

value of 13 nM that is in close agreement with the K_i value (12 nM) observed for QFS hydrolysis by purified endopeptidase 3.4.24.16 (Table 2). These data clearly indicate that this endopeptidase is a major contributor to neuronal neurotensin inactivation *in vitro*.

Neurotensin triggers naloxone-insensitive analgesia in mice after central administration (Clineschmidt *et al*, 1979; Coquerel *et al*., 1988). This paradigm was examined to assess the potential of P33 as a physiological blocker of central neurotensin degradation. Surprisingly, it appeared that the administration of P33 alone shortened the forepaw licking latency (Table 4). One possibility to explain such an observation could be that P33 protects an endogenous algesic substance from degradation by endopeptidase 3.4.24.16. That such an agent could be nociceptin/orphanin FQ, a recently discovered heptadecapeptide (Meunier *et al*., 1995; Reinscheid *et al*., 1995) with algesic properties appears unlikely because this peptide resists proteolysis by purified endopeptidase 3.4.24.16 (Montiel *et al*., 1997). Another possibility remains that the algesic effect of P33 was overestimated, due to an unusual high lick latency obtained after saline administration. In line with such a hypothesis, one should note the fact that in our previous study, P08, a mixed inhibitor of endopeptidases 3.4.24.15 and 3.4.24.16 did not modify the latency observed in saline conditions (Vincent *et al*., 1995).

Our analgesia experiments clearly established that the combined administration of P33 with an ineffective dose of neurotensin triggered a drastic analgesic effect that appeared maximal at 1 μg (Table 4). Although it is not possible to preclude the possibility that P33 also targeted ACE in our experimental conditions, the contribution of ACE is unlikely because the enzyme does not contribute to neurotensin catabolism by pure cultured neurones (Checler *et al*., 1986a). Furthermore, the specific ACE inhibitor, captopril (Ondetti *et al*., 1977) does not modify the neurotensin-induced hypothermia- and analgesia in mice (Coquerel *et al*., 1986 and our data not shown).

Altogether, our study describes a highly potent and selective endopeptidase 3.4.24.16 and establishes for the first time that this enzyme contributes to the central physiological inactivation of neurotensin. This inhibitor should prove useful to protect neurotensin from degradation in experiments aimed at further investigating the pharmacological and physiological roles of neurotensin in the central nervous system.

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References

BARELLI, H., DIVE, V., YIOTAKIS, A., VINCENT, J.P. & CHECLER, F. (1992). Potent inhibition of endopeptidase 24.16 and endopeptidase 24.15 by the phosphonamide peptide N-(phenylethylphosphonyl)-Gly-LPro-L-aminohexanoic acid. *Biochem. J*., **287,** 621 – 625.

BARELLI, H., FOX-THRELKELD, J.E.T., DIVE, V., DANIEL, E.E., VINCENT, J.P. & CHECLER, F. (1994). Role of endopeptidase 3.4.24.16 in the catabolism of neurotensin, *in vivo*, in the vascularly perfused dog ileum. *Br. J. Pharmacol*., **112,** 127 – 132.

BARELLI, H., VINCENT, J.P. & CHECLER, F. (1991). Rat brain endopeptidase 24-15: specificity towards opioids, tachykinins and neurotensin-related peptides. Comparison with the neurotensin degrading metallopeptidase endopeptidase 24-16. *Neurochemistry, Life Sci. Adv*., **10,** 115 – 124.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*., **72,** 248 – 259.

CHABRY, J., CHECLER, F., VINCENT, J.P. & MAZELLA, J. (1990). Colocalization of neurotensin receptors and of the neurotensin-degrading enzyme endopeptidase 24-16 in primary cultures of neurones. *J. Neurosci*.. **10,** 3916 – 3921.

CHECLER, F. (1991). Peptidases and neuropeptide-inactivating mechanisms in the circulation and in the gastrointestinal tractus. In *Neuropeptide Function in the Gastrointestinal Tract*. ed. Daniel, E.D. pp. 273 – 307. Boston: CRC Press.

CHECLER, F. (1993). Neuropeptide-degrading peptidases. In *Methods in Neurotransmitter and Neuropeptide Research*. Part 2. ed. Nagatsu, T., Parvez, H., Naoi, M. & Parvez, S. pp. 375 – 418. Amsterdam: Elsevier Science Publishers.

CHECLER, F., BARELLI, H., KITABGI, P. & VINCENT, J.P. (1988). Neurotensin metabolism in various tissues from central and peripheral origins. Ubiquitous involvement of a novel neurotensin degrading metalloendopeptidase. *Biochimie*, **70,** 75 – 82.

CHECLER, F., BARELLI, H. & VINCENT, J.P. (1989). Tissue distribution of a novel neurotensin-degrading metallopeptidase. An immunological approach using monospecific polyclonal antibodies. *Biochem. J*., **257,** 549 – 554.

CHECLER, F., MAZELLA, J., KITABGI, P. & VINCENT, J.P. (1986a). High affinity receptor sites and rapid proteolytic inactivation of neurotensin in primary cultured neurons. *J. Neurochem*., **47,** 1742 – 1748.

CHECLER, F., VINCENT, J.P. & KITABGI, P. (1986b). Purification and characterization of a novel neurotensin degrading peptidase from rat brain synaptic membranes. *J. Biol. Chem*., **261,** 11274 – 11281.

CHECLER, F., VINCENT, J.P. & KITABGI, P. (1983). Degradation of neurotensin by rat brain synaptic membranes. Involvement of a thermolysin-like metalloendopeptidase (enkephalinase), angiotensin-converting enzyme and other unidentified peptidases. *J. Neurochem*., **41,** 375 – 384.

CLINESCHMIDT, B.V., MCGUFFIN, J.V. & BUNTING, P.B. (1979). Neurotensin: antinocisponsive action in rodents. *Eur. J. Pharmacol*., **54,** 129 – 139.

COQUEREL, A., DUBUC, I., KITABGI, P. & COSTENTIN, J.A. (1988). Potentiation by thiorphan and bestatin of the naloxone-insensitive analgesic effects of neurotensin and neuromedin N. *Neurochem. Int*., **12,** 361 – 366.

COQUEREL, A., DUBUC, I., MENARD, J.F., KITABGI, P. & COSTENTIN, J. (1986). Naloxone-insensitive potentiation of neurotensin hypothermic effect by the enkephalinase inhibitor thiorphan. *Brain Res*., **398,** 386 – 389.

DAUCH, P., BARELLI, H., VINCENT, J.P. & CHECLER, F. (1991a). Fluorimetric assay of the neurotensin-degrading metalloendopeptidase, endopeptidase 24-16. *Biochem. J*., **280,** 412 – 426.

DAUCH, P., VINCENT, J.P. & CHECLER, F. (1991b). Specific inhibition of endopeptidase 24.16 by dipeptides. *Eur. J. Biochem*., **202,** 269 – 276.

DAVIS, T.P., GILLESPIE, T.J. & KONINGS, P.N.M. (1992). Specificity of neurotensin metabolism by regional rat brain slices. *J. Neurochem*., **58,** 608 – 617.

EDDY, N.B. & LEIMBLACH, D. (1991). Synthetic analgesics (II): dithienylbutenyl and dithienylbutylamines. *J. Pharmacol. Exp. Ther*., **107,** 385 – 393.

HUGGINS, C.G. & THAMPI, N.S. (1968). A simple method for the determination of angiotensin I converting enzyme. *Life Sci*., **7,** 633 – 639.

JIRÀCEK, J., YIOTAKIS, A., VINCENT, B., CHECLER, F. & DIVE, V. (1996). Development of the first potent and selective inhibitor of the zinc-endopeptidase 3.4.24.16 using a systematic approach based on combinatorial chemistry of phosphinic peptides. *J. Biol. Chem*., **271,** 19606 – 19611.

KITABGI, P. (1989). Neurotensin modulates dopamine neurotransmission at several levels along brain dopaminergic pathways. *Neurochem. Int*., **14,** 111 – 119.

KITABGI, P., DUBUC, I., NOUEL, D., COSTENTIN, J., CUBER, J.-C., FULCRAND, H., DOULUT, S., RODRIGUEZ, M. & MARTINEZ, J. (1992). Effects of thiorphan, bestatin and a novel metallopeptidase inhibitor JMV 390-1 on the recovery of neurotensin and neuromedin N released from mouse hypothalamus. *Neurosci. Lett*., **142,** 200 – 204.

KONKOY, C.S., WATERS, S.M. & DAVIS, T.P. (1994). Acute administration of neuroleptics decreases neurotensin metabolism on intact, regional rat brain slices. *J. Pharmacol. Exp. Ther.*, **269,** 555 – 563.

LITTLEWOOD, G.M., IVERSEN, L.L. & TURNER, A.J. (1988). Neuropeptides and their peptidases: functional considerations. *Neurochem. Int.*, **12,** 383 – 389.

MCKELVY, J.F. & BLUMBERG, S. (1986). Inactivation and metabolism of neuropeptides. *Ann. Rev. Neurosci.*, **9,** 415 – 434.

MENTLEIN, R. & DAHMS, P. (1994). Endopeptidases 24.16 and 24.15 are responsible for the degradation of somatostatin, neurotensin and other neuropeptides by cultivated rat cortical astrocytes. *J. Neurochem.*, **62,** 27 – 36.

MEUNIER, J.C., MOLLEREAU, C., TOLL, L., SUAUDEAU, C., MOISAND, C., ALVINERIE, P., BUTOUR, J.L., GUILLEMOT, J.C., FERRARA, P., MONSARRAT, B., MAZARGUIL, H., VASSART, G., PARMENTIER, M. & COSTENTIN, J. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature*, **377,** 532 – 535.

MONTIEL, J.L., CORNILLE, F., ROQUES, B.P. & NOBLE, F. (1997). Nociceptin/orphanin FQ metabolism. Role of aminopeptidase and endopeptidase 3.4.24.15. *J. Neurochem.*, **68,** 354 – 361.

NEMEROFF, C.B., LUTTINGER, D. & PRANGE, A.J. (1980). Neurotensin: central nervous system effects of a neuropeptide. *Trends Neurosci.*, **3,** 212 – 215.

ONDETTI, M.A., RUBIN, B. & CUSHMAN, D.W. (1977). Design of specific inhibitors of angiotensin-converting enzyme: new class of orally antihypertensive agents. *Science*, **196,** 441 – 444.

REINSCHEID, R.K., NOTHACKER, H.P., BOURSON, A., ARDATI, A., HENNINGSEN, R.A., BUNZOW, J.R., GRANDY, D.K., LANGEN, H., MONSMA, Jr, F.J. & CIVELLI, O. (1995). Orphanin FQ: a neuropeptide that activates an opioid-like G protein-coupled receptor. *Science*, **270,** 792 – 794.

SCHMIDT, C., PEYROUX, J., NOBLE, F., FOURNIÉ-ZALUSKI, M.C. & ROQUES, B.P. (1991). Analgesic responses elicited by endogenous enkephalins (protected by mixed peptidase inhibitors) in a variety of morphine-sensitive noxious tests. *Eur. J. Pharmacol.*, **192,** 253 – 262.

TURNER, A.J., MATSAS, R. & KENNY, A.J. (1985). Are there neuropeptide-specific peptidases? *Biochem. Pharmacol.*, **34,** 1347 – 1356.

VINCENT, B., BEAUDET, A., DAUCH, P., VINCENT, J.P. & CHECLER, F. (1996). Distinct properties of neuronal and astrocytic endopeptidase 3.4.24.16: a study on differentiation, subcellular distribution and secretion processes. *J. Neurosci.*, **16,** 5049 – 5059.

VINCENT, B., DIVE, V., YIOTAKIS, A., SMADJA, C., MALDONADO, R., VINCENT, J.P. & CHECLER, F. (1995). 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*. *Br. J. Pharmacol.*, **115,** 1053 – 1063.

VINCENT, B., VINCENT, J.P. & CHECLER, F. (1994). Neurotensin and neuromedin N undergo distinct catabolic processes in astrocytes and primary cultured neurons. *Eur. J. Biochem.*, **221,** 297 – 306.

YIOTAKIS, A., VASSILOU, S., JIRACEK, J. & DIVE, V. (1996). Protection of hydroxyphosphinyl function of phosphinic dipeptides by adamantyl. Application to the solid-phase synthesis of phosphinic peptides. *J. Org. Chem.*, **61,** 6601 – 6605.

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