The metabolism of neuropeptides

Neurokinin A (substance K) is a substrate for endopeptidase-24.11 but not for peptidyl dipeptidase A (angiotensin-converting enzyme)

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Both endopeptidase-24.11 and peptidyl dipeptidase A have previously been shown to hydrolyse the neuropeptide substance P. The structurally related peptide neurokinin A is also shown to be hydrolysed by pig kidney endopeptidase-24.11. The identified products indicated hydrolysis at two sites, Ser⁵–Phe⁶ and Gly⁸–Leu⁹, consistent with the known specificity of the enzyme. The pattern of hydrolysis of neurokinin A by synaptic membranes prepared from pig striatum was similar to that observed with purified endopeptidase-24.11, and hydrolysis was substantially abolished by the selective inhibitor phosphoramidon. Peptidyl dipeptidase A purified from pig kidney was shown to hydrolyse substance P but not neurokinin A. It is concluded that endopeptidase-24.11 has the general capacity to hydrolyse and inactivate the family of tachykinin peptides, including substance P and neurokinin A.

INTRODUCTION

Substance P and other structurally related peptides, such as kassinin, eliodisin and physalaemin, comprise a family of naturally occurring peptides referred to as the tachykinins (Table 1). These peptides share a common C-terminal sequence, Phe-Xaa-Gly-Leu-MetNH₂, and exhibit similar biological activities such as smooth-muscle contraction, reduction of blood pressure and stimulation of glandular secretion (Erspamer, 1981; Jessell & Womack, 1985). There is now ample evidence that substance P may serve as one of the sensory-nerve transmitters in the mammalian nervous system. Further, two other substance P-like peptides have also been described in mammalian central nervous system, and these have been termed neurokinin A (substance K, neuromedin L) and neurokinin B (neuromedin K) (Kangawa *et al.*, 1983; Nawa *et al.*, 1983, 1984; Minamino *et al.*, 1984). These peptides may also function as neurotransmitters in the brain.

A number of studies have investigated the metabolism of substance P *in vivo* and *in vitro*, and several enzymes have been implicated in this process (see, e.g., Turner *et al.*, 1985). Of characterized cell-surface peptidases, two specific enzymes have received particular attention: these are endopeptidase-24.11 (EC 3.4.24.11) (Matsas *et al.*, 1983, 1985) and peptidyl dipeptidase A (angiotensin-converting enzyme, EC 3.4.15.1) (Yokosawa *et al.*, 1983; Cascieri *et al.*, 1984; Skidgel *et al.*, 1984). We have shown that the patterns of metabolism of substance P by endopeptidase-24.11 and by synaptic membranes from pig striatum are similar, with hydrolysis occurring at the Gln⁶–Phe⁷, Phe⁷–Phe⁸ and Gly⁸–Leu¹⁰ bonds (Matsas *et al.*, 1983). Phosphoramidon, a selective inhibitor of endopeptidase-24.11, and an antiserum to the enzyme both inhibit the hydrolysis of substance P by synaptic membranes (Matsas *et al.*, 1983). Furthermore, the distribution of endopeptidase-24.11 in the brain (Matsas

et al., 1985) and its enrichment in synaptosomal preparations (de la Baume *et al.*, 1981) are not inconsistent with a role in the metabolism of substance P.

The primary specificity of peptidyl dipeptidase A is to release dipeptide fragments from the C-terminus of an oligopeptide. The observation (Yokosawa *et al.*, 1983; Cascieri *et al.*, 1984; Skidgel *et al.*, 1984) that this enzyme can hydrolyse substance P at the Phe⁸–Gly⁹ bond, releasing the tripeptide Gly-Leu-MetNH₂, was therefore somewhat unexpected and, since substance P is amidated at the C-terminus, clearly implies an endopeptidase attack on certain substrates. Inhibitors of peptidyl dipeptidase A such as captopril have been shown to potentiate certain of the peripheral actions of substance P, such as salivation (Cascieri *et al.*, 1984), suggesting that this enzyme might also play a role in substance P metabolism *in vivo*, at least in peripheral tissues.

In the present study we have compared the abilities of endopeptidase-24.11 and peptidyl dipeptidase A purified from pig kidney, a rich source of both enzymes, to hydrolyse neurokinins A and B, and have examined the effects of peptidase inhibitors on the hydrolysis of neurokinin A by synaptic membranes. Peptidyl dipeptidase A from pig kidney failed to hydrolyse either substrate, suggesting that only endopeptidase-24.11 has a general capacity to hydrolyse and inactivate tachykinins.

EXPERIMENTAL

Materials

Substance P was obtained from Cambridge Research Biochemicals (Harston, Cambridge, U.K.). Captopril (SQ14225, D-3-mercapto-2-methylpropanoyl-L-proline) was a gift from Squibb Institute for Medical Research, Princeton, NJ, U.S.A. Neurokinins A and B were gifts

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Fig. 1. Hydrolysis of neurokinin A by pig kidney endopeptidase-24.11

The peptide products of neurokinin A hydrolysis were resolved by h.p.l.c. and monitored at 214 nm. The peptides are numbered as in Table 2. Abbreviation: NKA, neurokinin A.

These sites are consistent with the known specificity of the enzyme, hydrolysing at bonds involving the amino groups of hydrophobic residues. The minor, unidentified, peptides (peaks 2 and 4) may correspond to hydrolysis at the Phe⁶-Val⁷ bond. The K_m for neurokinin A was 113 μ M and k_{cat}/K_m relative to substance P was 1.2. Because of limited availability of material, fewer studies were performed with neurokinin B. However, the substrate was efficiently hydrolysed by endopeptidase-24.11, and Leu-MetNH₂ was identified as one of the major products.

Neurokinin A is not hydrolysed by pig kidney peptidyl dipeptidase A

Substance P was hydrolysed by peptidyl dipeptidase A as previously described (Yokosawa *et al.*, 1983; Cascieri *et al.*, 1984; Skidgel *et al.*, 1984). Hydrolysis principally occurred at the Phe⁸-Gly⁹ bond, releasing the tripeptide Gly-Leu-MetNH₂. The time course of release of Gly-Leu-MetNH₂ is shown in Fig. 2. Hydrolysis was abolished by 1 μ M-captopril, a specific inhibitor of peptidyl dipeptidase A. In contrast, no release of Gly-Leu-MetNH₂ could be detected with neurokinin A as substrate (Fig. 2) even after an incubation time of 18 h at 37 °C. No other product peaks and no loss of substrate were detectable. Similarly no hydrolysis of neurokinin B by peptidyl dipeptidase A could be detected.

Fig. 2. Time course of the hydrolysis of tachykinins by pig kidney peptidyl dipeptidase A

Substance P or neurokinin A was incubated with peptidyl dipeptidase A (200 ng of protein) for various time intervals, and the release of the C-terminal tripeptide Gly-Leu-MetNH₂ was monitored by h.p.l.c. No release of Gly-Leu-MetNH₂ from substance P was detectable if the reaction was carried out in the presence of 1 μ M-captopril. ●, Substance P; ▲, neurokinin A.

Hydrolysis of neurokinin A by synaptic-membrane preparations

The N-terminal sequence of neurokinin A suggests that it might also be susceptible to hydrolysis by aminopeptidases, unlike substance P. The pattern of hydrolysis of neurokinin A by synaptic membranes (Fig. 3a) was similar to that observed with endopeptidase-24.11. A combination of 1 μ M-phosphoramidon (to inhibit endopeptidase-24.11) and 0.1 mM-bestatin (to inhibit aminopeptidases) completely abolished hydrolysis of neurokinin A (Fig. 3b). However, phosphoramidon alone produced substantial inhibition of the formation of most products. Captopril at 1 μ M (to inhibit peptidyl dipeptidase A) had little effect. Table 2 compares the effects of different combinations of peptidase inhibitors on the production of individual peptide peaks.

DISCUSSION

We have previously shown that endopeptidase-24.11 can hydrolyse substance P and physalaemin (Matsas *et al.*, 1983, 1984a). The present studies reveal that the endopeptidase can also hydrolyse the related tachykinins neurokinins A and B. In the case of neurokinin A, the susceptible bonds were identified as Ser⁵-Phe⁶ and Gly⁸-Leu⁹, consistent with the known specificity of endopeptidase-24.11. Peptidyl dipeptidase A from pig kidney, which is able to hydrolyse substance P, releasing the C-terminal tripeptide Gly-Leu-MetNH₂, appears unable to hydrolyse either neurokinin A or neurokinin B at a significant rate. Thus amino acid residues on the amino side of the scissile bond substantially affect the ability of peptidyl dipeptidase A to display endopeptidase activity. The replacement of phenylalanine-8 in substance

Fig. 3. Hydrolysis of neurokinin A by a pig striatal synaptic-membrane preparation

(a) No inhibitors present; (b) incubation in the presence of 1 μ M-phosphoramidon and 100 μ M-bestatin. The products were resolved by h.p.l.c. and monitored at 214 nm. The peptides are numbered as in Table 2. Abbreviation: NKA, neurokinin A.

Table 2. Effects of inhibitors on the hydrolysis of neurokinin A by pig striatal synaptic membranes

The inhibitors were added at the start of the incubation period. The concentrations of inhibitors were: phosphoramidon, 1 μ M; bestatin, 100 μ M; captopril, 1 μ M. Abbreviation: n.d., not defined.

Peak	Retention time (min)	Peptide	Effect of inhibitors on peak area [control (no inhibitors) = 100 for each peak]					
			Phosphoramidon (P)	Bestatin (B)	Captopril (C)	P+B	C+B	P+C+B
1	1.60	His-Lys-Thr-Asp-Ser	14	50	89	0	43	2
2	2.00	n.d.	22	19	100	0	33	22
3	3.74	Leu-MetNH ₂	0	60	96	0	58	0
4	5.08	n.d.	0	63	110	0	93	0
5	5.44	Phe-Val-Gly	0	342	69	0	265	0
6	6.76	His-Lys-Thr-Asp-Ser-Phe-Val-Gly	9	100	127	0	101	8

P by valine in neurokinins A and B may represent the critical change in sequence. The hydrolysis of neurokinin A by striatal synaptic membranes was substantially abolished by phosphoramidon, indicating that endopeptidase-24.11 may play a major role in its inactivation in those brain regions, such as striatum, that are relatively enriched in the enzyme. Complete inhibition of neurokinin hydrolysis required a combination of phosphoramidon and bestatin, indicating that a bestatin-sensitive aminopeptidase may also contribute to the hydrolysis of neurokinin A, as is the case for the inactivation of the

enkephalins (de la Baume *et al.*, 1982) and cholecystokinin-8 (Matsas *et al.*, 1984b). The major conclusion of the present study is that endopeptidase-24.11 has the general capacity to hydrolyse and inactivate the family of tachykinin peptides, including substance P and the neurokinins.

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