Neurokinin B is hydrolysed by synaptic membranes and by endopeptidase-24.11 ('enkephalinase') but not by angiotensin converting enzyme

Nigel M. Hooper and Anthony J. Turner*

MRC Membrane Peptidase Research Group, Department of Biochemistry, University of Leeds, Leeds LS2 9JT, England

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Endopeptidase-24.11 ('enkephalinase', EC 3.4.24.11) from pig kidney hydrolysed neurokinin B at 4 sites:

$$Asp\text{–}Met\text{–}His\text{–}Asp\downarrow Phe\text{–}Phe\downarrow Val\text{–}Gly\downarrow Leu\text{–}MetNH_2$$

The major site of hydrolysis was the Gly^8–Leu^9 bond. Angiotensin converting enzyme (peptidyl dipeptidase A, EC 3.4.15.1) from pig kidney hydrolysed substance P releasing the C-terminal tripeptide Gly–Leu–$MetNH_2$ but failed to hydrolyse neurokinin B. Pig brain striatal synaptic membranes hydrolysed neurokinin B producing a similar pattern of products as did endopeptidase-24.11. Substantial inhibition of this activity was achieved with the selective inhibitor phosphoramidon. A combination of phosphoramidon and bestatin abolished the hydrolysis of neurokinin B by synaptic membranes. Thus, a bestatin-sensitive aminopeptidase may play a role in the synaptic metabolism of neurokinin B in addition to endopeptidase-24.11. This aminopeptidase appears to be distinct from aminopeptidase N (EC 3.4.11.2).

Neurokinin B *Tachykinin* *Endopeptidase-24.11* *Enkephalinase* *Angiotensin converting enzyme* *Aminopeptidase*

1. INTRODUCTION

Neurokinin B (neuromedin K) is a recently discovered peptide present in mammalian CNS, where it may function as a neurotransmitter [1]. The peptide shares a common C-terminal sequence, Phe-Xaa-Gly-Leu-$MetNH_2$, with the tachykinin family of peptides which include substance P [2]. The synaptic inactivation of substance P has been extensively investigated and at least 2 major cell surface peptidases have been implicated in its hydrolysis: endopeptidase-24.11 ('enkephalinase', EC 3.4.24.11) and angiotensin converting enzyme (peptidyl dipeptidase A, EC 3.4.15.1) (review [3]). Synaptic membranes prepared from pig or human striatum hydrolyse substance P at the same sites as does endopeptidase-24.11 and this hydrolysis is inhibited by the selective inhibitor phosphoramidon and by a specific antiserum to the enzyme [4,5]. On the basis of such evidence we have concluded that endopeptidase-24.11 may play a significant role in the synaptic metabolism of substance P as well as enkephalins and certain other neuropeptides [4,6]. However, inhibitors of angiotensin converting enzyme such as captopril have also been shown to potentiate certain of the peripheral actions of substance P [7] suggesting that this enzyme might play a role in substance P metabolism in vivo, at least in peripheral tissues.

The metabolism of neurokinin B has not previously been reported. Here we show that pig striatal synaptic membranes and purified preparations of endopeptidase-24.11 hydrolyse neurokinin B in an identical fashion. Angiotensin converting enzyme purified from pig kidney appears unable to

* To whom correspondence should be addressed

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hydrolyse neurokinin B. We therefore conclude that, of identified cell-surface peptidases, only endopeptidase-24.11 has the general capacity to hydrolyse and inactivate tachykinin peptides.

2. EXPERIMENTAL

2.1. *Peptides and inhibitors*

Neurokinin B was a gift from Merck, Sharp & Dohme Research Laboratories, Harlow, England, and its purity was checked by reverse phase HPLC. Captopril (SQ14225, D-3-mercapto-2-methylpropanoyl-L-proline) was a gift from the Squibb Institute for Medical Research, Princeton, NJ. Lisinopril (*N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline, MK521) was a gift from Dr A.A. Patchett, Merck, Sharp and Dohme Research Laboratories, Rahway, NJ. Bestatin was purchased from Sigma. Other chemicals were from sources previously noted [4–6].

2.2. *Membranes and enzyme preparations*

Crude synaptosomal/mitochondrial (P_2) preparations and synaptic membranes from pig striatum were prepared as in [4]. Endopeptidase-24.11 was purified from pig kidneys, the richest source of the enzyme, by immunoaffinity chromatography [8]. Likewise angiotensin converting enzyme was purified from pig kidneys by affinity chromatography using Lisinopril-Sepharose [9]. Both enzymes were apparently homogeneous by SDS-polyacrylamide gel electrophoresis.

2.3. *Hydrolysis of neurokinin B*

Samples of synaptic membranes (290 μg protein), endopeptidase-24.11 (200 ng) or angiotensin converting enzyme (100 ng) were incubated with 0.5 mM neurokinin B for various times at 37°C (final volume 100 μl). The reaction was stopped and the products analysed by HPLC as described for substance P [4]. Products were identified either by using marker peptides or by collection of the individual product peaks and amino acid analysis of the corresponding peptides [4].

3. RESULTS

3.1. *Hydrolysis of neurokinin B by pig kidney endopeptidase-24.11*

Neurokinin B was hydrolysed by endopeptidase-24.11 and 7 peptide products were identified (table 1). The identity of these peaks was confirmed by amino acid analysis and, in the case of Val-Gly, by the use of a marker peptide. The identified products could be attributed to hydrolysis at 4 sites:

↓ ↓ ↓ ↓

Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-MetNH_2

Table 1

Products released by incubation of neurokinin B with endopeptidase-24.11

Peak no.	Retention time (min)	Peptide identified	Fragment of neurokinin B
1	2.10	V-G	7 – 8
2	2.32	D-M-H-D	1 – 4
3	3.42	L-Ma	9 – 10
4	4.84	F-V-G	6 – 8
5	7.64	D-M-H-D-F	1 – 5
6	9.76	F-F	5 – 6
7	10.42	F-F-V-G	5 – 8
8	12.64	D-M-H-D-F-F-V-G	1 – 8
9	17.46	D-M-H-D-F-F-V-G-L-Ma	neurokinin B

Data are from incubations achieving >80% degradation of neurokinin B. The identity of peak 1 was confirmed by co-elution with the authentic peptide. The other products were confirmed by amino acid analysis

Table 2

The effect of inhibitors on the hydrolysis of neurokinin B by pig striatal synaptic membranes

Peak	Peptide	Effect of inhibitors on peak area (control (no inhibition) = 100 for each peak)					
		Phosphor-amidon (P)	Bestatin (B)	Captopril (C)	P+B	C+B	P+C+B
1	V-G	48	66	98	0	8	0
2	D-M-H-D	0	108	76	0	50	0
3	L-Ma	11	67	75	11	67	0
4	F-V-G	0	224	122	0	237	0
5	D-M-H-D-F	0	78	95	0	84	0
6	F-F	0	a	0	0	0	0
7	F-F-V-G	52	b	105	b	b	b
8	D-M-H-D-F-F-V-G	10	93	89	14	104	11

The inhibitors were added at the start of the incubation period. Concentrations: phosphoramidon, 1 μM; bestatin, 100 μM; captopril, 1 μM. In the absence of any inhibitors, approx. 50% of the substrate was hydrolysed under the conditions used. a, peak 6 was not detectable in the absence of any inhibitors due to rapid hydrolysis by endogenous aminopeptidases. It was only detectable when bestatin was included. b, peak 7 and bestatin elute with very similar retention times so an estimate of inhibition was not possible in these cases

The most rapidly hydrolysed site was the Gly^8–Leu^9 bond releasing Leu-$MetNH_2$, as is the case for substance P [4]. Hydrolysis of the Phe^6–Val^7 bond was a relatively minor cleavage.

3.2. *Neurokinin B is not hydrolysed by pig kidney angiotensin converting enzyme*

Angiotensin converting enzyme hydrolyses substance P with release of the C-terminal tripeptide Gly-Leu-$MetNH_2$ [7,10,11]. Neurokinin B (0.5 mM) was incubated with a purified preparation of angiotensin converting enzyme at 37°C for periods of time up to 18 h at 37°C. No Gly-Leu-$MetNH_2$ was formed under these conditions nor was any other product detectable. Furthermore, there was no significant loss of substrate during the incubation. Under identical conditions substance P was hydrolysed as reported [7,10,11] and this hydrolysis was prevented by the selective inhibitor captopril (1 μM).

3.3. *Hydrolysis of neurokinin B by synaptic-membrane preparations from pig striatum*

Neurokinin B was hydrolysed by synaptic-membrane preparations. In some experiments selective peptidase inhibitors were included in the incubation mixture. The results are summarised in table 2. The pattern of products formed was similar to that observed when neurokinin B was incubated with endopeptidase-24.11. Substantial inhibition of the formation of most products was observed when phosphoramidon (1 μM) was included. Virtually complete inhibition of neurokinin B hydrolysis was achieved when a combination of phosphoramidon (1 μM) and the aminopeptidase inhibitor bestatin (0.1 mM) was included. Captopril (1 μM) had little effect. Similar results were obtained with a crude synaptosomal/mitochondrial (P_2) preparation suggesting that certain peptidases had not been selectively removed during the purification of synaptic membranes.

4. DISCUSSION

4.1. *Specificity of endopeptidase-24.11 and angiotensin converting enzyme*

Four bonds in neurokinin B were hydrolysed by endopeptidase-24.11. All the sites of hydrolysis are consistent with the previously reported specificity of the enzyme [6]. In all cases hydrolysis occurred at bonds involving the amino groups of

hydrophobic residues. The major cleavage point involved the Gly^8-Leu^9 bond releasing Leu-$MetNH_2$. Hydrolysis at this site would destroy the biological activity of neurokinin B which resides in the C-terminal portion of the peptide.

The previous report by a number of groups [7,10,11] that substance P could be hydrolysed by angiotensin converting enzyme releasing a C-terminal tripeptide was somewhat unexpected. Substance P is amidated at the C-terminus and angiotensin converting enzyme has been regarded as a peptidyl dipeptidase releasing dipeptides from an unblocked C-terminus [12]. Our preparation of angiotensin converting enzyme could also hydrolyse substance P in agreement with the previous reports [7,10,11]. Despite the similarity in C-terminus between neurokinin B and substance P, no hydrolysis of the former peptide could be observed with angiotensin converting enzyme from pig kidney. We therefore conclude that neurokinin B is a substrate for endopeptidase-24.11 but not for angiotensin converting enzyme. The precise structural features that can allow angiotensin converting enzyme to function as an endopeptidase with certain substrates will require further evaluation with model peptides. Of the 2 well-characterised cell-surface peptidases it would appear that only endopeptidase-24.11 has a general capacity to hydrolyse and inactivate tachykinins.

4.2. *Hydrolysis of neurokinin B by pig striatal synaptic membranes*

Phosphoramidon is a specific inhibitor of endopeptidase-24.11 [13]. Our results show that substantial inhibition of neurokinin B hydrolysis by synaptic membranes or with a crude synaptosomal (P_2) membrane preparation is achieved with phosphoramidon alone. Captopril had little effect. A combination of phosphoramidon and bestatin completely blocked the hydrolysis of neurokinin B as is the case for the hydrolysis of cholecystokinin-8 by synaptic membranes [14]. Thus, a bestatin-sensitive peptidase may also play a role in neurokinin B hydrolysis, although the endopeptidase appears to be the predominant activity. Aminopeptidase N (EC 3.4.11.2) is present in synaptic membranes [15] and is sensitive to bestatin. However, a purified preparation of aminopeptidase N failed to hydrolyse neurokinin B (unpublished observations). Both cholecystokinin-8 and neurokinin B possess an N-terminal aspartyl residue and may therefore be susceptible to the action of aminopeptidase A (EC 3.4.11.7) which is sensitive to bestatin and shows a preference for acidic residues. However, the presence of this enzyme has yet to be confirmed in synaptic membrane preparations.

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