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The metabolism of neuropeptides

Endopeptidase-24.11 in human synaptic membrane preparations hydrolyses substance P

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Synaptic membrane preparations from human striatum and human diencephalon were shown to contain a phosphoramidon-sensitive metalloendopeptidase that appeared identical with endopeptidase-24.11. The activity of endopeptidase-24.11 was determined with an enzymic assay employing [D-Ala[2],Leu[5]]enkephalin as substrate, and its distribution in human brain was similar to that in pig brain, with the striatum containing the highest levels. The choroid plexus and pons also contained substantial activity. A good correlation ($r = 0.97$) was obtained for the distribution of the endopeptidase in pig brain and pituitary by the enzymic assay and by an immunoradiometric assay specific for pig endopeptidase-24.11. Synaptic membrane preparations from human striatum and diencephalon hydrolysed substance P at the same sites as did preparations of pig striatal synaptic membranes, and hydrolysis was substantially abolished by phosphoramidon. These results suggest that endopeptidase-24.11 is the principal enzyme hydrolysing substance P in human synaptic membrane preparations.

We have previously reported that substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) is hydrolysed by synaptic membrane preparations from pig striatum at three sites, Gln[6]-Phe[7], Phe[7]-Phe[8] and Gly[9]-Leu[10]. The same bonds were hydrolysed by purified pig kidney endopeptidase-24.11 (EC 3.4.24.11), indicating that the endopeptidase activity in the synaptic membrane preparation had the same specificity. The identity of the two activities was further supported by the demonstration that they were similarly inhibited by phosphoramidon and by a polyclonal antiserum raised to the pig kidney enzyme (Matsas *et al.*, 1983). By using a monoclonal antibody to kidney endopeptidase-24.11 (Gee *et al.*, 1983) as an immunoadsorbent, we have purified the enzyme from pig striatal membranes and shown it to be identical, save for small differences in subunit M_r and in the pattern of glycosylation (Relton *et al.*, 1983). We and others have shown that the activity referred to as 'enkephalinase' (Schwartz, 1983) is identical with endopeptidase-24.11 (Fulcher *et al.*, 1982; Matsas *et al.*, 1983; Relton *et al.*, 1983; Almenoff & Orlowski, 1984) and we have suggested that the enzyme may play a key role in the inactivation of enkephalins, substance P and other regulatory peptides (Matsas *et al.*, 1983, 1984*a,b*; Turner *et al.*, 1985). A previous report (Lee *et al.*, 1981) on the metabolism of substance P by an enzyme prepared from a particulate fraction of human diencephalon revealed an activity that was not consistent with the action of endopeptidase-24.11. The bonds hydrolysed were Gln[6]-Phe[7], Phe[7]-Phe[8] and Phe[8]-Gly[9] (but not Gly[9]-Leu[10]), and phosphoramidon had no effect on this human enzyme preparation. The present paper provides evidence that an activity resembling endopeptidase-24.11 is indeed present in human brain. Synaptic membrane preparations from human brain hydrolysed substance P at the same sites as did the comparable preparation from pig brain (Matsas *et al.*, 1983), and this activity was sensitive to inhibition by phosphoramidon. We have also used an i.r.m.a. to study the distribution of endopeptidase-24.11 in the pig central nervous system and have compared these results with a survey

Abbreviations used: i.r.m.a., immunoradiometric assay; IC_{50}, concentration of inhibitor causing 50% inhibition; Met-NH_2, methioninamide.

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in human brain membranes by using an enzymic assay.

Experimental

Materials

[D-Ala^2,Leu^5]Enkephalin and substance P were purchased from Cambridge Research Biochemicals (Harston, Cambridge, U.K.). Captopril (SQ 14225; D-3-mercapto-2-methylpropanoyl-L-proline) was a gift from Squibb Institute for Medical Research, Princeton, NJ, U.S.A. Post-mortem human brains were obtained from the Department of Pathology, Leeds General Infirmary, within 48 h of death. Pig brains were removed immediately after death from 2–4-week-old animals obtained from the University of Leeds Field Station. Other materials were from sources previously noted (Fulcher *et al.*, 1982; Matsas *et al.*, 1983, 1984*a,b*).

Methods

Preparation of synaptic membranes from the human brain. The striatum and diencephalon were removed by dissection. If not processed immediately for the preparation of synaptic membranes (Matsas *et al.*, 1983), the tissue was stored overnight at 4°C in 0.32M-sucrose/10mM-Tris/HCl, pH 7.4. A crude membrane fraction (P_2; Matsas *et al.*, 1983) was also used in some experiments; this was prepared from tissue stored at −70°C.

Enzymic assays. Samples of synaptic membranes (15–30 μg of protein) were incubated with 0.5 mM-substance P (3 h) or [D-Ala^2,Leu^5]enkephalin (1 h) at 37°C (final volume 100 μl). The reaction was stopped, and the products analysed by h.p.l.c. as described previously (Matsas *et al.*, 1983).

I.r.m.a. of samples of the central nervous system from piglets

This was performed on crude P_2 fractions using a monoclonal antibody (GK7C2) as described by Gee *et al.* (1985). GK7C2 was diluted 1:2 × 10^5 in these assays.

Results

Characterization of synaptosomes and synaptic membranes prepared from human brain

Occluded lactate dehydrogenase activity was determined by studying the latency revealed by assaying in the presence and absence of 0.1% (w/v) Triton X-100. This treatment increased the enzyme activity by a factor of 10, less than that observed for pig brain synaptosomes (20–30-fold increase), and this is probably attributable to the use of tissue obtained some hours after death and stored overnight in sucrose at 4°C. The specific activity determined for the hydrolysis of [D-Ala^2,Leu^5]enkephalin by human striatal synaptic membranes was 4 nmol·min^{-1}·mg^{-1}, a value that is comparable with that determined for hydrolysis by membranes prepared from pig striata (5 nmol·min^{-1}·mg^{-1}; Matsas *et al.*, 1983).

Hydrolysis of [D-Ala^2,Leu^5]enkephalin by synaptic membranes prepared from human diencephalon is phosphoramidon-sensitive

The titration curve for the inhibition of the hydrolysis of 1 mM-[D-Ala^2,Leu^5]enkephalin is shown in Fig. 1. The curve, which shows the mean results for three experiments on different batches of membranes, gave an IC_{50} of 6 ± 2 nM, similar to that reported (8 nM) for pig striatal membranes (Matsas *et al.*, 1983). These assays were performed in the presence of 1 μM-captopril in order to exclude any contribution from peptidyl dipeptidase A (angiotensin-converting enzyme) to the hydrolysis of the Gly^3-Phe^4 bond of the substrate. In the absence of captopril, 1 μM-phosphoramidon caused 95% inhibition.

Distribution of endopeptidase-24.11 activity in human brain

From the results shown in Fig. 1, [D-Ala^2,-Leu^5]enkephalin was judged to be a suitable substrate with which to survey the regional distribution of endopeptidase-24.11. These assays were conducted in the presence of 1 μM-captopril,

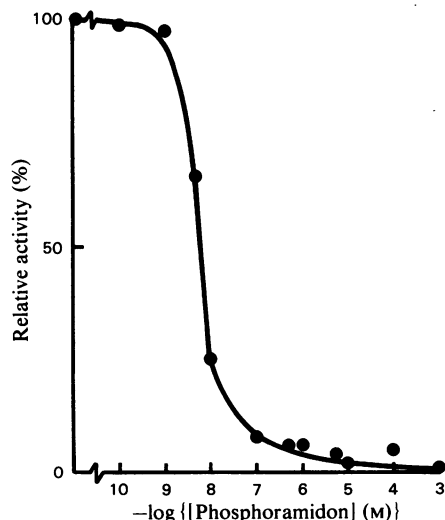

Fig. 1. *Phosphoramidon inhibition of the hydrolysis of* [D-*Ala*^{2},*Leu*^{5}]*enkephalin by a synaptic membrane preparation from human diencephalon*

See under 'Methods' for details. The substrate concentration was 1 mM and the membrane preparation contained 1.2 mg of protein/ml. Points on the curve are the means for three experiments.

and under these conditions all activity was fully inhibited by 1 μM-phosphoramidon. The results obtained from separate experiments on two human brains are shown in Table 1. For comparison, assays of similar regions in three pig brains are also shown. In general, the two surveys agree well. The caudate nucleus contained the highest activity in human brain, whereas in pig brain it was identical in activity with the globus pallidus. In both surveys, all regions of the cerebral cortex were low in activity, especially in the human. The pons was relatively rich in activity. Human choroid plexus gave a value not much lower than that given by the globus pallidus.

Comparison of i.r.m.a. with enzymic assay of regions of pig central nervous system

The monoclonal antibody, GK7C2, to pig endopeptidase-24.11 does not recognize the enzyme in other species (human, rat, rabbit; N. S. Gee & A. J. Kenny, unpublished work); hence an i.r.m.a. depending on GK7C2 (Gee *et al.*, 1985) could not be applied to human brain. However, comparable samples from the various regions of pig central nervous system (shown in Table 1) were also subjected to i.r.m.a. The correlation (Fig. 2) is good ($r = 0.97$), but the slope of the line of best-fit was slightly skewed towards the i.r.m.a. ($b = 0.7$).

Table 1. *Regional distribution of endopeptidase-24.11 in human brain and pig central nervous system*

See under 'Methods' for details. The substrate was 0.5 mM-[D-Ala[2],Leu[5]]enkephalin. The assays were performed on P_2 fractions, in the presence of 1 μM-captopril. In each region and in both species, the activity observed was fully inhibited by 1 μM-phosphoramidon. Shown are the values, normalized (100) for globus pallidus, obtained from assays on two human brains and the means (±S.E.M. where appropriate) from samples from three pigs, except for pituitary (two pigs) and lumbar spinal cord (one pig). The cord samples included attached nerve roots and ganglia. The specific activities of the pig and human globus pallidus membranes were 1.1 ± 0.3 and 0.5 nmol·min^{-1}·mg of protein^{-1} respectively.

Region Species ...	Distribution: Human	Pig
Globus pallidus	100, 100	100
Caudate nucleus	140, 136	97±6
Cerebral cortex		
Frontal	4, 3.5	15±2
Temporal	4, 3.5	16.9±0.3
Occipital	4, 5	16.2±0.6
Thalamus	10, 8.5	14±2
Pons	60, 56	33±1
Medulla	16, 19	26±3
Cerebellum	18, 19	24±1
Choroid plexus	84, 92	—
Spinal cord		
Cervical	—	106±17
Thoracic	—	147±8
Lumbar	—	158
Pituitary	—	192

Hydrolysis of substance P by synaptic membranes prepared from human striatum and diencephalon

Fig. 3 shows the h.p.l.c. analyses of the peptides released when substance P was incubated with human striatal synaptic membrane preparations. Apart from the substrate peak, seven products (peaks 1–7) were observed. The addition of bestatin enhanced peak 5, and the addition of phosphoramidon virtually abolished hydrolysis of substance P. The pattern of peptide products corresponds exactly with that observed with pig striatal synaptic membranes (Matsas *et al.*, 1983). The effect of bestatin was to reduce the attack on peak 5 by aminopeptidases present in the membranes. The identity of the peptides in peaks 1–7 was not determined, but the retention times were identical with those obtained in the same analytical system when substance P was incubated with purified kidney endopeptidase-24.11 (results not shown, but cf. Matsas *et al.*, 1983). When substance P was incubated with synaptic membrane preparations from human diencephalon (results not shown), peptides with the same

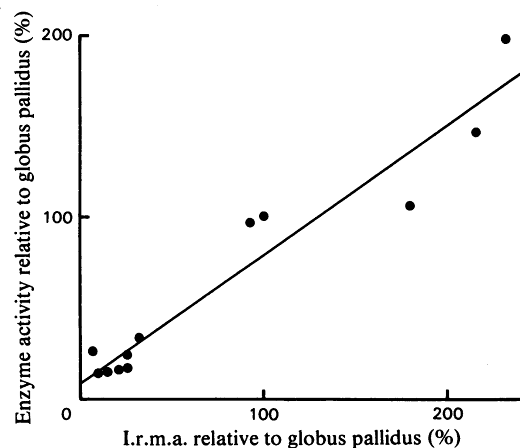

Fig. 2. *Correlation of enzymic assay with i.r.m.a. in various regions of pig central nervous system*

See under 'Methods' for details. [D-Ala[2],Leu[5]]Enkephalin (0.5 mM) was the substrate in the enzymic assay. The tissue samples are those listed in Table 1. Both assays have been normalized with respect to the values obtained for globus pallidus. The i.r.m.a. value for globus pallidus contained activity equivalent to 0.28% of a 10% homogenate of pig kidney cortex (Gee *et al.*, 1985).

Table 2. *Effect of inhibitors on the h.p.l.c. peak areas when substance P was incubated with synaptic membrane preparations from human striatum*
See under 'Methods' for details. Results are means for two experiments and show activities relative to the control (=100). Inhibitors were added at the start of the incubation (3h). Incubation mixtures contained 15 μg of membrane protein and 0.5 mM-substance P. Abbreviations used: the one-letter code is used for amino acids; Ma, methionineamide; PR, phosphoramidon; C, captopril; Dip-F, di-isopropyl fluorophosphate; '*' indicates that accurate assessment of the inhibition of product 7 was not possible by integration of peak area in these cases, but Fig. 3 reveals substantial inhibition.

		Relative activity							
Peak no.	Peptide content / Additions ...	None	PR (1 μM)	Bestatin (0.1 mM)	C (1 μM)	PR+C (each 1 μM)	1,10-Phenanthroline (0.1 mM)	EDTA (0.1 mM)	Dip-F (0.1 mM)
1	RPKPQQ (1–6)	100	14	78	87	12	0	14	100
2	LMa (10–11)+F	100	15	48	75	5	0	23	103
3	FG (8–9)	100	4	68	90	0	0	50	108
4	RPKPQQF (1–7)	100	12	84	81	9	20	0	100
5	FFG (7–9)	100	0	288	101	0	0	0	99
6	RPKPQQFFG (1–9)	100	17	96	111	24	0	0	117
7	FFGLMa (7–11)	100	*	89	81	*	0	0	117

Table 3. *Effect of inhibitors on the h.p.l.c. peak areas when substance P was incubated with synaptic membrane preparations from human diencephalon*
See under 'Methods' for details. Results are means for two experiments and show activities relative to the control (=100). Inhibitors were added at the start of incubation (3h). The incubation mixtures contained 24 μg of membrane protein and 0.5 mM-substance P. For abbreviations, see Table 2. '*' indicates that accurate assessment of the inhibition of product 7 was not possible by integration of peak areas.

		Relative activity						
Peak no.	Peptide content / Additions ...	None	PR (1 μM)	Bestatin (0.1 mM)	C (1 μM)	PR+C (each 1 μM)	1,10-Phenanthroline (0.1 mM)	Dip-F (0.1 mM)
1	RPKPQQ (1–6)	100	23	93	100	19	30	100
2	LMa (10–11)+F	100	26	60	83	9	10	103
3	FG (8–9)	100	8	73	91	3	0	104
4	RPKPQQF (1–7)	100	17	98	82	10	0	100
5	FFG (7–9)	100	0	397	135	0	0	101
6	RPKPQQFFG (1–9)	100	21	111	118	12	0	102
7	FFGLMa (7–11)	100	*	156	110	*	0	96

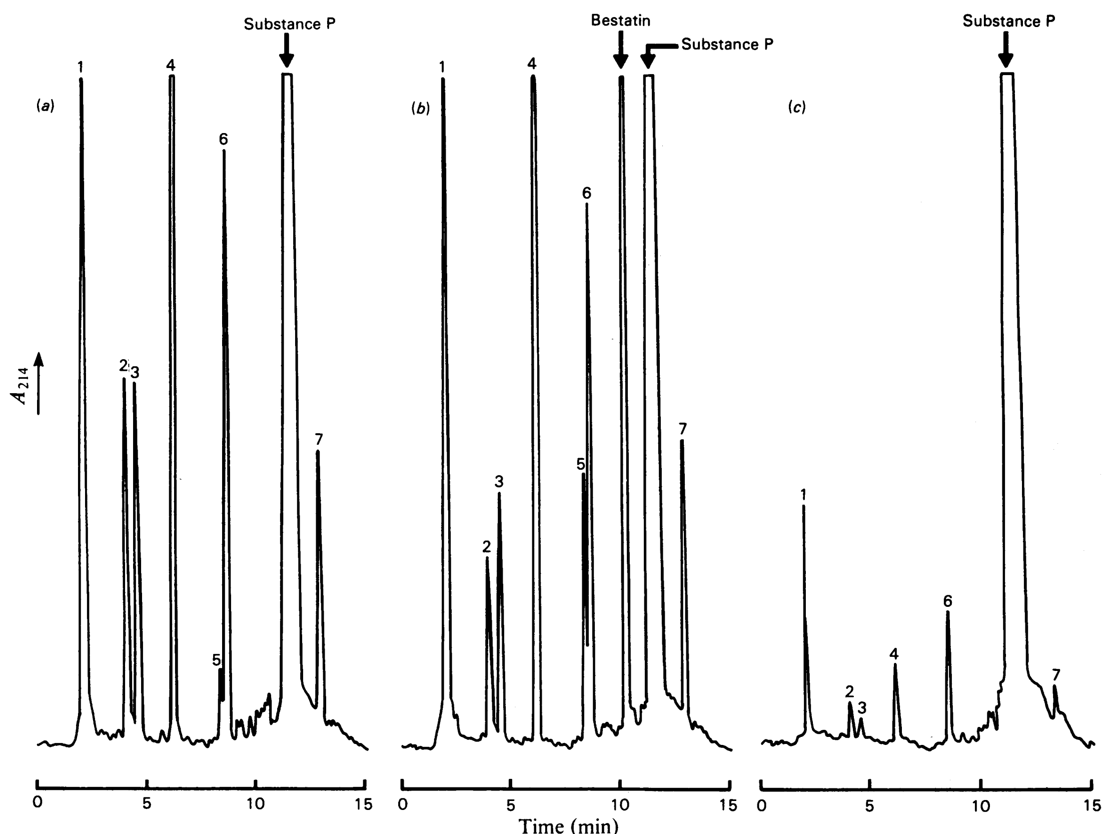

Fig. 3. *Hydrolysis of substance P by human striatal synaptic membrane preparations*
See under 'Methods' for details. The products of hydrolysis were resolved by h.p.l.c. and designated by the same numbers as those given in Tables 2 and 3. (*a*) Incubation without additions; (*b*) 0.1 mM-bestatin; (*c*) 1 μM-phosphoramidon.

retention times and reflecting enzymic responses to bestatin and phosphoramidon similar to those shown in Fig. 3 were found.

The extent to which different peptidase activities in the synaptic-membrane preparations contributed to the formation of the products observed was investigated by repeating the experiments in the presence of other inhibitors. One of these, captopril, is an inhibitor of peptidyl dipeptidase A (angiotensin-converting enzyme); it was included because this membrane enzyme can also hydrolyse substance P (Yokosawa *et al.*, 1983; Cascieri *et al.*, 1984; Skidgel *et al.*, 1984). Membranes prepared from human striatum (Table 2) and human diencephalon (Table 3) were investigated. In general, the membrane preparations from the two regions showed the same responses to the inhibitors. Bestatin enhanced peak 5 (Phe-Phe-Gly) by preventing its conversion into peak 3 (Phe-Gly) and to phenylalanine, which is eluted with peak 2. It had only small effects on the other peaks. Phosphoramidon caused strong inhibition affecting all peaks, slightly more potent in the striatal membranes. Captopril caused some inhibition, in the range 0–25%. However, the combination of captopril and phosphoramidon did not completely inhibit all activity. Under these conditions (0.5 mM-substance P, 3 h incubation) peptidyl dipeptidase A did not appear to play a major role in hydrolysing substance P. 1,10-Phenanthroline at a concentration of 0.1 mM inhibited more strongly than phosphoramidon, suggesting that the residual activity seen in the presence of the latter was the result of other metallopeptidases. Di-isopropyl fluorophosphate had no effect.

Discussion

The main conclusion from these studies is that synaptic membrane preparations from the human striatum and diencephalon contain a phosphoramidon-sensitive metalloendopeptidase that appears identical with endopeptidase-24.11. Thus both the IC_{50} value for phosphoramidon inhibition

of the hydrolysis of [D-Ala^2,Leu^5]enkephalin and the peptide products formed from substance P were very similar to those obtained with the pure enzyme. In human brain, as in the pig, striatum contained the highest activity of endopeptidase-24.11, but the choroid plexus and pons also contained substantial activity. Human spinal cord was not available, but in the pig, samples (which included attached nerve roots) of all regions of the cord, as well as the pituitary, contained higher activities than did brain. The availability of a specific i.r.m.a. for pig endopeptidase-24.11 enabled the same samples from the central nervous system and pituitary to be checked by this independent method and, in general, there was a good correlation between the two assays.

Several enzymes appear as candidates for inactivating substance P. The findings of Lee *et al.* (1981) suggest that their 'substance P-degrading enzyme' purified from human diencephalon was distinct from both endopeptidase-24.11 and peptidyl dipeptidase A on the basis of its specificity and its inhibitor-sensitivity. It is unclear, though, whether that activity represented a homogeneous enzyme preparation and whether it was located in the plasma membrane. A similar activity has been reported to be mitochondrial in location in rat brain (Horsthemke *et al.*, 1984). In our survey of human diencephalon and striatum, endopeptidase-24.11 appeared to be the principal membrane enzyme hydrolysing substance P and [D-Ala^2,-Leu^5]enkephalin. Furthermore, among a range of natural peptide substrates, substance P was the most efficiently hydrolysed by endopeptidase-24.11 (Matsas *et al.*, 1984*a*).

Although peptidyl dipeptidase A can hydrolyse substance P, the reported $k_{cat.}/K_m$ for the reaction at 37°C ($3.3 \times 10^3\,\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$; Cascieri *et al.*, 1984) is 800-fold lower than that for endopeptidase-24.11 ($2.6 \times 10^6\,\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$; Matsas *et al.*, 1984*a*). Thus only in those brain regions or tissues where the concentration of peptidyl dipeptidase A is in considerable excess over the endopeptidase is the former enzyme likely to participate significantly in substance P catabolism. The potentiation of certain of the peripheral actions of substance P by inhibitors of peptidyl dipeptidase A (Cascieri *et al.*, 1984) might be the result of inhibition at sites, such as lung endothelium, where the enzyme is especially abundant. In membranes prepared from human striatum and diencephalon, the major contributor to substance P hydrolysis was endopeptidase-24.11 although a minor component of activity attributable to peptidyl dipeptidase A cannot be excluded. All substance P-hydrolysing activity was inhibitable by 1,10-phenanthroline and none could be attributed to serine peptidases such as dipeptidyl peptidase IV.

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