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Substance P and [Leu]enkephalin are hydrolyzed by an enzyme in pig caudate synaptic membranes that is identical with the endopeptidase of kidney microvilli

(neuropeptide degradation/peptidase/phosphoramidon/high-performance liquid chromatography)

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ABSTRACT The hydrolysis of [Leu]enkephalin and substance P by purified pig kidney endopeptidase (EC 3.4.24.11) and synaptic membranes prepared from pig caudate nuclei has been compared. The hydrolysis of an enkephalin analogue (Tyr-D-Ala-Gly-Phe-Leu) at the Gly-Phe bond was completely inhibited by phosphoramidon. The IC_{50} concentration (8 nM) was similar to that reported for [Leu]enkephalin hydrolysis by the purified endopeptidase [Fulcher, I. S., Matsas, R., Turner, A. J. & Kenny, A. J. (1982) *Biochem. J.* 203, 519–522]. Seven peptides were produced when substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) was hydrolyzed by the kidney endopeptidase. These were formed by cleavage at bonds Gln-Phe (positions 6 and 7), Phe-Phe (positions 7 and 8), and Gly-Leu (positions 9 and 10). Synaptic membranes generated peptides with the same HPLC retention times and hydrolysis of substance P by either preparation was inhibited completely by 10 μM phosphoramidon. The most susceptible bond appeared to be Gly-Leu (positions 9 and 10). A specific polyclonal antibody raised in rabbits to purified pig endopeptidase inhibited the hydrolysis of [Leu]enkephalin and substance P by detergent-solubilized kidney microvilli or synaptic membranes; the titration curves were essentially identical. We conclude that the endopeptidase, which we suggest should be designated "endopeptidase-24.11," is present in caudate synaptic membranes and could play an important role in the hydrolysis of neuropeptides.

When the inactivation of biologically active peptides has been reported it has become commonplace to name the peptidase responsible in a way that implies the existence of peptide-specific enzymes. Although peptidases of high specificity do exist, such a close relationship between peptidase and substrate is unusual. The field of neuropeptide metabolism in brain has, in particular, been plagued by this restrictive nomenclature [e.g., enkephalinase and substance P-degrading enzyme (1–4)]—a limitation that has consequently hindered comparisons with well-characterized peptidases from other tissues. It is our contention (5) that a limited number of peptidases mediate a wide range of functions and that their cellular and subcellular localization, rather than peptide specificity, defines their roles at different sites.

Substance P and the enkephalins are believed to function as neurotransmitters in the central nervous system. Their postsynaptic actions are thought to be terminated by metabolism rather than by a transport mechanism (6, 7). Although cytosolic and lysosomal peptidases capable of hydrolyzing substance P have been reported (8, 9), enzymes localized in the synaptic membrane may have more relevance to the physiological inactivation of the neuropeptide. The amino acid sequence of substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) suggests that it would be susceptible to attack by two well-characterized membrane peptidases. The specificity of dipeptidyl peptidase IV (EC 3.4.14.5) (10, 11) suggests that it is capable of cleaving sequentially the Pro-Lys and Pro-Gln bonds. Neutral endopeptidase (EC 3.4.24.11) might be expected to hydrolyze bonds involving the amino groups of the four hydrophobic residues that substance P contains (12). Both of these enzymes have been purified in this laboratory from pig kidneys and characterized in some detail. Dipeptidyl peptidase IV is a serine peptidase, strongly inhibited by diisopropylphosphorofluoridate (13). The endopeptidase is a Zn^{2+} metalloenzyme, inhibited ($IC_{50} \approx 10$ nM) by phosphoramidon [*N*-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan] (14) and thiorphan [*N*-(DL-2-benzyl-3-mercapto-propionyl)-glycine] (5).

We have previously shown (5) that the hydrolysis of the Gly-Phe bond (positions 3 and 4) of [Leu]enkephalin (Tyr-Gly-Gly-Phe-Leu) by the enzyme in brain termed "enkephalinase" is attributable to this endopeptidase. This attribution was based on the similar response of synaptic membranes and the kidney enzyme to several inhibitors. In this paper we show that substance P is hydrolyzed by synaptic membranes prepared from caudate nucleus from pig brain in an identical manner to that achieved by the purified kidney endopeptidase. Furthermore, the hydrolysis of this peptide and that of enkephalin is inhibited by a monospecific antiserum raised to the pig kidney endopeptidase.

The name recommended by the Enzyme Commission for the endopeptidase is "kidney brush border neutral proteinase." This was appropriate while kidney was the only known source. We now know that it is in intestinal brush borders (15) and in the membrane fractions from various tissues, including lung, spleen, aorta, heart (16), pituitary (17), pancreas (18), and spermatozoa (19). It was originally called "neutral endopeptidase" to distinguish it from an acidic lysosomal peptidase, but because other neutral endopeptidases are present in some microvillar membranes (16), an unambiguous name is essential to avoid confusion in the literature. We propose that the phosphoramidon-sensitive endopeptidase should be designated "endopeptidase 24.11" from the last numerals of its EC number.

MATERIALS AND METHODS

Chemicals. Substance P and enkephalin analogues were obtained from Cambridge Research Biochemicals (Cambridge, United Kingdom). Bestatin was obtained from Sigma (London). Other materials, including radiolabeled enkephalin, were from sources previously noted (5).

Preparation of Membrane Fractions from Pig Kidney and Brain. Kidney microvillar membranes were prepared as de-

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scribed (20). Synaptic membranes were prepared from caudate nuclei (15–30 g) as previously described for cortex (5). A crude membrane fraction was also studied in some experiments. A 10% homogenate was prepared from 4 g of caudate nuclei in 50 mM Tris·HCl buffer, pH 7.4. This was centrifuged at 1,000 × g for 3 min and then centrifuged at 200,000 × g for 20 min. The latter pellet was resuspended and recentrifuged and the fraction was designated P_2.

Purified Kidney Endopeptidase. This was prepared by immunoadsorbent chromatography after solubilizing microvillar membranes with Triton X-100 (21). The specific activity in the standard assay with 5.7 μM [^{125}I]iodoinsulin B chain as substrate was 177 nmol·min^{-1}·mg^{-1} of protein. The V_{max} with glutaryl-Gly-Gly-Phe-2-naphthylamide as substrate was 484 nmol·min^{-1}·mg^{-1} of protein. It contained no detectable activity, by fluorescent assays, of aminopeptidase N (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), dipeptidyl peptidase IV (EC 3.4.14.5), or γ-glutamyl transferase (EC 3.2.2.2). Moreover, it was homogeneous on gel electrophoresis and in crossed immunoelectrophoresis against an antiserum raised to microvillar membranes (21).

Preparation of Antiserum to Kidney Endopeptidase. Antiserum was raised in a rabbit. It proved necessary to absorb traces of antibodies to other microvillar proteins. After this step, the antisera from two bleedings were shown to be monospecific by crossed immunoelectrophoresis (21).

Hydrolysis of Enkephalin. The hydrolysis of 20 nM [*tyrosyl*-3,5-^{3}H][Leu^5]enkephalin by synaptic membranes or kidney microvillar membranes was determined (5). Puromycin (0.1 mM) and 4-chloromercuribenzoate (1 mM), or bestatin (0.1 mM), were added to inhibit aminopeptidase activity in synaptic membranes. The products were resolved by TLC and the radioactivity present in the zone corresponding to Tyr-Gly-Gly was determined (5).

Hydrolysis of Substance P. Samples of synaptic membranes (30 μg of protein, 1-hr incubation) or kidney endopeptidase (30 ng, 15-min incubation) in 0.1 M Tris·HCl at pH 7.4 were incubated with substance P (0.5 mM) at 37°C in a total volume of 100 μl. The reaction was stopped by heating to 100°C for 4 min and protein was removed by centrifugation. A sample (60 μl) of the supernatant was then analyzed by HPLC to assess product formation.

Hydrolysis of [D-Ala^2,Leu^5]Enkephalin. The incubation conditions were those described above for substance P but using 1 mM substrate and terminating the reaction by the addition of 30 μl of 30% (vol/vol) acetic acid followed by centrifugation. Bestatin (0.1 mM) was included to inhibit aminopeptidase activity in synaptic membranes.

Peptide Separation by HPLC. The apparatus consisted of a μBondapak C_{18} column, 6000A and M-45 pumps, 660 gradient former, and a fixed wavelength (214 nm), model 441 UV detector (Waters Associates, Northwich, Cheshire, United Kingdom). Products of substance P hydrolysis were resolved with a 20-min linear gradient of acetonitrile (4.8–60%) in 0.08% H_3PO_4 at pH 2.5, followed by elution for 5 min with 60% acetonitrile/0.08% H_3PO_4, pH 2.5. The column was eluted at 2 ml/min. Under these conditions, substance P exhibited a retention time of 12 min. For [D-Ala^2,Leu^5]enkephalin, the conditions were modified: flow rate, 1.5 ml/min; 15-min gradient of 4.5–30% acetonitrile in 0.08% H_3PO_4 at pH 2.5, followed by 8-min elution at the final conditions. The enkephalin analogue was eluted at 16 min.

Amino Acid Analyses. When individual peptides were required for amino acid analysis, the fractions eluting from the HPLC column were collected in a Pharmacia Frac-100 fraction collector equipped with a peak sensor. After hydrolysis of peptides in 6 M HCl, amino acid analysis was performed in a Rank-Hilger Chromaspek J180 analyzer.

Immunotitration Procedure. Synaptic and kidney microvillar membranes were solubilized by Triton X-100 [detergent/protein, 5:1 (wt/wt)]. After centrifugation at 30,000 × g for 15 min, the supernatant fraction was incubated at 4°C for 18 hr with an IgG fraction from an antiserum raised to kidney endopeptidase. The hydrolysis of substance P and tritiated [Leu]-enkephalin was determined as above. In the latter case, puromycin (0.1 mM) and 4-chloromercuribenzoate (1 mM) were included to inhibit the action of aminopeptidases on enkephalin. Identical experiments employing equivalent amounts of preimmune IgG were carried out in parallel.

RESULTS

Enzyme Activities in Synaptic Membranes. The yield of synaptic membranes prepared from caudate nuclei was 200 μg/g of wet weight. When assayed for endopeptidase-24.11, by using 5.7 μM [^{125}I]iodoinsulin B chain as substrate, the specific activity was 0.014 nmol·min^{-1}·mg^{-1}, a value nearly 1/1,000th of that for kidney microvilli. This value refers to the phosphoramidon-sensitive activity. Synaptic membrane endopeptidase-24.11, like that in kidney microvilli (14), was not released from the membrane by papain treatment.

Cerebral cortex was an inferior source of endopeptidase. When synaptic membranes were assayed with 1 mM [D-Ala^2,Leu^5]enkephalin, the mean specific activities were: caudate nucleus, 5.0 ± 1.5 nmol·min^{-1}·mg^{-1} (±SEM; n = 3); cortex, 0.52 nmol·min^{-1}·mg^{-1}. By using a fluorimetric assay for dipeptidyl peptidase IV (21) in caudate membranes the hydrolysis of Gly-Pro-4-methyl-7-coumarylamide was <0.1 nmol·min^{-1}·mg^{-1}. The specific activity of lactate dehydrogenase (EC 1.1.1.27) was 0.038 μmol·min^{-1}·mg^{-1} of protein (specific activity = 0.07 relative to the homogenate).

Hydrolysis of [D-Ala^2,Leu^5]Enkephalin by Caudate Synaptic Membranes. This substrate has the advantage that it is resistant to attack by aminopeptidases (22). The only products resolved by HPLC were identified as Tyr-D-Ala-Gly and Phe-Leu when the incubation mixture contained 0.1 mM bestatin. In the absence of bestatin, the tripeptide peak was unchanged, but Phe-Leu was further hydrolyzed and only phenylalanine was detected by the monitor. This secondary attack is likely to be due to aminopeptidase activity. The hydrolysis of the Gly-Phe bond (positions 3 and 4) was inhibited by phosphoramidon, and the inhibition curve is shown in Fig. 1. The activity was completely inhibited; the IC_{50} value was 8 nM, comparable with the value reported previously for the hydrolysis of [Leu]enkephalin by kidney endopeptidase (5).

The hydrolysis of this substrate by the cruder P_2 fraction was also inhibited by phosphoramidon with a similar sensitivity and almost to the same extent. A comparable result was also achieved with the P_2 fraction by using the radiochemical assay (Fig. 1).

Hydrolysis of Substance P. The purified kidney endopeptidase converted substance P to at least seven resolvable products (Fig. 2*A*). No aminopeptidase activity had been found in our preparation (21) and the presence of 0.1 mM bestatin in the incubation mixture did not affect the formation of the products (Fig. 2*C*). When substance P was incubated with caudate synaptic membranes in the presence of 0.1 mM bestatin, seven peptides were produced with retention times identical to those observed with the kidney endopeptidase (Fig. 2*D*). When bestatin was omitted, peptide 5 was not detected (Fig. 2*B*), an effect that we attribute to the activity of one or more aminopeptidases in the synaptic membranes. Phosphoramidon (10 μM) totally abolished the hydrolysis of substance P by kidney en-

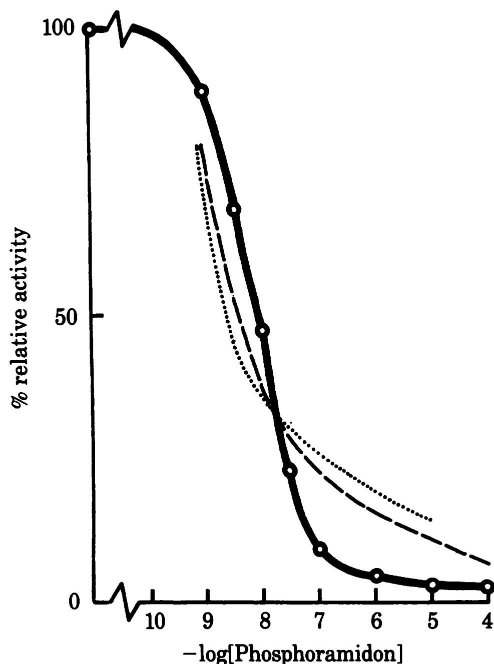

FIG. 1. Inhibition of [Leu]enkephalin hydrolysis by caudate membranes. ○, Synaptic membranes with substrate [D-Ala^2,Leu^5]enkephalin; ——, P_2 fraction with same substrate; ······, P_2 fraction with substrate [*tyrosyl*-3,5-^{3}H][Leu^5]enkephalin.

dopeptidase (Fig. 2*E*) and synaptic membranes (Fig. 2*F*), indicating that the active site of the synaptic membrane enzyme is probably identical to that of the kidney endopeptidase. It also shows that the membrane exopeptidases could not initiate hydrolysis of this substrate.

In two experiments, substance P was incubated with a crude membrane fraction (P_2) prepared from caudate. Phosphoramidon (10 μM) inhibited only 38% of the degradation (estimated by decrease in substance P concentration). Furthermore, at least three additional peptides were formed in these conditions. This implies that peptidases insensitive to phosphoramidon are present in other subcellular compartments. Although the preparation of purified membranes was more time-consuming and yielded less material than the crude P_2 fraction, it is essential to use the purer preparation if the hydrolysis of substance P is to be mediated only by endopeptidase-24.11. This limitation does not apply to experiments with [D-Ala^2,Leu^5]enkephalin, for which endopeptidase-24.11 is the only enzyme hydrolyzing the substrate, even in the P_2 fraction.

Some other inhibitors were tested. Captopril (1 μM) and diisopropylphosphorofluoridate (1 mM) had no effect. 1,10-Phenanthroline (1 mM) caused 95% inhibition; 1 mM EDTA, 27% inhibition; and 1 mM puromycin, 36% inhibition of substance P hydrolysis.

The peptide products formed by the action of the kidney endopeptidase were identified by collecting the fractions corresponding to peaks 1–7 and subjecting them to acid hydrolysis and amino acid analysis. The results are shown in Table 1 and Fig. 3. The analyses are consistent with the hydrolysis at three bonds, Gln-Phe (positions 6 and 7), Phe-Phe (positions 7 and 8), and Gly-Leu (positions 9 and 10). All fit well with the known specificity of endopeptidase-24.11. The possibility of hydrolysis at Leu-Met-NH_2 (positions 10 and 11) was also considered. Only those products absorbing at 214 nm were detected by the monitor, and methionineamide (retention time, 1.86 min) absorbs very weakly. No peak was seen at this time; though it is possible that it was not resolved from peptide 1 (2.18 min), no

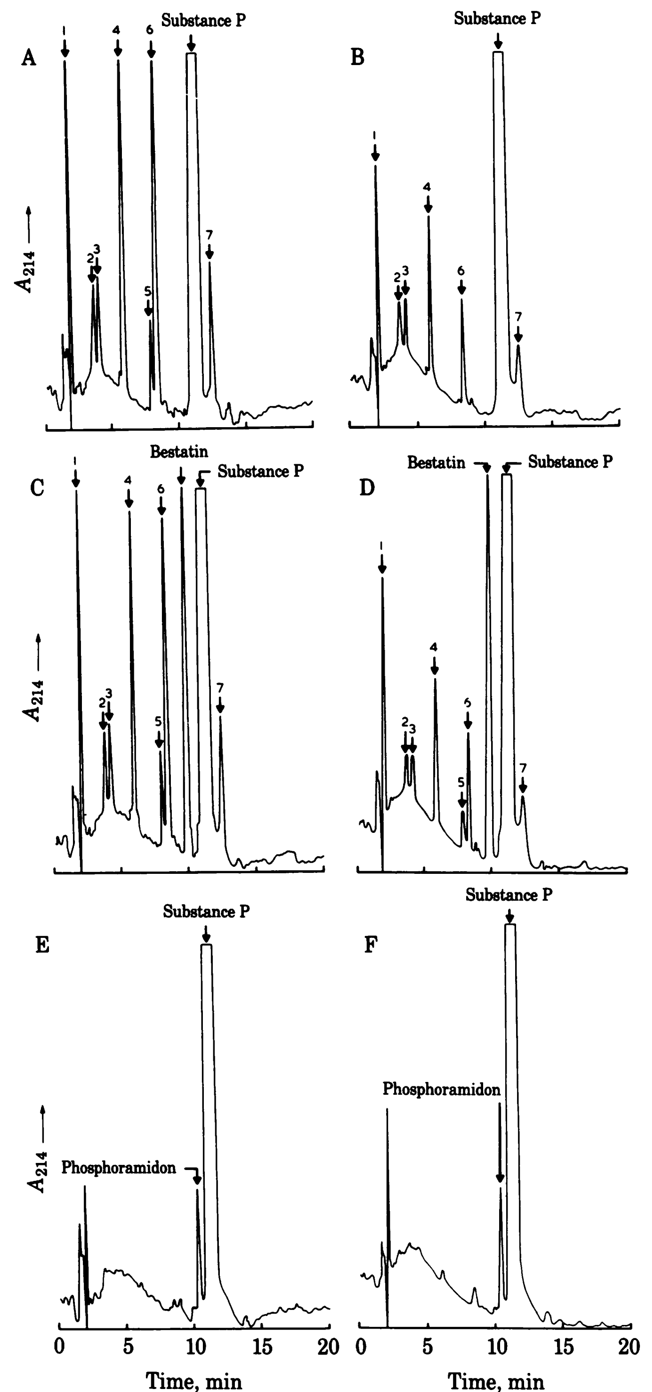

FIG. 2. Hydrolysis of substance P by caudate synaptic membranes and kidney endopeptidase-24.11. The peptide products of substance P hydrolysis were resolved by HPLC. The peptides are numbered as in Table 1. *A*, *C*, and *E*, Kidney endopeptidase; *B*, *D*, and *F*, synaptic membranes; *A* and *B*, no inhibitors; *C* and *D*, 0.1 mM bestatin; and *E* and *F*, 10 μM phosphoramidon. The uninhibited hydrolysis values were 20% for kidney endopeptidase and 9.5% for synaptic membranes.

methionine was detected in the analysis of this peptide.

The progress of substance P hydrolysis by kidney endopeptidase was followed over a period of 5 hr. Peptides 1–5 increased linearly. Peptides 6 and 7 reached plateau values after 1 hr, suggesting further degradation. Peptide 2 (Leu-Met-NH_2) was generated twice as fast as the next (peptide 4), indicating rapid attack at the bond at position 9–10.

Immunotitration of Endopeptidase Activity from Caudate Synaptic Membranes and Kidney Microvillar Membranes. Membrane fractions, solubilized with Triton X-100, were ti-

Table 1. Identification of products of substance P hydrolysis by kidney endopeptidase-24.11

Peak number	Arg R	Pro P	Lys K	Gln Q	Phe F	Gly G	Leu L	Met-NH_2 M-NH_2	Yield, %	Peptide sequence identified*
1	1.3	1.6	1.0	1.8	—	—	—	—	11.5	RPKPQQ (1–6)
2	—	—	—	—	—	—	1.2	1.0	35.5	LM-NH_2 (10–11)
3	—	—	—	—	1.0	1.1	—	—	14.5	FG (8–9)
4	1.1	2.1	1.1	2.2	1.1	—	—	—	18.2	RPKPQQF (1–7)
5	—	—	—	—	2.0	1.0	—	—	15.5	FFG (7–9)
6	1.0	2.0	1.2	2.3	2.0	1.0	—	—	1.4	RPKPQQFFG (1–9)
7	—	—	—	—	1.7	1.0	0.8	0.3	4.2	FFGLM-NH_2 (7–11)
Substance P	1.0	2.4	1.1	2.2	2.4	1.2	1.1	1.0	—	RPKPQQFFGLM-NH_2 (1–11)

The peaks are numbered as in Fig. 2. The data (expressed as mol of amino acid/mol of peptide) are from two experiments achieving 56% and 76% hydrolysis of the substrate. The yields have been normalized to 100% hydrolysis. Peaks 6 and 7 were only quantified in the second experiment because the amounts were too small in the first. The sum of the normalized yields was 100.8%.

* Positions are shown in parentheses.

trated with antisera raised to kidney endopeptidase. The hydrolysis of [Leu]enkephalin and substance P was inhibited by these antibodies (Fig. 4). Although the inhibition was incomplete in the range studied, the titration curves for each substrate were very similar for the two membrane preparations. Preimmune serum was not inhibitory. Aminopeptidase activity on enkephalin was not affected by the immune serum. The antisera used in these experiments were from different bleedings of the same immunized rabbit, hence the slight difference in titer in the two titrations. Thermolysin, an enzyme possessing a similar catalytic mechanism, was not inhibited by these antibodies over the same titration range (data not shown).

DISCUSSION

Synaptic Membrane Endopeptidase. Our results indicate that endopeptidase-24.11 is present in synaptic membranes prepared from the caudate nucleus of pig brain. The evidence for this statement is as follows. (*i*) Phosphoramidon inhibits the hydrolysis of the Gly-Phe bond (positions 3 and 4) of enkephalin with an IC_{50} value (8 nM) similar to that reported for the hydrolysis of this substrate and [^{125}I]iodoinsulin B chain by the purified kidney enzyme (5). (*ii*) The hydrolysis of substance P by synaptic membranes and kidney endopeptidase was completely inhibited by 10 μM phosphoramidon. (*iii*) The effect of chelating agents on substance P hydrolysis by synaptic and microvillar membranes was similar for the two tissues. (*iv*) The bonds hydrolyzed in substance P and enkephalin are those involving the amino groups of hydrophobic amino acid residues, a specificity previously established for the enzyme purified from rabbit kidneys (12). (*v*) The pattern of peptides resolved by HPLC after hydrolysis of substance P by synaptic membranes was identical to that observed for the purified endopeptidase (provided aminopeptidase activity was inhibited). (*vi*) A specific polyclonal antiserum raised to the kidney endopeptidase inhibited the hydrolysis of both enkephalin and substance P when either synaptic or kidney microvillar membranes were titrated with the antiserum and the titration curves were almost superimposable.

Points *i*–*v* establish that our synaptic membranes contain a peptidase that appears to be identical with the enzyme classified as EC 3.4.24.11. The antibody experiments show that the proteins in the two membranes have some similar antigenic determinants. The brain endopeptidase also has topological features in common with the kidney enzyme, in that the active site is exposed at the external surface and that the protein cannot be released from the membranes by papain treatment. However, confirmation that the two enzymes are identical in all structural details must await studies on the isolated endopeptidase from brain.

Neuropeptide Metabolism. In synaptic membranes from caudate nucleus endopeptidase-24.11 was the only significant

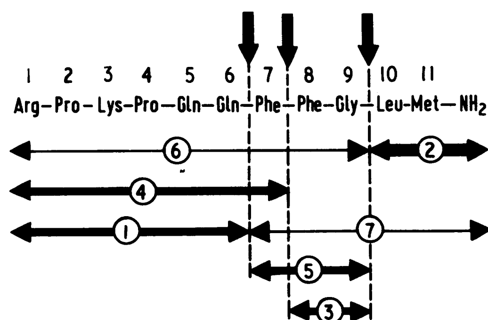

FIG. 3. Peptides identified after hydrolysis of substance P by kidney endopeptidase-24.11. The peptides are numbered as in Table 1 and Fig. 2. The thickness of the horizontal lines relates to the yield of each peptide in experiments in which 56–76% hydrolysis of substrate had been achieved. With only 20% hydrolysis, the yield of peptides 6 and 7 had reached their maxima. Peptides 1–5 continued to increase to at least 76% hydrolysis.

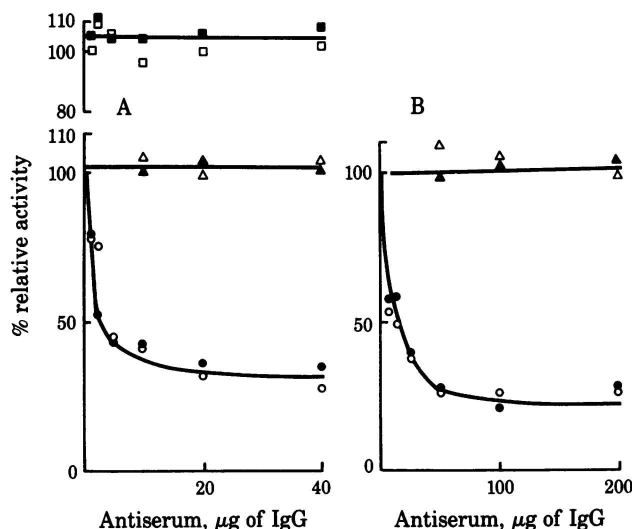

FIG. 4. Inhibition of endopeptidase activity in caudate synaptic membranes and kidney microvilli by antisera raised to kidney endopeptidase. (*A*) Hydrolysis of [*tyrosyl*-3,5-^{3}H][Leu^5]enkephalin; (*B*) hydrolysis of substance P; open symbols, kidney microvilli (0.5 μg of protein); closed symbols, synaptic membranes (200 μg of protein) (both preparations were solubilized by Triton X-100). ● and ○, Immune serum; ▲ and △, preimmune serum; ■ and □, immune serum, aminopeptidase activity determined by release of [^{3}H]tyrosine from [Leu]enkephalin.

enzyme in hydrolyzing substance P. Phosphoramidon completely protected the peptide from attack. No hydrolysis by dipeptidyl peptidase IV, releasing Arg-Pro (positions 1 and 2) and Lys-Pro (positions 3 and 4), occurred. Peptides produced by attack at bonds 2–3 and 4–5 were not detected and diisopropylphosphorofluoridate had no significant effect on substance P hydrolysis. Aminopeptidase attack was probably implicated in further degradation of some of the endopeptidase-produced peptides—e.g., peptides 3 (Phe-Gly) and 5 (Phe-Phe-Gly)—but this enzyme was impotent in initiating the attack on substance P. However, the natural enkephalins are very susceptible to aminopeptidase attack at the Tyr-Gly bond (positions 1 and 2). The analogue [D-Ala^2,Leu^5]enkephalin resists such attack and was hydrolyzed only at the Gly-Phe bond (positions 3 and 4) and this attack was wholly attributable to endopeptidase-24.11.

The metabolism of substance P by an enzyme purified from human diencephalon has been described (4). Although this enzyme was a metallo-endopeptidase it was insensitive to phosphoramidon. In addition, the mode of attack was different—only primary products were identified, indicating that only intact substance P was a substrate. Although hydrolysis at bonds 6–7 and 7–8 occurred, no attack at the bond at position 9–10 was observed; moreover hydrolysis at Phe-Gly (positions 8 and 9) indicates an enzyme of unusual specificity and is incompatible with the action of endopeptidase-24.11. These discrepancies might arise from species or regional variations.

The Role of Endopeptidase-24.11. The very wide distribution (16) in membranes of an enzyme with catalytic and immunological similarities to the microvillar endopeptidase-24.11 deserves some comment. The specificity of the endopeptidase is rather broad and relates to bonds -X-Y-, in which Y is any of seven hydrophobic amino acid residues (12). It is not peptide-specific and the best substrates appear to be peptides of small and intermediate size; more structured proteins seem to resist attack. Many biologically active peptides are protected from exopeptidase attack—e.g., 5-oxoproline is the NH_2-terminal residue of neurotensin, caerulin, bombesin, gastrin, and others, whereas α-melanocyte-stimulating hormone and endorphins are *N*-acetylated. The COOH-terminal α-carboxyl group is amidated in many cases and several peptides, such as gastrin, luteinizing hormone-releasing hormone, and α-melanocyte-stimulating hormone, are protected at both termini. These considerations emphasize the potential role of an endopeptidase with broad specificity. Therefore, we suggest that endopeptidase-24.11, located in plasma membranes and facing the extracytoplasmic space, may play a key role in the inactivation of many peptides—hormones, neuropeptides, and others—after they have been released from the cell.

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