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Mechanism of Degradation of LH-RH and Neurotensin by Synaptosomal Peptidases

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McDERMOTT, J. R., A. I. SMITH, P. R. DODD, J. A. HARDY AND J. A. EDWARDSON. *Mechanism of degradation of LH-RH and neurotensin by synaptosomal peptidases.* PEPTIDES 4(1) 25–30, 1983.—The products of degradation of LH-RH and neurotensin by synaptosomes isolated from rat hypothalamus and cortex have been identified. LH-RH is cleaved at Tyr^5-Gly^6 and Pro^9-Gly^{10} giving rise to LH-RH (1–5), LH-RH (6–10) and LH-RH (1–9). Neurotensin is cleaved at Arg^8-Arg^9, Pro^{10}-Tyr^{11} and Ile^{12}-Leu^{13}, giving neurotensin (1–8), neurotensin (1–10), neurotensin (1–12) and neurotensin (9–13) as major products. While most of the peptidase activity is localized in the cytoplasmic fraction, a small but significant proportion is membrane bound. For LH-RH, the specificity of the membrane-bound activity is similar to that in the cytosol fraction; for neurotensin, the membrane fraction preferentially gives rise to the (1–10) and (1–11) peptides. The most potent inhibitors of the LH-RH and neurotensin degrading enzymes in synaptosomes are heavy metal ions (mercury and copper), p-chloromercuribenzoate and 1,10 phenanthroline.

LH-RH Neurotensin Neuropeptide Synaptosomes Synaptosomal membrane Peptidases HPLC

THERE is good evidence that a number of brain peptides are involved in neuronal signalling either as transmitters per se or as modulators of synaptic processes [8]. Little is yet known of the metabolic processes controlling peptide levels at the nerve terminal and, in particular, how neuropeptides are inactivated after release. Several brain peptidases, both soluble and membrane-bound, are capable of degrading neuropeptides and have at least some of the properties expected of transmitter-related enzymes. They include proline endopeptidase [3, 11, 20, 21], a dipeptidylcarboxypeptidase which degrades enkephalin [14] and a membrane-bound endopeptidase which preferentially degrades substance-P [13].

We have previously described the mechanism of inactivation of two neuropeptides, luteinizing hormone releasing hormone (LH-RH) (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) and neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), by crude soluble and particulate fractions from rat brain [18,19]. The relevance of results from such studies involving crude subcellular fractions to the physiological mechanisms of inactivation which occur at the nerve terminal is uncertain. In the present study we have therefore examined the breakdown of LH-RH and neurotensin by peptidases in purified synaptosomes from rat brain, using high performance liquid chromatography (HPLC) and amino acid analysis to separate and identify the products. Some of the properties of the enzymes involved have also been examined.

METHOD

Synaptosomes and Synaptosomal Fractions

Synaptosomes were prepared from rat hypothalamus or cortex by a recent modification of the Gray and Whittaker method which gives a much shorter preparation time [5]. By overlaying the supernatant from the first centrifugation step directly onto 1.2 M sucrose and centrifuging at 160,000 g for 15 min, mitochondria and lysosomes were removed as a pellet while synaptosomes and myelin were retained at the gradient interface. The interface material was removed, diluted approximately three-fold with 0.32 M sucrose and layered onto 0.8 M sucrose. Centrifugation at 160,000 g yielded synaptosomes as the pellet while myelin was retained at the interface.

Crude synaptic membrane and synaptic cytoplasm fractions were prepared by resuspending the synaptosomal pellet in distilled water followed by centrifugation (bench centrifuge, 3 min, room temperature). The crude membrane fraction containing synaptic mitochondria and undisrupted synaptosomes was obtained as the pellet, and the cytoplasmic fraction as the supernatant.

Purified synaptic membranes were prepared by a modification of the method of Jones and Matus [9]. The synaptosome pellet was resuspended in 5 mM tris-HCl (pH 7.4; 2.5 ml), sonicated for 15 secs, and then mixed with 1.75 M sucrose (5 ml) giving a final sucrose concentration of 1.17 M. Onto this suspension was layered 0.93 M sucrose (2.5 ml). After centrifugation at 160,000 g for 15 min, the membrane fraction was collected from the interface. This fraction was diluted 5-fold with 5 mM tris-HCl, sonicated and centrifuged again as above. The interface material was again collected, diluted with 5 mM tris-HCl and membranes again collected by centrifugation. This procedure removes mitochondria as a pellet.

Lactate dehydrogenase (LDH) was determined by the method of Kornberg [12] and protein by the method of Lowry *et al.* [15].

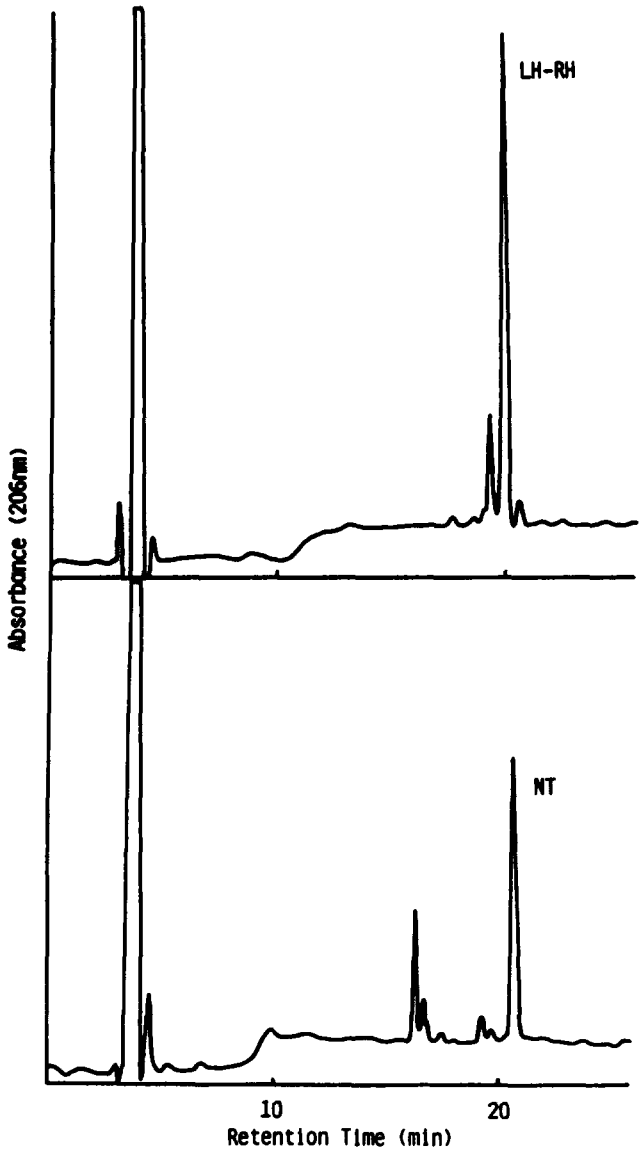

FIG. 1. HPLC separation of products of degradation of LH-RH and neurotensin by synaptosomes. Intact synaptosomes from rat hypothalamus (1 hypothalamic equivalent/ml of Krebs Ringer bicarbonate) were incubated with LH-RH (21 μM) or neurotensin (15.3 μM) at 37° for 30 min. Products were separated by HPLC on a μBondapak C18 column using an acetonitrile gradient containing 0.08% TFA (see text). An amount corresponding to 10 μg of original LH-RH was injected.

Incubations

Peptide (Peninsula, San Carlos, CA; 50 or 100 μg/ml) was incubated with synaptosomes or synaptosomal fractions from hypothalamus (1 hypothalamus equivalent/ml) or cortex (0.2 equivalent/ml) in Krebs Ringer bicarbonate (pH 7.3; final volume 200 μl) at 37° for varying times up to 1 hr. Inhibitors were added at the concentrations shown in Table 2 and incubations carried out for 30 min. The reaction was stopped by adding methanol (2 vol) and the mixture centrifuged (12,000 g; 5 min). The supernatant liquid was removed, evaporated, and the residue dissolved in 225 μl of 0.1% trifluoroacetic acid (TFA) for HPLC.

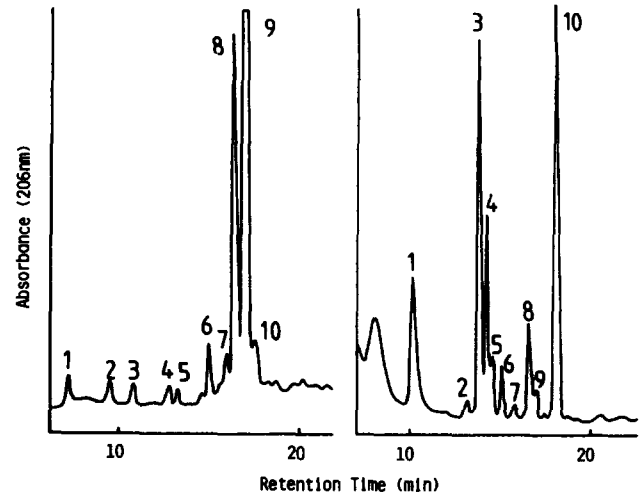

FIG. 2. HPLC separation of products of LH-RH (left) and neurotensin (right) degradation for amino acid analysis. Incubation (conditions as in Fig. 1) of 5, 15, 30 and 60 min duration were combined, the products separated by HPLC (as in Fig. 1) and collected for amino acid analysis. The peaks are identified in Table 1.

HPLC

Separations were carried out on a μBondapak C18 column using Waters Associates (Northwich, Ches.) HPLC equipment as described previously [17] but incorporating a Wisp autosampler. Solvents were: A—11 mM TFA, 2.5 mM acetic acid; B—11 mM TFA in 70% acetonitrile. A linear 20 min gradient, flow rate 1 ml/min, from 5—70% B was used; uv monitoring was at 206 nm.

Amino Acid Analysis

Peaks were collected, the solvent evaporated and the peptide hydrolyzed in 6 M HCl in vacuo at 110° for 18–20 hr. Amino acids were determined using a Chromaspek analyzer (Rank Hilger, Margate, Kent) fitted with o-phthaldehyde detection.

RESULTS

Degradation of LH-RH and Neurotensin by Intact Synaptosomes

Typical HPLC profiles of the degradation products obtained when LH-RH and neurotensin were incubated with intact hypothalamic synaptosomes are shown in Fig. 1. Similar profiles were obtained with cortex synaptosomes. The rates of degradation were: LH-RH—0.25 nmol/min/mg protein; neurotensin—0.57 nmol/min/mg protein. Incubations over several time intervals (5, 15, 30 and 60 min) were combined and subjected to HPLC (Fig. 2). The products, shown in Table 1, were identified by amino acid analysis and, in some cases, by comparison of their retention times with those of previously identified peptide fragments [18,19]. The major product of LH-RH degradation at all time points was LH-RH (1–5). The corresponding C-terminal peptide, LH-RH (6–10), was also present, but in smaller amounts, probably because this fragment is rapdily degraded further. This suggests that a primary cleavage point for LH-RH is the Tyr^5-Gly^6 bond. LH-RH (1–9) was also identified, arising

TABLE 1

Peak	Amino Acid Composition*	Assignment
(LH-RH)		
2	Tyr	Tyr
3	$Pro_1Gly_2Leu_1Arg_1$	LH-RH (6–10)
4		Trp†
5	$Ser_1Glu_1His_1$	LH-RH (1–4)
6	Glu_1His_1	LH-RH (1–3)
8	$Ser_1Glu_1Tyr_1His_1$	LH-RH (1–5)
9	$Ser_1Glu_1Pro_1Gly_2Leu_1Tyr_1His_1Arg_1$	LH-RH
10	$Ser_1Glu_1Pro_1Gly_1Leu_1Tyr_1His_1Arg_1$	LH-RH (1–9)
(NT)		
1	Tyr	
2	Ile_1Leu_1	NT (12–13)
3	$Asp_1Glu_2Pro_1Leu_1Tyr_1Lys_1Arg_1$	NT (1–8)
4	$Asp_1Glu_2Pro_2Leu_1Tyr_1Lys_1Arg_2$	NT (1–10)
5	$Asp_1Glu_2Pro_1Leu_1Tyr_1Lys_1$	NT (1–7)
6	$Asp_1Glu_2Pro_2Leu_1Tyr_2Lys_1Arg_2$	NT (1–11)
8	$Asp_1Glu_2Pro_2Ile_1Leu_1Tyr_2Lys_1Arg_2$	NT (1–12)
9	$Pro_1Ile_1Leu_1Tyr_1Arg_1$	NT (9–13)
10	$Asp_1Glu_2Pro_2Ile_1Leu_2Tyr_2Lys_1Arg_2$	NT

Peaks 1 and 7 (LH-RH) and 7 (NT) did not correspond to any peptide fragment.

*To nearest whole number. Trp was not determined since it is destroyed by acid hydrolysis.

†Co-elutes with Trp standard.

from the cleavage at Pro^9-Gly^{10}. The minor products LH-RH (1–4), LH-RH (1–3), Trp and Tyr indicate that the primary product LH-RH (1–5) is slowly degraded by sequential removal of C-terminal amino acids. The main cleavage point of neurotensin is the Arg^8-Arg^9 bond since neurotensin (1–8) was the major product and neurotensin (9–13) was the only C-terminal peptide identified. Other products were formed by proline endopeptidase-like activity (i.e. cleavage at Pro^7-Arg^8 and Pro^{10}-Tyr^{11}) and cleavage at Tyr^{11}-Ile^{12} and Ile^{12}-Leu^{13}. These pathways are summarized in Fig. 3.

Subcellular Distribution of Degrading Activity

A 3–4 fold increase in both LH-RH and neurotensin degrading activity occurred on lysis of the synaptosomes, and after centrifugation, most of the activity resided in the supernatant cytoplasmic fraction. The crude membrane fraction was clearly contaminated with cytoplasmic elements as shown by the activity of the cytoplasmic marker enzyme LDH. In the purified membrane fraction where LDH contamination was low (~1.7% of that in the original lysed synaptosomes) a small but significant amount of peptide degrading activity remained (Table 2). The products of degradation of LH-RH were the same in the membrane and cytoplasmic fractions although LH-RH (1–3) was present in greater quantities after degradation by the membrane fraction (Fig. 4A). Neurotensin, however, gave a quite different ratio of products (Fig. 4B). The 1–10 and 1–11 fragments were more prominent in the degradation by membrane peptidases whereas neurotensin (1–8) was the major product in the cytoplasmic fraction.

Inhibitors

The effect of a number of inhibitors on the degradation of the two peptides by lysed synaptosome preparations is summarized in Table 3. Inhibitors of serine dependent peptidases (PMSF and trypsin inhibitor) and pepstatin had little effect. The most potent inhibitors were p-chloromercuribenzoate (PCMB), heavy metal ions (mercury and copper) and 1,10-phenanthroline. The inhibitory action of phenanthroline and EDTA was also accompanied by a change in the ratio of the products formed. In the case of LH-RH, the further breakdown of LH-RH (1–5) was markedly inhibited. With neurotensin, the 1–12 and 9–13 fragments were present in greater amounts in the presence of EDTA, again indicating that the breakdown of primary products was inhibited.

pH Dependence

LH-RH degradation by lysed synaptosomes was maximal at pH 7.5–8.0, whereas neurotensin degradation had no clear pH dependence (Table 4). However, the products of neurotensin degradation changed as the pH increased. At low pH neurotensin (1–12) was the major product while at pH 7.5 neurotensin (1–8) predominated (Fig. 5).

DISCUSSION

In this study we have determined the pathways of LH-RH and neurotensin degradation by purified rat synaptosomes. Most of the LH-RH and neurotensin degrading activity in

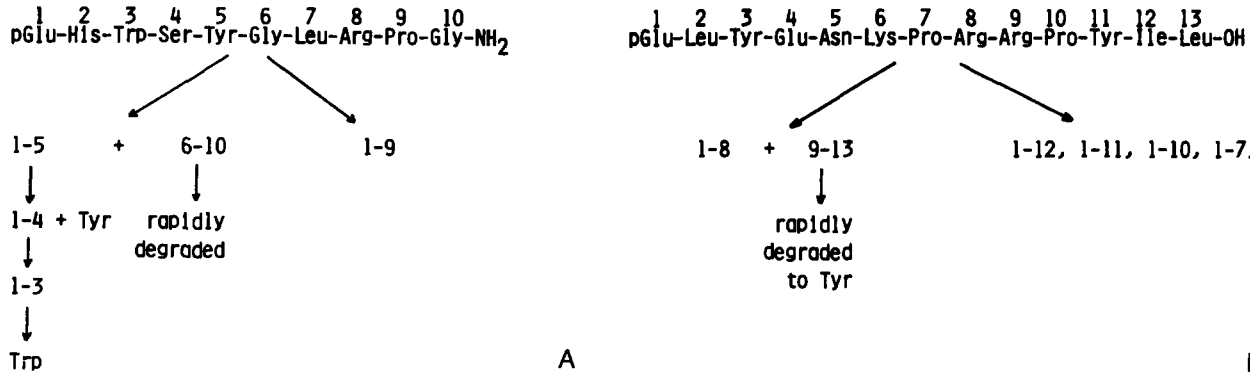

FIG. 3. Degradation pathways of (A) LH-RH and (B) neurotensin on incubation with intact synaptosomes.

TABLE 2

DISTRIBUTION OF LH-RH AND NEUROTENSIN DEGRADING ACTIVITY IN HYPOTHALAMIC SYNAPTOSOME FRACTIONS

Fraction	% of Neurotensin degrading activity	% of LH-RH degrading activity	% LDH activity
Intact synaptosomes	30.2	25.0	N.D.
Lysed synaptosomes	100	100	100
Cytoplasm	70.0	65.0	N.D.
Crude membrane	20.8	24.1	7.6
Purified membrane	12	7	1.7

N.D. not determined.

TABLE 3

EFFECT OF INHIBITORS ON THE DEGRADATION OF LH-RH AND NEUROTENSIN BY LYSED RAT HYPOTHALAMIC SYNAPTOSOMES

Inhibitor	Conc	% inhibition LH-RH	NT
PMSF	1 mM	13	10
Trypsin inhibitor	0.1 mg/ml	10	0
DTT and EDTA	2 mM	31	49
EDTA	2 mM	41	30
1, 10-Phenanthroline	1 mM	68	81
p-Chloromercuribenzoate	1 mM	100	68
Hg^{++}	2 mM	100	100
Cu^{++}	2 mM	100	100
Pepstatin	1 μg/ml	11	4

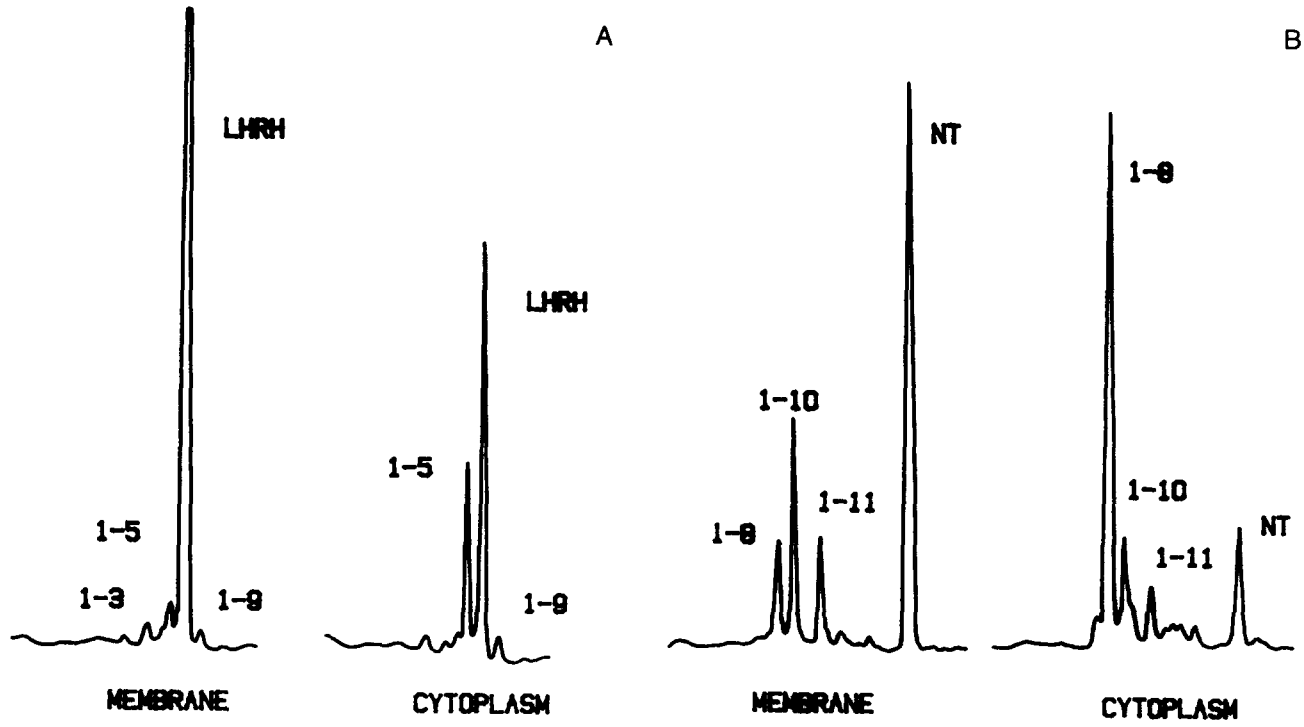

FIG. 4. Comparison of (A) LH-RH and (B) neurotensin degradation by rat cortex synaptosomal membrane and cytoplasm fractions. 30 min incubation.

synaptosomes was localized in the cytoplasmic fraction, in agreement with a previous study which used radioimmunoassay to follow the degradation of LH-RH by synaptosomal fractions [10]. Some peptidase activity was associated with the purified membrane fraction. This is unlikely to be due entirely to contamination from cytoplasmic elements as shown by the low LDH activity. Degradation of neurotensin by the membrane fraction produced a markedly different ratio of products than did the cytoplasmic or intact synaptosomal fractions. While neurotensin (1–8) was the major peak from the cytoplasmic fraction, neurotensin (1–10) and (1–11) predominate in the membrane degradation. Recently, the membrane bound peptidase, enkephalinase, has been shown to cleave a number of neuropeptide bonds in addition to Gly-Phe in enkephalin; these include the Pro^{10}-Tyr^{11} and Tyr^{11}-Ile^{12} bonds of neurotensin [2]. It is therefore possible that enkephalinase-like activity is responsible for the production of neurotensin (1–10) and (1–11) by the membrane fraction. The maximum overall contribution of this enkephalinase-like activity to neurotensin degradation by intact synaptosomes can be inferred from the effect of EDTA, which is an inhibitor of enkephalinase, and is of the order of 35%. The difference in specificity between the membrane-bound and cytoplasmic LH-RH degrading activity is less pronounced than with neurotensin, slightly more LH-RH (1–3) being formed by the membrane peptidases. For both

TABLE 4

pH DEPENDENCE OF LH-RH AND NEUROTENSIN DEGRADING ACTIVITY IN RAT HYPOTHALAMIC SYNAPTOSOMES

	5.5	6.0	6.5	7.0	7.5	8.0
% Degradation of LH-RH	18	8	15	33	34	48
% Degradation of NT	53	63	57	60	64	65

Lysed synaptsomes were incubated in 0.05 M sodium phosphate buffer with peptide for 30 min. The degree of degradation was determined from the peak height of LH-RH and NT after HPLC.

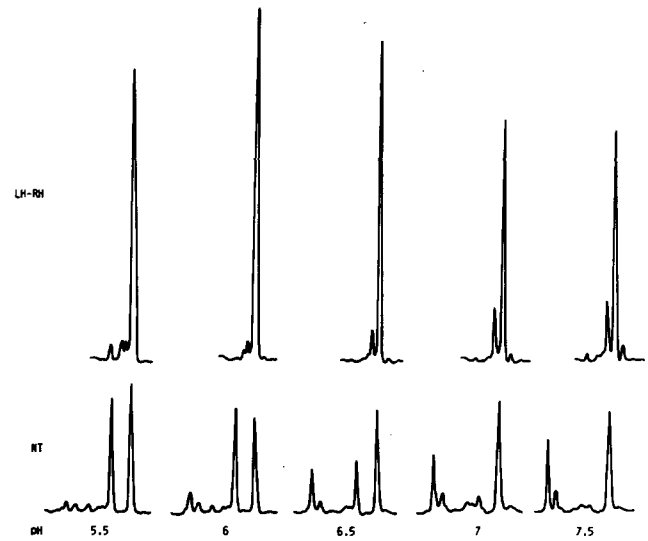

FIG. 5. Effect of pH on products of LH-RH and neurotensin degradation by lysed rat hypothalamic synaptosomes. 30 min incubation.

peptides, there was no evidence for a significant contribution by pyroglutamyl peptidase activity to the degradation pathway. However, activity due to this enzyme did appear to be present in our synaptic membrane preparations as in other preparations [7] since on incubation with thyrotrophin-releasing hormone (TRH), the diketopiperazine, cyclo (His-Pro), was produced (unpublished results).

The cytoplasmic peptidase activity in the synaptosomal fraction is directed against four types of peptide bond: Pro-X; Arg-Arg; Tyr-X and Ile-Leu. Part of this activity may be due to previously identified enzymes. The thiol-dependent endopeptidase, post proline cleaving enzyme, will hydrolyse the Pro-Gly bond in LH-RH and the Pro-Tyr and Pro-Arg bonds in neurotensin and is mainly cytoplasmic [21]. Hydrolysis of the Arg-Arg bond in neurotensin is unlikely to be due to simple trypsin-like activity since other Arg-X and Lys-X bonds remain intact. The enzyme responsible may be related to the endopeptidase which cleaves paired basic residues in polypeptide precursor molecules (e.g., pro-opiomelanocortin; [14]). The metabolism of neurotensin in the circulation may be mediated by a similar enzyme since both 1–8 and 9–13 fragments are formed, along with the 1–11 fragment, after intravenous injection of [^{3}H]-neurotensin [2]. A cation-sensitive neutral endopeptidase present in pituitary and brain has been shown to cleave LH-RH at Tyr^5-Gly^6 and neurotensin at Ile^{12}-Leu^{13} [22]. This enzyme, which has a broad substrate specificity and is inhibited by divalent cations and PCMB, may therefore be a component of the synaptosomal peptidase activity. In rabbit brain, both post proline cleaving enzyme and cation-sensitive neutral endopeptidase have subcellular distributions similar to LDH and distinct from the synaptosomal marker choline acetyl transferase [6]. Our previous studies on crude subcellular fractions of rat brain indicated that most of the LH-RH and neurotensin degrading activity was present in the soluble 25,000 g supernatant fraction [18,19] and the specificity of this peptidase activity was similar to that seen in the present study. This raises doubts about the particular function of these enzymes in neuropeptide metabolism at the nerve terminal, and suggests they may have a more widespread role in the inactivation and possibly synthesis of neuropeptides. We have recently demonstrated that pituitary colloid, a proteinaceous material accumulating in the pituitary cleft after a variety of physiological stimuli (e.g., adrenalectomy or oestrogen treatment), contains, among other enzymes, post-proline cleaving enzyme and peptidase activity similar to cation-sensitive neutral endopeptidase, and there is evidence that the pituitary secretes these enzymes [4]. The possibility that soluble endopeptidases such as these are secreted and have an extracellular function in the CNS must be considered.

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