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DIFFERENCE IN SUSCEPTIBILITY OF ARGININE-VASOPRESSIN AND OXYTOCIN TO AMINOPEPTIDASE ACTIVITY IN BRAIN SYNAPTIC MEMBRANES

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Arginine-vasopressin and oxytocin, peptides which serve as putative precursors for neurotrophic fragments, were digested in the presence of the respective ^{14}C-Tyr^{2}- and ^{14}C-GlyNH$_2$^{9}-labeled nonapeptides with a purified synaptic membrane preparation of rat brain. In this preparation aminopeptidase activity predominates in the conversion of these peptides. The disappearance of intact peptide and the release of free ^{14}C-Tyr and ^{14}C-GlyNH$_2$ was followed simultaneously with time by HPLC. Oxytocin was about four times more resistant to proteolysis than arginine-vasopressin as measured by slower disappearance of intact oxytocin, and reflected by the slower release of ^{14}C-Tyr, but not of ^{14}C-GlyNH$_2$ from oxytocin. Comparison of degradation rates of structure analogues showed that peptides having Ile in position 3, as oxytocin, were more resistant than analogues having Phe in position 3, as arginine-vasopressin. The data demonstrate that arginine-vasopressin and oxytocin differ markedly in susceptibility to the aminopeptidase activity in brain synaptic membranes, and indicate that this difference resides primarily in the amino acid residue in position 3. It is suggested that the difference in susceptibility may affect the pattern of neurotrophic metabolites in brain.

INTRODUCTION

The nonapeptides arginine-vasopressin (AVP) and oxytocin (OXT) modulate a variety of brain functions (1-4). One aspect of their functioning in brain is attributed to their role as precursors for smaller peptide fragments with central activities of their own (5,6). Consequently, the proteolytic enzymes in brain which are involved in the processing of AVP and OXT may have a regulatory role. Previously, we have considered synaptosomal plasma membranes as sites of functional proteolytic processing of neuropeptides (7-9). Proteolytic activities in brain synaptic membrane fractions which cleave OXT, have been investigated (10) and metabolites of OXT and AVP characterized (11,

Abbreviations: AVP = arginine-vasopressin, OXT = oxytocin, LVP = lysine-vasopressin, AVT = arginine-vasotocin, OXP = oxypressin, PA = pressinamide, TA = tocinamide, HPLC = high-pressure liquid chromatography.

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12). These studies have demonstrated that aminopeptidase activity predominates in the conversion of both AVP and OXT.

The present paper describes that, although the mechanism of conversion is similar for OXT and AVP, the peptides differ markedly in susceptibility to the aminopeptidase activity in brain synaptic membranes. It is indicated that this difference is dictated by the nature of the amino acid residue in position 3 in the peptides, and may have quantitative consequences for the profile of neurotrophic fragments of the peptides generated by the aminopeptidase activity.

MATERIALS AND METHODS

Oxytocin (OXT), arginine-vasopressin (AVP), lysine-vasopressin (LVP), arginine-vasotocin (AVT), oxypressin (OXP), pressinamide (PA), and tocinamide (TA) were synthesized and kindly provided by Drs. H.M. Greven and J.W. van Nispen (Organon, Oss, The Netherlands). [^{14}C-Tyr^{2}]-OXT, [^{14}C-GlyNH$_2$^{9}]-OXT, [^{14}C-Tyr^{2}]-AVP, and [^{14}C-GlyNH$_2$^{9}]-AVP were generous gifts of the late Dr. R. Walter (University of Illinois, Chicago, USA) and Dr. W.H. Simmons (Loyola University Medical Center, Maywood, Ill., USA). ^{14}C-Labeled peptides were purified by high-pressure liquid chromatography (HPLC) before use. The primary structures of the peptides are presented in table I.

The preparation and characteristics of synaptic membranes of rat forebrain tissue have been described previously (9).

Peptides were incubated in a concentration of 2 x 10^{-5} M with synaptic membranes (2.5 mg protein/ml) in 40 mM sodium phosphate, pH 7.0, at 37°C in a total volume of 1300 µl. ^{14}C-Labeled peptides (10,000 - 40,000 dpm) were made up to the final concentration with cold peptide and incubated under identical conditions. At time intervals aliquots of 200 µl were taken. The reaction was terminated by addition of 50 µl 2.5 N acetic acid and heating at 80°C for 5 min. The membranes were removed by centrifugation at 10,000 g_{av} for 20 min.

HPLC was performed on a µBondapak C18 column (Waters Associates, Milford, USA) eluted with mixtures of 10 mM ammonium acetate, pH 4.15, and acidified methanol (0.15% acetic acid) at a flow rate of 2.0 ml/min. Digests containing ^{14}C-labeled compounds were fractionated by gradient elution: for ^{14}C-OXT's the gradient ran linearly from 5% to 40% acidified methanol in 25 min; the concave gradient for ^{14}C-AVP's from 0% to 40% organic solvent in 30 min. Fractions of 1.0 ml were collected and subjected to liquid scintillation counting. Digests of non-labeled peptides were fractionated under the following isocratic conditions: OXT, 37% methanol, retention time 10.4 min; AVT, 26%, 6.7 min; TA, 26%, 9.8 min; OXP, 40%, 12.0 min; AVP, 30%, 7.2 min; LVP, 26%, 7.8 min; PA, 32%, 8.8 min. Intact peptide substrates, thus separated from metabolites, were quantitated by the peak height in the UV absorbance (210 nm) profile of the column effluent.

RESULTS

The time course of proteolysis of AVP and OXT by brain synaptic membranes is shown in fig. 1. The conversion process was monitored by the disappearance of intact ^{14}C-Tyr- and ^{14}C-GlyNH$_2$-labeled AVP's and OXT's simultaneously with

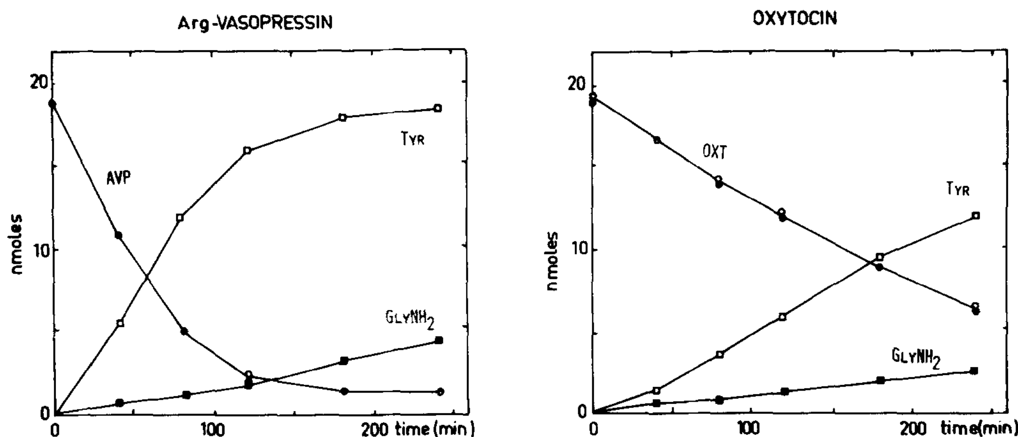

Figure 1. Time course of conversion of AVP and OXT by brain synaptic membranes. [^{14}C-Tyr^2]-AVP, [^{14}C-GlyNH$_2$^{9}]-AVP, [^{14}C-Tyr^2]-OXT and [^{14}C-GlyNH$_2$^{9}]-OXT (2×10^{-5} M) were separately incubated with the membrane preparation (2.5 mg protein/ml) in 40 mM sodium phosphate buffer pH 7.0 at 37°C. Aliquots taken at various time intervals were made membrane-free and subjected to HPLC. The separated products were quantitated by radioactivity content in collected 0.5 min fractions. The disappearance of ^{14}C-Tyr^2-labeled (o) and ^{14}C-GlyNH$_2$^{9}-labeled (●) nonapeptides and the formation of free ^{14}C-Tyr (□) and ^{14}C-GlyNH$_2$ (■) from AVP and OXT is shown.

the release of the free amino acids ^{14}C-Tyr and ^{14}C-GlyNH$_2$ employing HPLC. AVP was markedly more susceptible to proteolysis than OXT. This difference in conversion rate was reflected by the formation of free ^{14}C-Tyr which is released during aminopeptidase cleavage: the initial rate of ^{14}C-Tyr released from AVP was 6.7 µM/h incubation medium, while that from OXT was 2.7 µM/h. The release of ^{14}C-GlyNH$_2$ was relatively low as compared to that of ^{14}C-Tyr and was not significantly different between the peptides (AVP: 0.58 µM/h, and OXT: 0.50 µM/h).

Peptides with structures related to AVP and OXT (see table I) were compared for their conversion rates during exposure to the synaptic membrane preparation. The time courses of disappearance of intact peptides during incubation is shown in fig. 2. Of all peptides tested PA was most susceptible to proteolysis; AVP, LVP and OXP were less rapidly degraded. The time course of LVP was similar to that of AVP and OXP (not shown). AVT was significantly more resistant, while OXT and TA were the most resistant peptides.

The disappearance of OXT, TA, and AVT was linear with time over 2 h, demonstrating zero-order kinetics of the conversion process. For OXP, AVP, LVP, and PA the conversion process only initially followed zero-order kine-

Table I

STRUCTURES OF NEUROHYPOPHYSEAL HORMONES AND ANALOGUES

peptide		structure[a]
oxytocin	OXT	H-Cys¹-Tyr-*Ile*-Gln-Asn-Cys-Pro-*Leu*-GlyNH₂⁹
arginine-vasotocin	AVT	H-Cys¹-Tyr-*Ile*-Gln-Asn-Cys-Pro-*Arg*-GlyNH₂⁹
tocinamide	TA	H-Cys¹-Tyr-*Ile*-Gln-Asn-Cys⁶-NH₂
oxypressin	OXP	H-Cys¹-Tyr-*Phe*-Gln-Asn-Cys-Pro-*Leu*-GlyNH₂⁹
arginine-vasopressin	AVP	H-Cys¹-Tyr-*Phe*-Gln-Asn-Cys-Pro-*Arg*-GlyNH₂⁹
lysine-vasopressin	LVP	H-Cys¹-Tyr-*Phe*-Gln-Asn-Cys-Pro-*Lys*-GlyNH₂⁹
pressinamide	PA	H-Cys¹-Tyr-*Phe*-Gln-Asn-Cys⁶-NH₂

[a] The structural differences are indicated by the residues in italics.

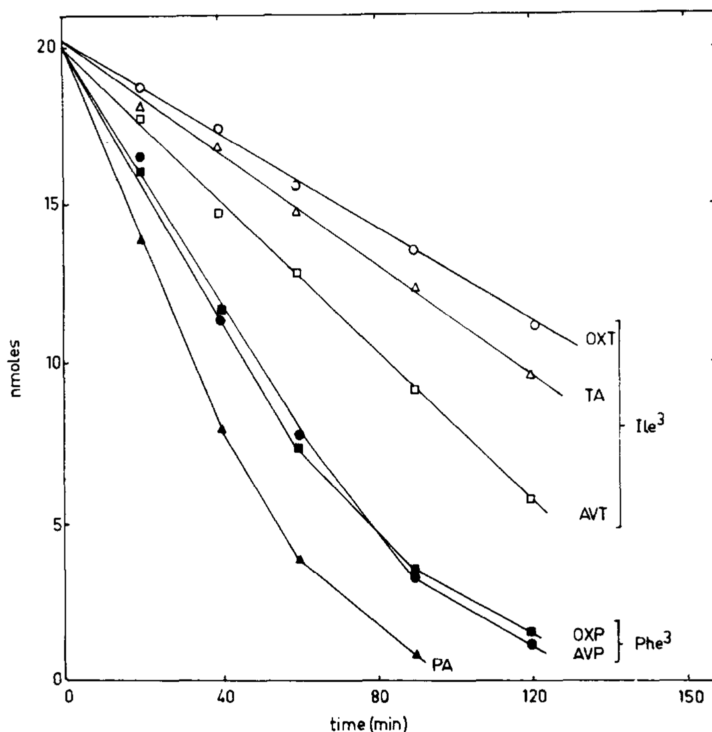

Figure 2. Comparison of the conversion of AVP, OXT and related analogues by brain synaptic membranes. The peptides OXT (o), TA (Δ), AVT (□), OXP (■), AVP (●) and PA (▲) were separately incubated under identical conditions and quantitated by their peakheights in chromatograms obtained by HPLC and monitoring of the eluate at 210 nm. The amount of peptide is plotted against the incubation time. The structures of the peptides are presented in table I.

Table II

RATE CONSTANTS OF CONVERSION OF NEUROHYPOPHYSEAL HORMONES AND ANALOGUES

peptide	rate constant[a] (μM/h)	correlation coefficient of linearity r^2	number of time points n
OXT	4.4	0.9964	6
TA	5.2	0.9949	6
AVT	7.2	0.9978	6
OXP	11.4	0.9889	5
AVP	11.6	0.9946	5
LVP	11.5	0.9960	5
PA	16.2	0.9919	4

[a] Rate constants of zero-order enzyme reaction were calculated by linear regression analysis using time points of the complete disappearance curve or time points in the linear phase.

tics. The rate constants of degradation of these peptides calculated during the zero-order phases are given in table II.

DISCUSSION

In general the structures of AVP and OXT are accessible to proteolytic attack at three sites (13), i.e. 1) the NH_2-terminus by aminopeptidases, 2) the COOH-terminal portion by enzymes cleaving off the COOH-terminal dipeptides or the $GlyNH_2$ residue, and 3) the disulfide bridge by reductases. In brain three types of cleavages have been recognized as well, depending on the subcellular fraction used (10,14,15). However, in synaptic membrane preparations, which are considered to contain the membrane structures involved in the functional proteolytic processing of secreted neuropeptides (7-9), aminopeptidase activity predominates largely in the conversion of oxytocin (10). Ultimate demonstration of the aminopeptidase nature of the AVP and OXT converting enzyme(s) has been obtained by identifying the early split products (12, and, in preparation). Moreover, the observation that the aminopeptidase cleavage of ^{14}C-OXT is similarly inhibited by cold OXT, AVP, and LVP suggests that the aminopeptidase activity involved is the same enzyme

for both peptides (Burbach and Scholtens, unpublished). Although this similarity in mechanism of conversion exists, a marked difference in conversion rates of AVP and OXT was found in the present experiments. This difference was reflected in the release of ^{14}C-Tyr, but not of ^{14}C-GlyNH$_2$, demonstrating that the peptides differ in susceptibility to the aminopeptidase activity specifically.

Comparison of structure analogues of AVP and OXT with regard to degradation rate provided clues to the structural features underlying this difference in susceptibility. Based on the conversion rates the peptides could be divided into two groups: peptides which were most rapidly degraded (PA, AVP, LVP, and OXP) had Phe in position 3; the most resistant group of peptides (OXT, TA, and AVT) had Ile at position 3. These data demonstrate that the amino acid residue in position 3 determines primarily the degree of susceptibility to the aminopeptidase activity. This finding is likely related to the marked conformational differences between OXT and AVP, which are mainly due to the residues in position 3 (16-19).

Within each group smaller differences were observed. For the Ile^3-containing peptides the amino acid residue in position 8 influenced the conversion rate. Replacement of Leu^8 in OXT by Arg^8 resulted in a significantly more rapid conversion, while deletion of the COOH-terminal tripeptide did not influence the conversion rate. In the group of Phe^3-containing peptides replacement of Leu^8 in OXP by Arg^8 or Lys^8 did not influence the conversion rate. Here, deletion of the COOH-terminal tripeptide increased the conversion rate. These observations suggest that the COOH-terminal tripeptide of AVP, OXT, and analogues contributes additionally to the susceptibility in the NH$_2$-terminal region. The extent of this contribution depends on the nature of the residue in position 3.

It is concluded that the different susceptibilities of AVP and OXT to conversion by brain synaptic membranes are primarily dictated by the nature of the amino acid residue in position 3: the aminopeptidase activity prefers to cleave Phe^3- over Ile^3-containing peptides. The efficiency of amino-

peptidase attack is secondarily influenced by the COOH-terminal tripeptide moiety. This influence differs for Phe^3 and Ile^3-containing peptides. The observed difference between AVP and OXT in susceptibility to brain aminopeptidase activity may indicate that endogenous AVP and OXT in brain have different turnover rates. Consequently, the pattern of generated AVP and OXT metabolites may differ quantitatively in brain and this could have functional implications for the activity profile of these peptides.

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