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OXYTOCIN BIOTRANSFORMATION IN THE RAT LIMBIC BRAIN: CHARACTERIZATION OF PEPTIDASE ACTIVITIES AND SIGNIFICANCE IN THE FORMATION OF OXYTOCIN FRAGMENTS

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SUMMARY

The enzymatic conversion of oxytocin by brain peptidases has been studied. Oxytocin was incubated with synaptosomal plasma membranes (SPM) isolated from the rat brain. Qualitative studies using a microdansylation technique revealed two types of oxytocin converting peptidases, e.g. aminopeptidase and C-terminal cleaving peptidase activities. Both enzyme activities were quantitated using [^{14}C]oxytocin labeled at either the tyrosine-2 or the glycinamide-9 residue. Radiolabeled products were separated by high-voltage paper electrophoresis or high-pressure liquid chromatography. The aminopeptidase activity was optimally active at pH 6.9 with a Michaelis constant (K_m) of 6.1 $\times$ 10^{-5} M. The pH optimum of the C-terminal cleaving peptidase activity was pH 6.0 with K_m= 1.3 $\times$ 10^{-5} M. Subcellularly, highest amino-peptidase activities were associated with SPM, synaptosomal and microsomal preparations, while the C-terminal cleaving peptidase prevailed in the cytosol and mitochondrial fractions. The regional distribution of both peptidases showed differences between several brain areas and indicated the medial basal hypothalamus as a locus of high oxytocin biotransformation. In the course of this investigation an oxytocin fragment of unknown structure was detected in the digests and its accumulation was studied together with the determination of peptidase activities. It is suggested that the SPM-associated peptidases may have a role in the modulation of oxytocin action in the brain.

INTRODUCTION

Oxytocin and vasopressin containing fiber systems have been identified in the brain. These fibers originate from the paraventricular, supraoptic and suprachiasmatic

nuclei of the hypothalamus and they innervate the limbic system, the medulla oblongata and the spinal cord[1,2,17,25,27]. These areas are known to be involved in the regulation of neuroendocrine, behavioural and cardiovascular responses.

Oxytocin and vasopressin are potent neuropeptides. Behavioural studies have indicated that these peptides are involved in the control of brain mechanisms related to learning and memory[24,38,39]. Catecholaminergic neurotransmission seems to mediate such effects of the neurohypophyseal hormones[13,28]. Vasopressin affects the turnover of noradrenaline and dopamine in several discrete brain regions as was shown by an altered disappearance rate of the amines after synthesis inhibition with α-methyl-para-tyrosine[26]. Oxytocin influences the turnover rate in a limited number of brain areas, which are distinct from the vasopressin affected regions (Versteeg, unpublished data). Local application of the neurohypophyseal hormones in these particular brain areas has provided further evidence that these peptide effects on adaptive behaviour are mediated by catecholaminergic neurotransmission[14].

It has been recognized that the CNS effects of the neurohypophyseal peptides are not restricted to the hormonal principles themselves; structure–activity studies have revealed that peptide fragments of oxytocin and vasopressin elicit strong central effects. Several of these peptides display behavioral activities distinct from those of their parent molecules[31,34,36,39,40]. In addition to effects in memory processes oxytocin fragments seem to be involved in the regulation of pituitary melanocyte-stimulating hormone (MSH) release. Pro-Leu-GlyNH_2 has been proposed to be the naturally occurring release inhibiting factor for MSH[4,21], while tetra- and pentapeptides from the ring portion of oxytocin may exhibit MSH releasing activity[3,5]. From these behavioral and endocrine observations it has been postulated that oxytocin and vasopressin can act as precursor molecules which are enzymatically converted into peptides with specific functions in the CNS[30,37].

Little is known about the mechanisms and the sites of conversion of the neurohypophyseal hormones into bioactive fragments. In the present study the conversion of oxytocin was investigated at its putative central site of action, the synaptosomal plasma membranes (SPM) of the limbic brain.

Isolated SPM preparations were analyzed for the presence of peptidases and the detected enzyme activities were further characterized for their kinetic parameters as well as subcellular and regional distribution. In the course of these experiments an oxytocin fragment of unknown structure was detected in the digests. Some aspects of the enzymatic generation of this peptide are reported.

MATERIALS AND METHODS

Dansylchloride was obtained from Fluka AG (Buchs SG, Switzerland) and dansylated amino acids were from Sigma (St. Louis, U.S.A.). Double-faced micropolyamide F-1700 sheets were purchased from Schleicher and Schüll (Dassel, G.F.R.). 1-Heptane sulfonic acid was a product from Waters Associates (Milford, Mass., U.S.A.). Synthetic oxytocin and related peptides were donated by Dr. H. M. Greven (Organon International, Oss, The Netherlands). [2-Tyr-1-^{14}C]oxytocin and [9-

$GlyNH_2$-1-^{14}C]oxytocin were generous gifts of Dr. R. Walter (University of Illinois, Chicago, U.S.A.).

Preparation of SPM fraction from limbic brain tissue

Hippocampus, hypothalamus and septum were dissected from male Wistar rats, weighing 170–190 g, according to the method of Gispen et al.[6]. The combined tissues were homogenized in 0.32 M sucrose (9 ml/g tissue) with 10 strokes of a Teflon–glass homogenizer. The homogenate was centrifuged at 1000 g_{av} for 10 min. A crude mitochondrial–synaptosomal fraction was obtained by centrifugation of the supernatant at 10,000 g_{av} for 20 min. This fraction was lysed by homogenization of the pellet in distilled water (5 ml/g tissue) and the suspension was kept gently stirred at 0 °C for 30 min. The supernatant obtained after centrifugation of the suspension at 10,000 g_{av} for 20 min was layered over a discontinuous sucrose density gradient consisting of 1.0 M abd 0.4 M sucrose. Synaptosomal plasma membranes which accumulated at the 1.0–0.4 M sucrose interface during a run at 100,000 g_{max} for 80 min, were collected, 5 times diluted with distilled water and spun down at 48,000 g for 30 min. In experiments concerning the regional distribution of oxytocin converting enzymes the gradient was omitted in the preparation of SPM fractions due to the small size of the tissue samples.

Subcellular fractionation of limbic brain tissue

A 10% homogenate of combined hippocampal, hypothalamic and septal tissue was obtained as described above and used as starting preparation for the isolation of nuclei, mitochondria, synaptosomes, synaptosomal plasma membranes, microsomes and cytosol. A myelin fraction was prepared in the course of the isolation procedure. The isolated fractions were dialyzed overnight against 2 mM sodium phosphate buffer.

Nuclei. The nuclear-debris pellet (P_1) which was obtained by centrifugation of the 10% homogenate at 1000 g_{av} for 10 min was taken up in 2 M sucrose (15 ml/g tissue). Nuclei were obtained as a pellet after centrifugation of the suspension in a swing-out rotor at 12,500 g_{av} for 90 min[20].

Mitochondria, synaptosomes, myelin. The supernatant obtained in the first centrifugation step (S_1) was centrifuged at 10,000 g_{av} for 20 min and the resulting pellet (P_2) was resuspended in 0.32 M sucrose. According to the procedure of Grey and Whittaker[7] mitochondria, synaptosomes and myelin were separated by centrifugation on a 1.2 M–1.0 M–0.85 M sucrose density gradient spun at 100,000 g_{av} for 60 min.

SPM. A SPM fraction was prepared as described above.

Microsomes, cytosol. The supernatant obtained in the second centrifugation step (S_2) was submitted to a series of centrifugation steps adopted from Whittaker[35] to yield the microsomal and cytosol fractions. These consisted of centrifugation of S_2 at 17,000 g_{av} for 60 min followed by centrifugation of the resulting supernatant at 100,000 g_{av} for 60 min. In the latter step microsomes were spun down; the supernatant was the cytosol fraction.

Microdansylation

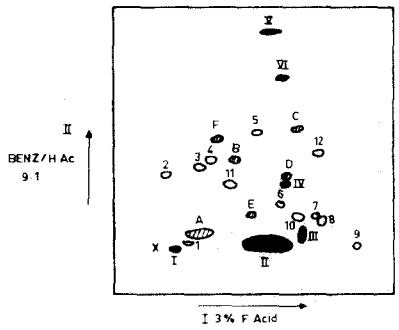
Peptides (5 nmol), oxytocin, vasotocin, or arginine–vasopressin, were incubated

with SPM preparations (20–60 μg protein) in 20 mM sodium phosphate buffer pH 7.2. The total volume was 32 μl. After stopping the reaction by heating at 95 °C for 5 min membranes were removed by centrifugation. Dansylation was carried out by incubation of a mixture of 10 μl sample, 10 μl 0.5 M sodium bicarbonate and 20 μl dansyl-chloride (1 mg/ml) at 50 °C in the dark for 45 min. The solvents were evaporated under reduced pressure and 2 μl 96 % ethanol was added to the residue. Reaction products (approximately 1 μl) were chromatographed on 5 × 5 cm polyamide sheets in two dimensions together with intrinsic markers (dansyl–methionine and dansyl–hydroxyproline). Identification of products was facilitated by co-chromatography of a mixture of reference dansyl–amino acids. Thin layer chromatography was performed as described in Fig. 1. Amino acids and peptides related to the C-terminal part of oxytocin could be separated in this system. Peptides containing the covalent ring structure remained close to the origin.

Enzyme assays

Enzyme preparations (50 μg protein) were incubated with oxytocin, ^{14}C-labeled at either the tyrosine-2 or the glycinamide-9 residue (3×10^{-6} M; 20,000 dpm) at 37 °C in a total vol. of 29 μl 25 mM phosphate buffer pH 6.9 or pH 6.0 respectively for 3 h. The reaction was stopped by boiling (5 min) and the mixture was centrifuged at 6000 g_{av} for 10 min. After fractionation of the reaction mixture the amounts of products were expressed as percentage of the total radioactivity and, if necessary, corrected to 50 μg protein in the incubation mixture, except when stated otherwise.

The relation of product formation and the amount of enzyme preparation behaved in a linear manner up to about 50 % conversion of the initial amount of oxytocin (not shown). This allowed the comparison of enzyme activities.



reagent	aminoacids	peptides
I DNS-pol	1 cys	A OXT
II DNS-OH	2 tyr	B PLG-OH
III " "	3 leu	C PLG-NH_2
IV DNS-NH_2	4 ile	D LG-NH_2
V DNS-X_1	5 pro	E LG-OH
VI DNS-X_2	6 gly	F PL-OH
	7 gln	
	8 asn	
	9 arg	
	10 pro-OH	
	11 met	
	12 gly-NH_2	

Fig. 1. Chromatography of a standard mixture of dansylated (DNS) amino acids and peptides related to oxytocin on micropolyamide thin layers. The plates were developed in the first dimension in formic acid (3 %, v/v), dried carefully and subsequently developed in the second dimension in benzine–acetic acid (9:1, v/v).

Separation of radiolabeled products

Separation of products was achieved by high-voltage paper electrophoresis or by high-pressure liquid chromatography (HPLC). Protein-free samples (25 μl) were applied to Whatman 3 MM paper together with carrier amounts of oxytocin, glycinamide, Leu-$GlyNH_2$ and Pro-Leu-$GlyNH_2$ (5–10 μg each). High voltage electrophoresis was performed in acetic acid–formic acid–H_2O (87 : 25 : 888), pH 1.9, at 1700 V during 180 min for digests containing ^{14}C-Tyr-labeled products or during 80 min for digests containing ^{14}C-$GlyNH_2$-labeled products. After drying the paper, reference peptides were visualized by ninhydrin staining and strips were cut into pieces for elution and scintillation counting.

The HPLC system for the fractionation of [^{14}C-Tyr]oxytocin digests was designed to separate [^{14}C]tyrosine and other possible labeled products such as [^{14}C]oxytocin$_{1-8}$ and [^{14}C]oxytocin$_{1-7}$ from [^{14}C]oxytocin. These peptides and tyrosine were added to the digests before HPLC fractionation. A rapid separation was achieved using a μBondapak C_{18} reversed-phase column, which was eluted with a linear gradient of 35–40% acidified methanol (1.5 ml acetic acid per liter methanol) in 10 mM ammonium acetate, pH 4.15, over 5 min followed by isocratic elution at final conditions.

Paired-ion HPLC was applied for the fractionation of the [^{14}C-$GlyNH_2$]oxytocin digests in order to obtain a suitable separation between glycinamide, Leu-$GlyNH_2$, Pro-Leu-$GlyNH_2$ and oxytocin. The μBondapak C_{18} reversed-phase column was eluted with a concave gradient over 10 min of 25–75% methanol in water. The solutions contained 0.2% 1-heptane sulfonic acid. The flow rate in both systems was 2.0 ml/min and the eluate was monitored continuously by UV absorbance at 210 nm. Fractions of 1.0 ml were collected and subjected to scintillation counting.

RESULTS

Microdansylation

SPM preparations were analyzed for the presence of oxytocin converting pepti-

TABLE I

Time-dependent release of amino acids upon incubation of oxytocin (OXT), arginine–vasopressin (AVP) and vasotocin (AVT) with SPM preparations of rat limbic brain as identified by microdansylation

> Indicates 'amounts larger than'; amounts were semi-quantitatively estimated from the relative fluor-escence of the DNS-amino acid spots.

Substrate structure		*Amino acids released after incubation periods of 1 and 6 h*	
		1 h	*6 h*
OXT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-$GlyNH_2$	Tyr	Tyr, Ile > Leu, $GlyNH_2$
AVCP	ys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-$GlyNH_2$	Tyr, Phe	Tyr, Phe > $GlyNH_2$
AVT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-$GlyNH_2$	Tyr	Tyr, Ile > $GlyNH_2$

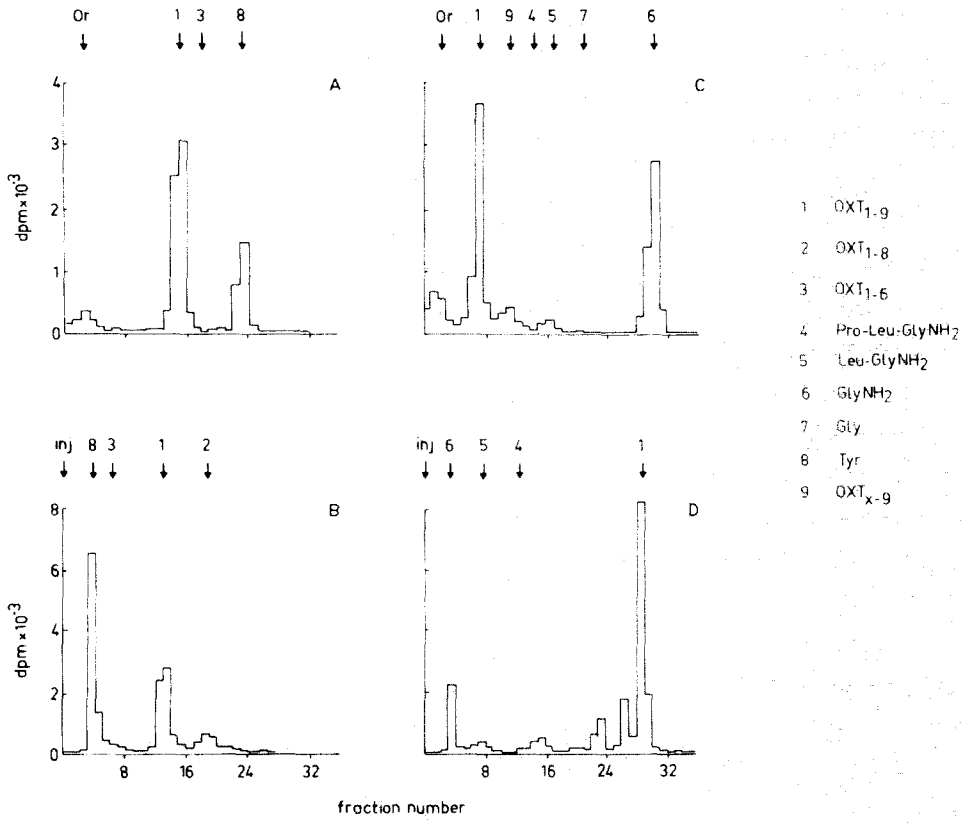


Fig. 2. Fractionation of digests of [^{14}C-Tyr]oxytocin (A and B) and [^{14}C-GlyNH$_2$]oxytocin (C and D) which were obtained by incubation with SPM preparations of rat limbic tissue by high-voltage paper electrophoresis (A and C) and high-pressure liquid chromatography (B and D). Incubation of oxytocin, sample handling and separation procedures were carried out as described in the text. The mobility of a number of standard peptides and amino acids has been indicated at the top of the graphs. The electrophoretograms and chromatograms demonstrate the presence of tyrosine as the major radiolabeled metabolite of [^{14}C-Tyr]oxytocin and the presence of intact oxytocin, glycinamide, OXT$_{x-9}$ and minor amounts of Leu-GlyNH$_2$ in digests of [^{14}C-GlyNH$_2$]oxytocin. In these systems no Pro-Leu-GlyNH$_2$ was detected.

dases by using a microdansylation technique. Incubation of oxytocin with the preparations resulted in a time-dependent release of amino acids. During short incubation time tyrosine and isoleucine were detected in the incubation mixtures. In addition to these amino acids, prolonged incubation yielded glycinamide and leucine (Table I). Small peptide fragments were not detected in the digests. Comparison of oxytocin conversion with that of arginine–vasopressin and vasotocin showed that these peptides were degraded by a similar mechanism (Table I). The results indicated that the pathway of oxytocin conversion in the SPM preparations involved two main routes: (a) cleavages in the N-terminal part of the hormone by aminopeptidase-like activities releasing the amino acid residues at the position 2 and 3; and (b) cleavages in the C-terminus of the peptides leading to the release of the C-terminal glycinamide, and in the case of oxytocin, the leucine residue at position 8.

Products

Based on the above described data, studies were undertaken to quantify both the aminopeptidase and the C-terminal cleaving peptidase activities in limbic SPM preparations. The release of [^{14}C]tyrosine from [^{14}C-Tyr-2]oxytocin and of [^{14}C]glycinamide from [^{14}C-GlyNH$_2$-9]oxytocin respectively were considered to be a reliable measure for these enzyme activities. Separation of products in the digests of [^{14}C-Tyr]oxytocin by high-voltage paper electrophoresis and HPLC indicated tyrosine as the main radiolabeled product of oxytocin conversion. Only a minor amount of a product comigrating with oxytocin$_{1-8}$ was detectable in the HPLC system (Fig. 2A and B). Using [^{14}C-GlyNH$_2$]oxytocin as substrate mainly glycinamide was present in the digests; the dipeptide Leu-GlyNH$_2$ was only a minor component.

In addition, a ^{14}C-labeled peptide fragment not comigrating with any glycinamide containing reference peptide was detected. This oxytocin fragment was not detectable when [^{14}C-Tyr]oxytocin was used as substrate; thus, it is devoid of the tyrosine-2 residue. As this peptide contains the C-terminal glycinamide residue but is of unknown structure we designate it as oxytocin$_{x-9}$ (OXT$_{x-9}$). Its formation was studied in the following experiments concomitantly with the determination of aminopeptidase and C-terminal cleaving peptidase activities.

pH dependency

The pH dependency of the two types of peptidase activities and the formation of OXT$_{x-9}$ is depicted in Fig. 3. The tyrosine releasing activity displayed a narrow pH optimum at about pH 6.9. This pH curve resembled that of the formation of OXT$_{x-9}$ closely. The C-terminal cleaving enzyme was active over a broader pH range. Its optimum was between pH 5.0 and 6.5.

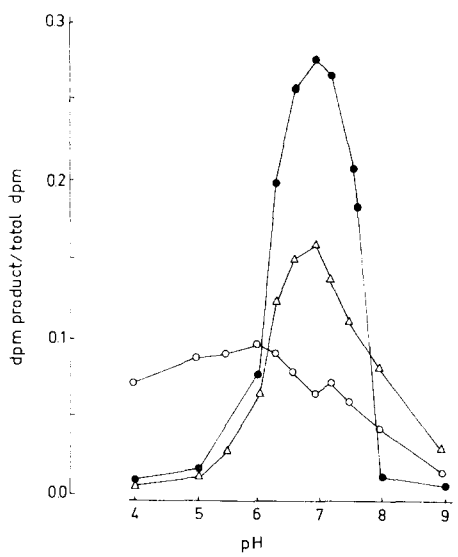


Fig. 3. pH dependency of the release of [^{14}C]tyrosine (●) and [^{14}C]glycinamide (○) and the accumulation of the [^{14}C]-glycinamide labeled oxytocin fragment OXT$_{x-9}$ (△) upon digestion of [^{14}C]oxytocin preparations by SPM associated peptidases.

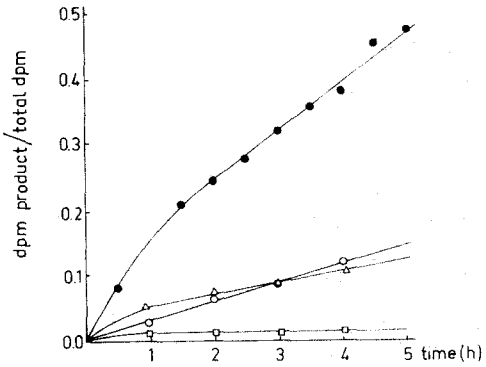


Fig. 4. Time course of the release of [^{14}C]tyrosine (●) and [^{14}C]glycinamide (○) and the accumulation of [^{14}C]OXT_{x-9} (△) upon incubation of [^{14}C-Tyr]oxytocin and [^{14}C-GlyNH_2]oxytocin respectively with SPM preparations from the rat limbic brain. The accumulation of [^{14}C]Leu-GlyNH_2 is indicated by □.

Kinetic parameters

From time course studies it could be seen that the release of tyrosine is the most rapid process in the conversion of oxytocin by SPM associated peptidases. The progressive increase in liberated glycinamide and the formation of the oxytocin fragment OXT_{a-9}, proceeded at a much slower rate (Fig. 4).

The velocity of the release of tyrosine and glycinamide and the formation of OXT_{x-9} were determined as a function of the oxytocin concentration. The experiments were performed at substrate concentrations ranging from 0.2 to 33 μM oxytocin. From these data Lineweaver–Burk plots were constructed (Fig. 5). From these plots,

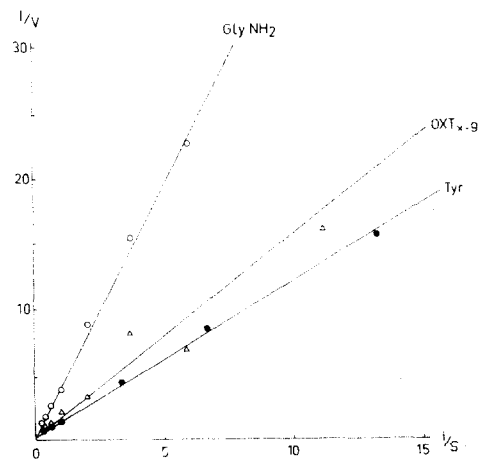


Fig. 5. Lineweaver–Burk plots of the enzymatic conversion of oxytocin by SPM associated peptidases as found for the release of [^{14}C]tyrosine (●) and [^{14}C]glycinamide (○) and for the accumulation of [^{14}C]OXT_{x-9} (△). A constant amount of SPM preparation (50 μg) was incubated with varying concentrations of [^{14}C-Tyr]oxytocin or [^{14}C-GlyNH_2]oxytocin. The oxytocin concentration S is expressed as μM and the velocity V as pmol/50 μg protein per 3 h incubation.

TABLE II

Kinetic parameters of enzymatic processes in the conversion of oxytocin by SPM preparations

Process	K_m *10* $^{-5}$ *M*	V_{max} *(pmol/50 µg/3 h)*
Release of tyrosine	6.1	487
Release of glycinamide	1.3	38
Accumulation of OXT_{x-9}	2.4	142

which were linear over the tested concentration range, the maximal velocity of the enzymatic processes (V_{max}) and the Michaelis constant (K_m) were calculated (Table II).

Subcellular distribution

In line of our working hypothesis that the limbic SPM is a target site for oxytocin action and that functional conversion may take place at the cell surface we compared the SPM preparation with other subcellular compartments of the limbic brain tissue. A marked dissociation of the aminopeptidase and C-terminal cleaving enzyme activity in subcellular distribution was found (Fig. 6). Highest aminopeptidase activities were measured in microsomal, SPM, and synaptosomal preparations, while relatively low activities were present in the mitochondrial and soluble fractions. Also the purified cell nuclei preparation contained considerable aminopeptidase activity. In contrast, the C-terminal cleaving peptidase prevailed in the mitochondrial and soluble fractions, while its activity was relatively low in the SPM, microsomal and myelin preparations.

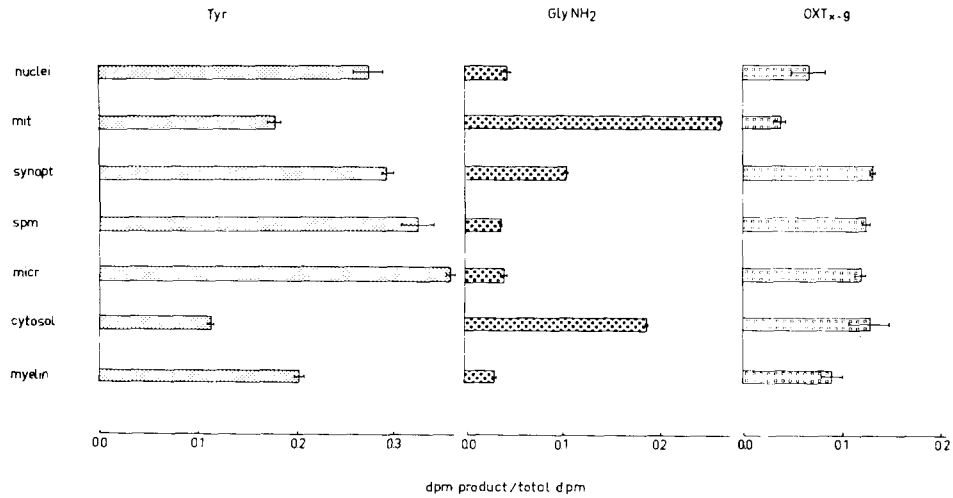


Fig. 6. Subcellular distribution of oxytocin converting peptidase activities which lead to the release of tyrosine and glycinamide and to the accumulation of OXT_{x-9} in rat limbic brain tissue.

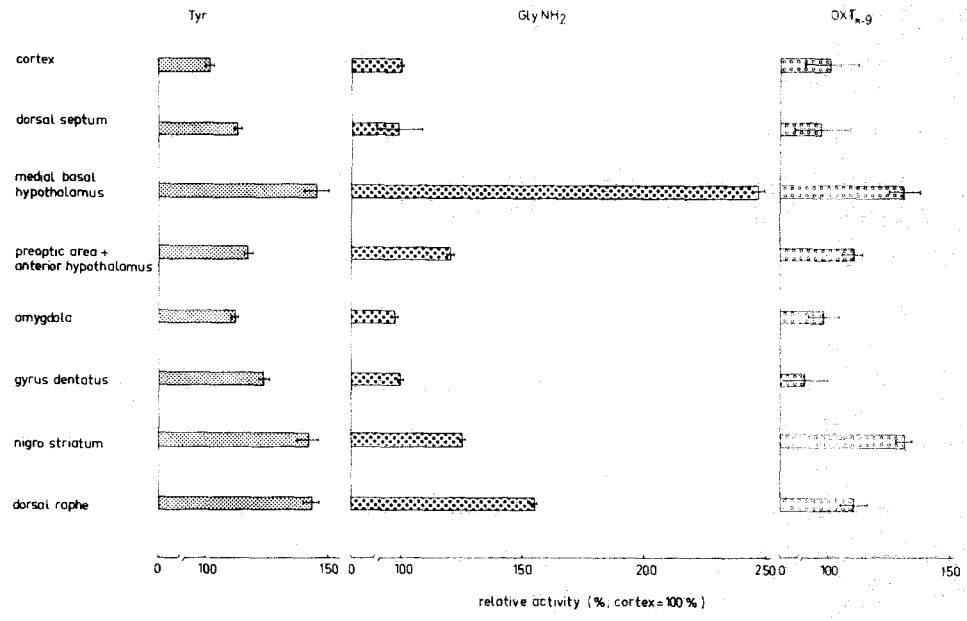


Fig. 7. Regional distribution of oxytocin converting peptidase activities in crude SPM preparations of the rat brain (see Methods section). The activities in the various brain areas are compared with those in the parietal cortex. The 100% value of the activities in the cortex correspond to 42.7 ± 3.0% release of [^{14}C]tyrosine, 35.0 ± 1.0% release of [^{14}C]glycinamide and 9.1 ± 1.1% OXT_{x-9} of the total radioactivity in the digests.

The distribution of the formation of OXT_{x-9} showed some similarities with that of the aminopeptidase. Although the amounts of Leu-$GlyNH_2$ were consistantly low in these experiments, its accumulation appeared to be subcellularly differentiated. The highest amounts (expressed as percentage of the total radioactivity) were found in the synaptosomal (3.3%) and microsomal fractions (3.1%). In mitochondria, SPM, cytosol and myelin preparations the percentage ranged from 1.2 to 1.6%; in the nuclear fraction no significant amount of Leu-$GlyNH_2$ (< 0.1%) was detected.

Regional distribution

Both tyrosine and glycinamide releasing peptidase activities were measured separately in a number of brain areas, which have been implicated in the mediation of oxytocin and vasopressin action. Omission of the gradient step in the preparation of the SPM fractions from these areas did not affect the aminopeptidase activity, but increased the release of glycinamide about two-fold. Consequently, accumulation of OXT_{x-9} was diminished. For the aminopeptidase activity, the relatively highest level was found in the medial basal hypothalamus, nigro-striatal area and the region of the dorsal raphe. The distribution was comparable with that of OXT_{a-9} formation. Highest activity of the C-terminal cleaving peptidase was detected in the medial basal hypothalamus and to a lesser extent in the dorsal raphe (Fig. 7).

DISCUSSION

The present studies concerning the biotransformation of oxytocin in SPM preparations of the rat limbic brain demonstrates the presence of two types of oxytocin converting peptidase activities. These enzymes were shown to act on peptide bonds adjacent to the tyrosine and isoleucine residues in the covalent ring structure and on the C-terminal acyclic region of the oxytocin molecule. Such mechanistic properties are supportive evidence for the involvement of aminopeptidase and C-terminal cleaving peptidase activities in the biotransformation of oxytocin at its putative site of action, the synaptosomal plasma membrane.

In addition to well documented investigations on the fate of oxytocin in its peripheral target organs (cf. refs. 29 and 32), there is only a limited literature available concerning its biotransformation in the brain. Using minced cerebral cortex tissue Plíska et al. have indicated the cysteinyl-tyrosyl bond as a point of proteolytic attack[22]. Others have considered this process as subsidiary to cleavages in the acyclic portion of the oxytocin molecule[10,19,33]. Our experiments show that in SPM preparations of the rat limbic brain aminopeptidase action is the major enzymatic mechanism in the conversion of oxytocin. The prevalance of this enzyme activity over the action of C-terminal cleaving peptidases in the SPM preparations is reflected by the time course of oxytocin digestion, which shows only a slow accumulation of glycinamide compared to the rapid release of tyrosine. The aminopeptidase activity has a 12-fold higher V_{max}. The subcellular distribution of this aminopeptidase activity showed enrichment in SPM, microsomal and synaptosomal preparations; its activity was less pronounced in the soluble and mitochondrial preparations. In the latter fractions the C-terminal cleaving activities prevailed.

The distribution of oxytocin converting enzyme activities appeared regionally differentiated in the brain. Both highest C-terminal cleaving peptidase activity and aminopeptidase activity were observed in the medial basal hypothalamus. Further experiments are required to establish whether these enzyme activities correlate with sites of oxytocin action.

Apart from the direct demonstration of an oxytocin converting aminopeptidase activity in the brain by Plíska et al.[22], several studies exist, which seem to suggest that oxytocin can serve as a substrate for L-cystine arylamidase, an enzyme with aminopeptidase-like action[15,16]. This enzyme is competitively inhibited by oxytocin and has been implicated in the degradation of luteinizing hormone–releasing hormone (LH–RH). Competition between oxytocin and LH–RH for the same enzyme system has been reported[8,11]. Preliminary data indicate, however, that the aminopeptidase described in this report requires the total oxytocin structure for full activity (Burbach, unpublished observations).

The action of the C-terminal cleaving peptidase in the SPM preparations resulted mainly in the release of glycinamide. Only minor amounts of the peptide Leu-GlyNH_2 were present in the digests. Koida and Walter have demonstrated that a post-proline cleaving enzyme isolated from lamb kidney cleaves specifically the prolyl-leucyl bond of oxytocin to form Leu-GlyNH_2[12]. In the brain, production of this dipeptide was

mainly detectable in the soluble cell fraction and in the microsomal fraction[19,20,33]. In our experiments with SPM preparations a minor amount of Leu-GlyNH$_2$ was consistently present at all time intervals tested, while the amount of glycinamide accumulated. This may support the suggestion that the formation of Leu-GlyNH$_2$ is the rate-limiting step in the cleavage of oxytocin in its C-terminal portion followed by a rapid cleavage of the dipeptide by an exopeptidase[18,19]. Formation of Leu-GlyNH$_2$ in the brain may be of physiological significance since this peptide and its enzymatically stable derivative cyclo-(Leu-Gly) display activities in several behavioral paradigms[9,23].

Walter et al. observed that hypothalamic particulate fractions contained an activity which could release the C-terminal tripeptide Pro-Leu-GlyNH$_2$ from oxytocin[33]. The involvement of a particulate bound aminopeptidase was suggested[4,33]. It was proposed that this enzyme mediated the intracellular processing of oxytocin in the biosynthesis of the MSH-release inhibiting factor[30]. We did not detect formation of Pro-Leu-GlyNH$_2$ during incubation of oxytocin with SPM preparations. Our experiments, however, were designed to investigate the conversion of oxytocin at its putative target sites on the brain cell membranes. Accordingly, a different population of enzymes can be assumed to function at these target sites from those in intracellular biosynthetic mechanisms.

Peptidase action at the SPM leads to the formation of an oxytocin fragment of intermediate size. This peptide contains the C-terminal glycinamide, but is devoid of the tyrosine residue. The accumulation of this product, termed OXT$_{x-9}$, in oxytocin digests resembles the release of tyrosine with regard to pH dependency, subcellular and regional distribution. This similarity suggests the involvement of the tyrosine releasing aminopeptidase in the formation of OXT$_{x-9}$. However, no strict data on its mere formation were obtained in these experiments since subsequent degradation by C-terminal cleaving enzymes results in the loss of its radioactive label. This phenomenon is reflected by the time course of its accumulation, which proceeds slower than that of the release of tyrosine and by the K_m and V_{max} values. Therefore the enzyme(s) responsible for its formation may have a role in the modulation of oxytocin action in the limbic brain. The chemical identification and the characterization of its CNS activities are in progress.

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