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OXYTOCIN BIOTRANSFORMATION IN THE RAT LIMBIC BRAIN: CHEMICAL CHARACTERIZATION OF TWO OXYTOCIN FRAGMENTS AND PROPOSED PATHWAY FOR OXYTOCIN CONVERSION

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SUMMARY

Two peptide fragments of oxytocin were isolated by high-pressure liquid chromatography from digests of oxytocin obtained after exposure to a SPM preparation of the rat limbic brain. The structures of these peptides, being Gln-Asn-Cys(O)$_x$-Pro-Leu-GlyNH$_2$ and Gln-Asn-Cys(-S-S-Cys)-Pro-Leu-GlyNH$_2$, were assessed by quantitative amino acid analysis, combined with the determination of N-terminal end groups and cysteic acid residues after performic acid treatment. The fragments comprised the 4-9 and 1,4-9 sequences of oxytocin, respectively. The types of proteolytic enzymes involved in their formation are discussed and a pathway for the conversion of oxytocin by SPM is proposed.

INTRODUCTION

The neurohypophyseal hormones, oxytocin and vasopressin, have been shown to affect brain mechanisms related to learning and memory (1,2). Limbic and midbrain structures are thought to mediate these processes. Fragments of both hormones, which are devoid of the classical endocrine effects of their parent molecules, elicit central effects as well. Therefore, proteolytic fragmentation of the neurohypophyseal hormones might be a regulating factor of their activities in the central nervous system.

In previous studies, we have partially characterized SPM associated peptidases which are involved in the proteolytic conversion of oxytocin (3,4).

Abbreviations: SPM = synaptosomal plasma membrane(s), HPLC = high-pressure liquid chromatography, DNS- = dansyl-, 1-dimethylaminonaphthalene-5-sulfonyl-. The amino acid residue numbers refer to the primary structure of oxytocin: Cys^1-Tyr-Ile^3-Gln-Asn^5-Cys-Pro^7-Leu-GlyNH$_2$^{9}.

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In these experiments the accumulation of an oxytocin fragment of unknown structure was observed in the digests. This peptide contained the C-terminal glycinamide residue, it was devoid of the tyrosine-2 residue and chromatographically distinct from the C-terminal tri- and dipeptides, Pro-Leu-$GlyNH_2$ and Leu-$GlyNH_2$. We termed this fragment tentatively oxytocin x-9 (OXT x-9).

The present paper describes the isolation of multiple OXT x-9 components from oxytocin digests and their chemical characterization. These and the previously described data on oxytocin converting peptidase activities allowed to point our routes in a pathway for oxytocin conversion mediated by SPM preparations of the rat limbic brain.

MATERIALS AND METHODS

Synthetic oxytocin, Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-$GlyNH_2$, and its fragments 1-8, 1-7, 1-6, 7-9 and 8-9 were kindly provided by Dr. H.M. Greven and Dr. J.W. van Nispen (Organon International B.V., Oss, The Netherlands). [9-$GlyNH_2$-1-^{14}C]-oxytocin was a generous gift of the late Dr. R. Walter (University of Illinois, Chicago, USA).

A SPM fraction from rat limbic brain tissue (hippocampus, hypothalamus plus septum) was prepared by the method of Zwiers et al. (5).

Oxytocin ($2x10^{-5}$ M; $4x10^4$ dpm of [^{14}C-$GlyNH_2$]-oxytocin was incubated with SPM preparations (1.7 mg protein/ml) at 37°C for 3 h in 1.5 ml 25 mM sodium phosphate buffer, pH 6.9 . The reaction was terminated by heating the suspension in a boiling water bath for 10 min and membranes were removed by centrifugation. The supernatant was subjected to HPLC fractionation.

Reversed-phase HPLC of the oxytocin digests was carried out on a μBondapak C_{18} column (0.39 x 30 cm), which was eluted with a linear gradient over 25 min of 5% to 40% acidified methanol (1.5 ml acetic acid per liter methanol) in 10 mM ammonium acetate, pH 4.15, at a flow rate of 2 ml/min. The eluate was monitored continuously by UV absorbance at 210 nm and fractions of 1.0 ml were collected. Aliquots of the fractions were subjected to scintillation counting. At the guidance of the radioactivity distribution fractions were pooled and lyophilized after removal of methanol in vacuo at 60°C.

Freeze dried samples (circa 2 nmoles) were hydrolyzed with 6 M hydrogen chloride containing 1 μM thioglycolic acid in evacuated glass tubes in a constant boiling toluene bath (110°C) for 22 h as described by Zwiers et al. (6). The amino acid composition was determined by automated analysis (TSM, Technicon) using a stainless steel column (0.21 x 18 cm) packed with Durrum DC-5A resin and fluorescence detection with o-phtaldialdehyde (7). Quantification using a computing integrator (Pye Unicam, DP101) was achieved by comparison to norleucine as internal standard and to standard mixtures of amino acids.

The oxidative cleavage of disulfide bridges with performic acid was carried out as described previously (8,9). Briefly, performic acid was prepared by mixing 1 volume of 30% hydrogen peroxide and 19 volumes of 99% formic acid and was allowed to stand at room temperature for 2 h in a closed vessel. Performic acid (50 μl) was added to the peptide material (3-4 nmoles) which was dissolved in 25 μl 99% formic acid containing 5 μl anhydrous methanol.

The reaction was carried out at -5°C to -10°C for 2.5 h and was terminated by adding 400 µl distilled water. The samples were subsequently lyophilized.

Dansylation of N-terminal amino acids was carried out essentially according to Gray and Smith (10). After hydrolysis and lyophilization the residues routinely were taken up in 25 µl distilled water and extracted with 10 µl ethylacetate. For the identification of DNS-cysteic acid 10 µl pyridine-water (1 : 1, v/v) or ethanol-water-acetic acid (100 : 5 : 3, v/v/v) was added to the dried residues. DNS-amino acids were identified on double-faced micropolyamide F-1700 sheets (5 x 5 cm; Schleicher & Schüll), which were developed in a three solvent system (11). DNS-cysteic acid was resolved from DNS-OH with a fourth solvent, 1 M ammonium hydroxyde-ethanol (1 : 1, v/v), run in the second dimension (11).

RESULTS

Incubation of [^{14}C-GlyNH$_2$]-oxytocin with a SPM preparation of the rat limbic brain yielded products retaining the C-terminal ^{14}C-glycinamide, as was seen from the distribution of the radioactivity after HPLC fractionation of the digests (fig. 1B). Several components were also detectable by UV absorbance at 210 nm (fig. 1A). Fractions were pooled as indicated in fig. 1B. Free glycinamide eluted in the void volume of the column (fractions 3-6), while intact oxytocin was collected in fraction E (fractions 53-57). OXT x-9, which was previously detected as a single peak by high-voltage paper electrophoresis, appeared heterogenous after HPLC fractionation. It was resolved into two components, fraction B and fraction C (fig. 1B). These fractions represented the principal digestion products. In order to prepare sufficient peptide material for structural analyses, incubations of oxytocin, omitting radiolabeled oxytocin, were carried out and fractions B and C were obtained as indicated by the HPLC distribution of radiolabeled products from parallel ^{14}C-oxytocin incubations. As calculated from the specific activity of ^{14}C-oxytocin, the yields of fraction B and fraction C from a single incubation were 1.6 nmoles and 1.8 nmoles, respectively.

Dansyl end group determination of fractions B and C showed a single DNS-glutamic acid residue. This demonstrated the purity of peptides in the respective fractions and indicated that the N-terminal amino acid of both peptides was the glutamine-4 residue of oxytocin. In fraction C, however, an additional, faintly fluorescent spot was visible, which did not co-

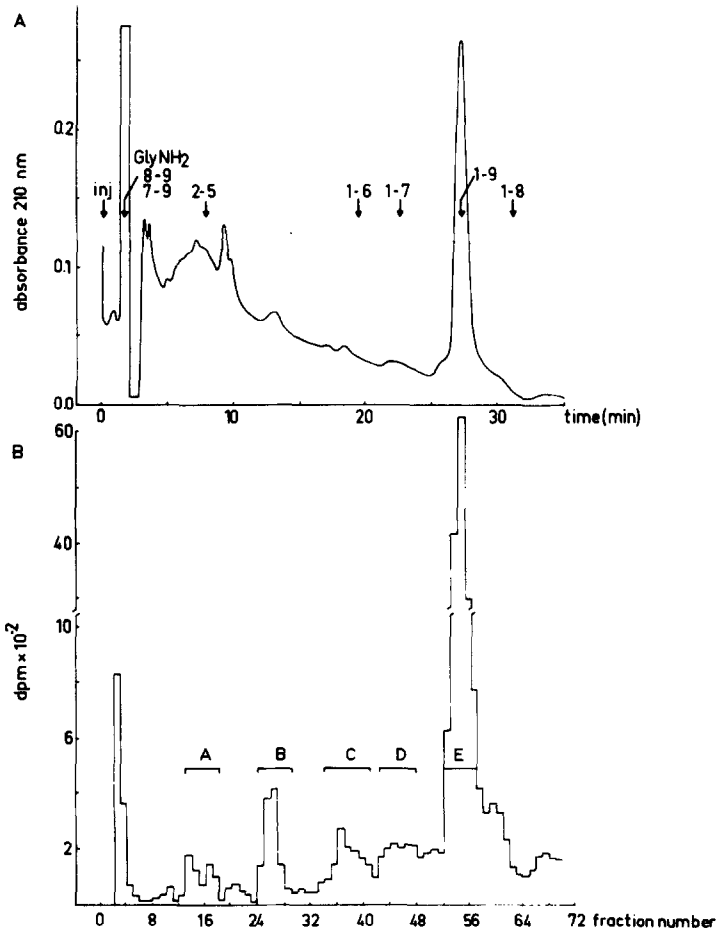

Figure 1. HPLC fractionation of a digest of oxytocin which was obtained by incubation with a SPM preparation for 3 h at pH 6.9. Chromatography was performed by gradient elution with acidified methanol and ammonium acetate buffer. The HPLC eluate was monitored continuously by UV absorbance (A) and aliquots of the collected fractions were counted for radioactivity (B). For experimental details see Methods. Fractions were pooled as indicated by the horizontal bars (fractions A to E). The vertical arrows show the elution position of synthetic oxytocin and related fragments.

migrate with one of the reference DNS-amino acids. The amino acid compositions of fraction B and fraction C shown in table I indicated that the peptide in fraction B as well as that in fraction C comprised the 4 to 9 sequence of oxytocin, Gln-Asn-(Cys)-(Pro)-Leu-GlyNH_2. This finding was surprising as the two peptides differed in mobility in the HPLC system. However, cysteine, cystine and proline residues were not detected with the o-phtaldialdehyde

Table I

AMINO ACID COMPOSITION OF HPLC FRACTIONS

amino acid	position in oxytocin	fraction B	fraction C
Cys+	1,6	-	-
Tyr	2	0.0	0.1
Ile	3	0.2	0.3
Glx	4	1.0	1.0
Asx	5	0.7	0.8
Pro+	7	-	-
Leu	8	0.6	0.7
Gly	9	1.3	1.2

+Cysteine and proline are not detected by the o-phtaldialdehyde fluorescence method.
The indicated numbers of residues were calculated by dividing the pmole amounts obtained in the analyses by the highest common factor.
The amino acids are those contained in the primary structure of oxytocin.

reagent in amino acid analyses. It was anticipated that the difference between the components could be located in the cysteine residues.

To investigate the possibility of a disulfide bridge in these peptides, samples were subjected to the oxidative cleavage reaction with performic acid prior to dansylation and acid hydrolysis. In these experiments fractions B and C were compared with synthetic oxytocin, oxytocin 1-6, arginine-vasopressin, vasotocin, cystine and cysteic acid. Thin layer chromatography was used to identify DNS-cysteic acid as described in the method section. In this experimental design, in addition to the DNS-glutamic acid residues, fraction C contained a fluorescent spot, which co-migrated with that obtained from the reference peptides. This spot was identified as DNS-cysteic acid and it was absent in fraction B. As expected, the treated reference peptide samples showed additionally the ortho-DNS-tyrosine derivative.

Based on amino acid compositions, radioactivity, N-terminal end groups and cysteic acid content, the following structures are proposed:

fraction B: Gln-Asn-Cys(O)$_x$-Pro-Leu-GlyNH$_2$, (x = 0,1,2,3),

fraction C: Gln-Asn-Cys(S-S-Cys)-Pro-Leu-GlyNH$_2$.

DISCUSSION

Oxytocin is converted by peptidases, which are associated with SPM preparations obtained from the rat limbic brain (3,4). During this proteolytic process amino acid residues are released and several peptide fragments accumulate. In the present study two main fragments, which have been detected previously as a single component after high-voltage paper electrophoresis (4), were resolved and isolated by HPLC and their structures were assessed.

The combined data from different techniques allowed to determine the primary structure of the two peptides. The 4 to 9 sequence of both peptides, which was indicated by their amino acid composition, was in agreement with the presence of the N-terminal glutamic acid and the C-terminal glycinamide residues as detected by dansylation and ^{14}C-radioactivity, respectively. However, the peptides differed in their cysteine residues. The presence of cysteic acid in fraction C after treatment with performic acid, dansylation and acid hydrolysis suggested that this residue was attached to the peptide by a disulfide bridge. Therefore, it may represent the cysteine-1 residue of oxytocin, which is linked to cysteine-6. This cysteine-1 residue was absent in fraction B, indicating that the disulfide bridge of this peptide was cleaved off prior to isolation. The remaining sulfhydryl group of cysteine-6, however, is probably very susceptible to oxidation. As in the experiments no precautions have been made to prevent oxidation, it is suggested that the cysteine-6 residue occurs in an oxidized form, indicated as $Cys(O)_x$ in the proposed structure.

The two isolated peptides lack the tyrosine and isoleucine residues of the oxytocin molecule. In previous studies the successive release of these amino acids from oxytocin has been observed and the involvement of an aminopeptidase activity has been suggested (3,4). The conserved disulfide bridge of the peptide oxytocin 1,4-9 indicates that the aminopeptidases involved in its formation do not require prior reduction of this bond. Little is known about brain aminopeptidases with such a specificity. Pliska et al.

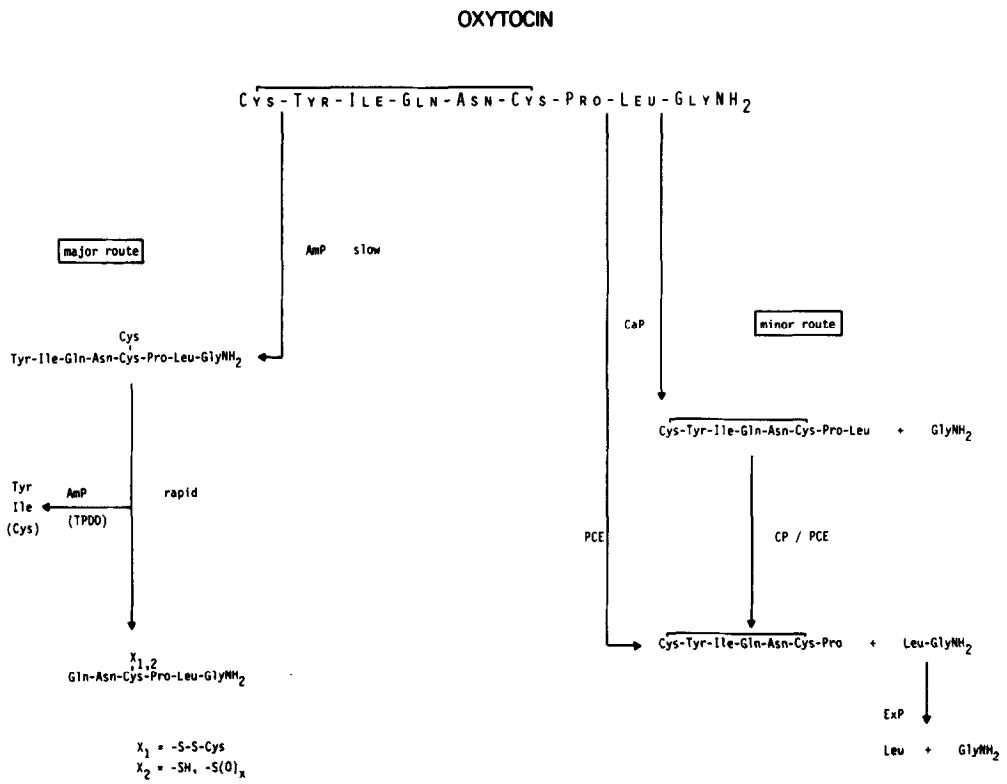

Figure 2. Pathway for oxytocin conversion by SPM preparations of rat brain. The proteolytic events occurring in the N-terminal and C-terminal parts of the oxytocin molecule are presented as separate routes. Several types of activities, which may be involved in the conversion process, are indicated:
AmP = aminopeptidase (12)
CaP = carboxamidopeptidase (18,19)
PCE = post-proline cleaving enzyme (20)
CP = carboxypeptidase
ExP = exopeptidase
TPDO = thiol:protein-disulfide oxidoreductase (16,17)

have demonstrated the cleavage of the cysteinyl-tyrosyl bond in the intact ring portion of oxytocin by minced cerebral cortex preparations (12). Other studies suggest that arylamidases, enzymes with aminopeptidase-like activity, can act on oxytocin (13,14,15). One such enzyme, purified from brain, released slowly the tyrosine and isoleucine residues from oxytocin in the presence of sulfhydryl-reducing agents and acted rapidly on open nona- and octapeptides, suggesting that the reduced hormone served as substrate (15).

Under our experimental conditions the degradation of oxytocin proceeds relatively slowly, while the isoleucine residue appears rapidly after the

release of tyrosine (3,4). In these experiments we did not detect the open nonapeptide oxytocin 1,2-9. These arguments may be indicative of a slow initial step, being the cleavage of the cysteinyl-tyrosyl bond, and of rapidly proceeding aminopeptidase action till the glutamine residue is encountered. This mechanism does not require reduction of the disulfide bridge. However, the detection of oxytocin 4-9 in equimolar amounts to oxytocin 1,4-9 indicates that reduction occurs and suggests that a thiol:protein-disulfide oxidoreductase-like enzyme (16,17) is present.

Recently, we have demonstrated that in isolated SPM preparations of rat brain aminopeptidase action prevails over cleavages in the C-terminal portion of oxytocin (3,4). In these studies constant low amounts of the C-terminal dipeptide Leu-GlyNH_2 and accumulating amounts of glycinamide were found, supporting the view of Leu-GlyNH_2 formation by a post-proline cleaving enzyme activity (20) followed by rapid cleavage of the dipeptide by unidentified exopeptidases (15). Moreover, oxytocin 1-8 was a minor component in the digests, which is in favour of the involvement of a carboxamidopeptidase activity (18,19). The various convertive routes are summarized in a proposed pathway and several types of proteolytic enzymes, which are known to act on oxytocin, are indicated (fig. 2).

It has been postulated that the neurohypophyseal hormones may serve as precursor molecules for peptides with specific functions in the brain (21,22). Several synthetic fragments retain behavioural activities, which in some cases are distinctly different from oxytocin itself, such as the dipeptide Leu-GlyNH_2 (1,2). Therefore, the enzymes, involved in the conversion of oxytocin may have a modulatory role in the central effects of this neuropeptide.

REFERENCES

1. van Ree, J.M., Bohus, B., Versteeg, D.H.G., and de Wied, D. (1978) Biochem. Pharmacol. 27, 1793-1800.
2. de Wied, D., and Versteeg, D.H.G. (1979) Fed. Proc. 38, 2348-2354.
3. Burbach, J.P.H., de Kloet, E.R., and de Wied, D. (1979) Acta Endocrinol. Suppl. 225, 250.

4. Burbach, J.P.H., de Kloet, E.R., and de Wied, D. (1980) Brain Res., in press.
5. Zwiers, H., Veldhuis, H.D., Schotman, P., and Gispen, W.H. (1976) Neurochem. Res. 1, 669-677.
6. Zwiers, H., Schotman, P., and Gispen, W.H. (1980) J. Neurochem. 34, 1689-1699.
7. Zwiers, H., Verhoef, J., Schotman, P., and Gispen, W.H. (1980) FEBS Lett. 112, 168-172.
8. Hirs, C.H.W. (1967) In: Methods in Enzymology, Vol. XI, Enzyme Structure (Hirs, C.H.W., ed.), pp. 197-199, Academic Press, New York and London.
9. Scoffone, E., and Fontana, A. (1975). In: Molecular Biology, Biochemistry and Biophysics, Vol. 8, Protein Sequence Determination (Needleman, S.B., ed.), pp. 162-201, Springer Verlag, Berlin.
10. Gray, W.R., and Smith, J.F. (1979) Anal. Biochem. 33, 36-42.
11. Narita, K., Matsuo, H., and Nakajima, T. (1975) In: Molecular Biology, Biochemistry and Biophysics, Vol. 8, Protein Sequence Determination (Needleman, S.B., ed.), pp. 30-103, Springer Verlag, Berlin.
12. Pliska, V., Thorn, N.A., and Vilhardt, M. (1971) Acta Endocrinol. 67, 12-22.
13. Kuhl, H., and Taubert, H-D. (1975) Acta Endocrinol. 78, 634-648.
14. Kuhl, H., Sandow, J., Krauss, B., and Taubert, H-D. (1979) Neuroendocrinology 28, 339-348.
15. Marks, N., Abrash, L., and Walter, R. (1973) Proc. Soc. Exp. Biol. Med. 142, 455-460.
16. Branda, L.A., Ferrier, B.M., and Celhoffer, L. (1972) Can. J. Biochem. 50, 507-509.
17. Small, C.W., and Watkins, W.B. (1974) Nature 251, 237-239.
18. Glass, J.D., Schwartz, I.L., and Walter, R. (1969) Proc. Natl. Acad. Sci. USA 63, 1426-1430.
19. Simmons, W.M., and Walter, R. (1980) Biochemistry 19, 39-48.
20. Koida, M., and Walter, R. (1976) J. Biol. Chem. 251, 7593-7599.
21. Walter, R. (1974) In: Psychoneuroendocrinology (Hatotani, N., ed.), pp. 285-294, Karger, Basel.
22. de Wied, D. (1977) Life Sci. 20, 195-204.