Biologically Active Macromolecular Forms of Oxytocin

[8-LYSINE]OXYTOCIN AS A SUITABLE LIGAND

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[8-Lysine]oxytocin was synthesized on a solid support and possessed an oxytocic activity of 100 ± 6 units/ μ mol on the isolated rat uterus. The ϵ -carbamoyl, ϵ -3-carboxypropionyl and ϵ -3-carboxybutyryl derivatives were prepared and had uterotonic activities of 400, 55 and 50 units/ μ mol respectively. [8-Lysine]oxytocin was coupled unambiguously through the ϵ -amino group to the carboxyl groups of carboxymethylated dextrans or ϵ -3-carboxypropionyl-gelatin. The macromolecular oxytocins were water-soluble and retained significant oxytocic activity. [8-Lysine]oxytocin should prove a useful ligand for affinity chromatography of oxytocin-binding proteins.

Peptide hormones coupled to soluble or insoluble macromolecules have been used as antigens for raising antibodies to low-molecular-weight hormones (Williams & Chase, 1967; Vaneckova *et al.*, 1975), for affinity chromatography of solubilized receptors (Cuatrecasas & Parikh, 1974), and to determine the accessibility of a hormone receptor to high-molecular-weight forms of the hormone which are unable to traverse the plasma membrane (Cuatrecasas & Hollenberg, 1976). For application in receptor studies it is essential that the macromolecular hormone retains biological activity, but the difficulty in obtaining an active product generally increases as the molecular weight of the hormone decreases. Consequently the peptide hormones successfully conjugated to macromolecules are those of molecular weight greater than 3000, which are less sensitive to modification, e.g. insulin (Cuatrecasas, 1973), glucagon (Johnson *et al.*, 1972), corticotropin (Selinger & Given, 1971), nerve-growth factor (Frazier *et al.*, 1973) and growth hormone (Bolanger & Fellows, 1975). The most widely used method of coupling has involved activation of polysaccharides by CNBr at alkaline pH (Axen *et al.*, 1967); the linkage formed, however, is not completely stable and in the presence of nucleophiles can give rise to leakage of hormone (Cuatrecasas & Hollenberg, 1976; Wilchek *et al.*, 1975). If the hormone becomes detached from the support in an active form, and indeed some have been reported to be super-active (Wilchek *et al.*, 1975), an erroneous estimate of the activity of the macromolecular hormone may be made. Thus a useful macromolecule-coupled hormone should be linked to its support via a stable covalent bond and retain biological activity.

Here we report an investigation of the use of [8-lysine]oxytocin for the immobilization of oxy-

tocin. It was already known that the leucine residue at position 8 can be replaced without marked effect on biological potency (Schwartz & Livingston, 1964), and this position was therefore considered a possible point for attachment of oxytocin to a macromolecule. When coupled through the ϵ -amino group to 3-carboxypropionyl-gelatin or carboxymethylated dextran via a stable amide bond, the 8-lysine analogue retained significant biological activity. The results support the view that the myometrical receptor is located in the plasma membrane.

Experimental and Results

Materials

Merrifield resin (2% cross-linked, 200–400 mesh, 1.6 mmol of chloride/g) was purchased from Bachem Fine Chemicals, Marina del Ray, CA, U.S.A. [3 H]-Isoleucine (12 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. T40 and T500 dextrans were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. Gelatin was obtained from Leine Bros., London, U.K., synthetic oxytocin standard (450 units/ μ mol; 10 units/ml) from Sandoz, Feltham, Middx., U.K., and stilboestrol (5 mg/ml in almond oil) from Macarthy Ltd., Romford, Essex, U.K.

Trifluoroacetic acid, trimethylamine, dichloromethane, chloroform, dimethylformamide and dimethyl sulphoxide were dried and redistilled before use.

Isolated uterus assay

Oxytocin assays were performed on the isolated rat uterus by the method of Holton (1948), Mg^{2+} -free Van Slyke–Hastings solution being used in the bathing medium (Munsick, 1960). Sprague–Dawley

rats were maintained in oestrus by prior injection of stilboestrol.

Synthesis of α -Boc-[8-lysine]oxytocin*

[8-Lysine]oxytocin was synthesized by the solid-phase method (Merrifield, 1963, 1969). The α -amino group of all amino acids was protected by the Boc substituent (Schnabel, 1967), the side chains of tyrosine and cysteine were protected by the benzyl group and the ϵ -amino group of lysine as the benzyloxycarbonyl derivative. Boc-glycine (0.59 g; 3.6 mmol) was esterified to the chloromethylated resin (4 g; 3.6 mmol) as the caesium salt (Gisin, 1973) to give a glycine substitution of 0.2 mmol/g of resin. The *N*-terminal Boc group of the peptide resin was removed during each cycle by 25% (v/v) trifluoroacetic acid in dichloromethane by using a 5 min pretreatment followed by a 30 min exposure. A wash with 10% (v/v) triethylamine in chloroform was included before the next coupling to remove residual acid. Boc-amino acids were coupled in 4-fold molar excess, based on the glycine content of the resin. Dicyclohexylcarbodi-imide (Sheehan & Hess, 1955) was used in 4.4-fold excess as coupling agent and each coupling was allowed to proceed for 4 h. Boc-asparagine and Boc-glutamine were coupled as the *p*-nitrophenyl esters (Bodanszky & du Vigneaud, 1959a,b) in dimethylformamide by using a 4-fold molar excess of reagent. After each di-imide coupling and during the course of the active ester couplings the extent of reaction was monitored by using the Kaiser ninhydrin test (Kaiser *et al.*, 1970). In most cases two 4-fold couplings were required, although isoleucine and *O*-benzyltyrosine required an additional coupling. [^3H]Isoleucine was used to facilitate subsequent purifications, the first coupling using 1 molar equivalent of Boc-isoleucine containing approx. 250 μCi of ^3H . To complete the reaction two further couplings were carried out with carrier Boc-isoleucine. The active ester reactions were complete within 12 h. After each amino acid had been incorporated, unchanged amino groups were masked by acetylation by using a 3-fold excess of *N*-hydroxysuccinimide acetate in dimethylformamide for 6 h.

At the end of the synthesis the fully protected peptide was removed from the resin as the amide by a 24 h treatment with methanol (20 ml/g of resin) saturated at -5°C with ammonia (Stewart & Young, 1969). The resin was filtered off, washed with dimethylformamide and the combined filtrates were evaporated to dryness. The residue was chromatographed on Sephadex LH 20 (column dimensions 140 cm $\times$ 2.5 cm) in dimethylformamide. The eluted peptides were detected by monitoring the absorbance of the eluate at 277 nm, the fractions constituting the major peak were combined and the protected peptide was precipitated with ether as an amorphous powder.

* Abbreviation: Boc, *t*-butoxycarbonyl.

The benzyloxycarbonyl and benzyl protecting groups were removed by reduction with sodium in liquid ammonia (Sifferd & du Vigneaud, 1935; du Vigneaud *et al.*, 1954), care being taken to avoid excessive reaction. The ammonia was removed by evaporation *in vacuo*, the residue dissolved in 0.1 M-acetic acid and the pH of the solution adjusted to 6.8 with conc. ammonia. The cysteine side chains were oxidized to the corresponding disulphide by titration with 0.028 M-potassium ferricyanide at high dilution (peptide concentration 0.1 mM) to favour the formation of intramolecular disulphide, the end point being determined by testing samples for excess of ferricyanide from Prussian Blue formation on addition of FeSO_4 . The reaction mixture was passed through a column (4 cm $\times$ 2 cm) of Bio-Rad AG3 (X4; Cl^- form) to remove ferricyanide and ferrocyanide ions. The resin was washed with water, and the eluate and washings were concentrated and chromatographed on Sephadex G-25 (column dimensions 40 cm $\times$ 1.5 cm) in 50% (v/v) acetic acid at 4°C . The absorbance of the eluate at 277 nm was monitored to detect the eluted peptides. Two peaks, (elution volumes 58 ml and 46 ml) were obtained corresponding to the required monomer and its dimer; the monomer was combined and evaporated *in vacuo*. The peptide was chromatographed on Sephadex CM-25 (column dimensions 122 cm $\times$ 1.5 cm) eluted with 0.5 M-ammonium acetate, pH 6.8 (Fig. 1). The fractions of the major peak were combined, concentrated by evaporation *in vacuo* at 0.5 mmHg pressure, and desalted on Sephadex G-25 (column dimensions 40 cm $\times$ 1.5 cm) in 50% (v/v) acetic acid at 4°C , and then chromatographed on Sephadex G-15 (column dimensions 150 cm $\times$ 2 cm) in 0.2 M-acetic acid to give the required α -Boc-[8-lysine]oxytocin.

Fig. 1. Chromatography of [8-lysine]oxytocin on Sephadex CM-25

The column (122 cm $\times$ 1.5 cm) was eluted with 0.5 M-ammonium acetate, pH 6.8 (0.5 M-acetic acid adjusted with conc. ammonia), with a flow rate of 18 ml/h; fractions (2.25 ml) were collected.

A sample hydrolysed in 6M-HCl for 16h at 110°C and analysed by the procedure of Spackman *et al.* (1958) gave the following molar proportions: aspartic acid; 1.00; glutamic acid, 0.96; proline, 0.97; glycine, 1.02; half-cysteine, 1.98; isoleucine, 0.86; tyrosine, 0.93; lysine, 1.03. After removal of the Boc group, the homogeneity of the preparation was checked by high-voltage paper electrophoresis at pH 6.5 in 2.5M-pyridine/acetate, at 20°C. The product had a mobility of 0.62 relative to lysine.

Synthesis of α -Boc- ϵ -carbamoyl-[8-lysine]oxytocin

α -Boc-[8-lysine]oxytocin (0.5mg) was dissolved in 0.5M-potassium cyanate (1ml) and the reaction mixture was maintained at pH 8 for 16h at 50°C (Stark & Smyth, 1963). The solution was acidified with 3M-HCl to pH 4.5 and maintained at 25°C for 1h to destroy excess of cyanate. The pH was re-adjusted to 8.5 with 4M-NaOH, and after 4h the reaction mixture was concentrated *in vacuo* and desalted on Sephadex G-25 (column dimensions 25cm $\times$ 1cm) in 50% (v/v) acetic acid at 4°C. Chromatography was carried out on Sephadex CM-25 (column dimensions 100cm $\times$ 1cm) in 0.5M-ammonium acetate, pH 6.8. The purified α -Boc- ϵ -carbamoyl-[8-lysine]oxytocin (elution volume 24ml; 85% yield) was desalted on Sephadex G-25 (column dimensions 25cm $\times$ 1cm) in 50% (v/v) acetic acid at 4°C.

Synthesis of α -Boc- ϵ -3-carboxypropionyl- and α -Boc-3- ϵ -carboxybutyryl-[8-lysine]oxytocin

α -Boc-[8-lysine]oxytocin (0.5mg) was dissolved in 0.1M-acetic acid (1ml) the pH adjusted with 1M-NaOH to 8, and succinic or glutaric anhydride (10mg) was added in dioxan (1ml). The pH was maintained for 16h by automatic addition of 0.1M-NaOH at 25°C. The reaction mixture was concentrated and desalted on Sephadex G-25 (column dimensions 25cm $\times$ 1cm) in 50% acetic acid at 4°C and chromatographed on Sephadex CM-25 (column dimensions 100cm $\times$ 1cm) in 0.5M-ammonium acetate, pH 6.8. The purified α -Boc- ϵ -3-carboxypropionyl- or α -Boc- ϵ -3-carboxybutyryl-[8-lysine]oxytocin (elution volumes 26ml and 23ml; yields 90 and 83% respectively) was desalted on Sephadex G-25 (column dimensions 25cm $\times$ 1cm) in 50% acetic acid at 4°C. To confirm purity, the peptides, after removal of the Boc group, were submitted to high-voltage electrophoresis. The products were shown to be free from ϵ -acetyl-[8-lysine]oxytocin, which could have resulted from exposure to a mixed anhydride formed during the reaction.

Oxytocic activity of [8-lysine]oxytocin derivatives

Each α -Boc peptide (1mg) was treated with trifluoroacetic acid (1.5ml) for 45min, and the acid was removed by evaporation *in vacuo*. The deprotected peptides were taken up in deionized water and dilu-

tions performed in Mg²⁺-free Van Slyke-Hastings solution (Munsick, 1960) for assay (Table 1).

Carboxymethylation of T40 and T500 dextrans

A stock solution of dimsyl (methylsulphonyl-carbonium) sodium was prepared by addition of ether washed, 50% (w/w) mineral oil-dispersed NaH (2.64g) to dimethyl sulphoxide (10ml) (Hakanori, 1964; Corey & Chaykosky, 1962). The solution was stirred under an atmosphere of N₂ at 75°C for 40min to give a light-brown solution. T40 or T500 dextran (5g) was dissolved in dimethyl sulphoxide (20ml) by warming and the dimsyl sodium solution added. After 3h iodoacetic acid (5.7g) in dimethyl sulphoxide (10ml) was added and the reaction allowed to proceed for a further 16h. The solution was diluted with an equal volume of water and dialysed overnight against tap water. A little insoluble material was removed by filtration and the dextran derivatives were freeze-dried. I.r. spectroscopy confirmed the presence of substantial amounts of carboxyl groups in both dextrans.

3-Carboxypropionylation of gelatin

Gelatin (5g) was dissolved in water (100ml), the pH of the solution was adjusted to 8 and succinic anhydride (10g) was added in portions with vigorous stirring, the pH being maintained at 8 by automatic addition of 1M-NaOH. When the reaction was complete the solution was dialysed against tap water, and then against distilled water, and finally freeze-dried.

Coupling of [8-lysine]oxytocin to macromolecules

The coupling method was the same for each macromolecule: 3-carboxypropionyl-gelatin or carboxymethyl-dextran (7mg) was dissolved in 0.2M-NaHCO₃ buffer, pH 10 (0.1ml), and α -Boc-[8-lysine]oxytocin (1mg) and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodi-imide-toluene-*p*-methosulphonate (4.2mg) added. To remove unchanged starting material the 24h reaction mixture was chromatographed on Sephadex G-50 (column dimensions 50cm $\times$ 1cm; Sephadex G-75 was used for T500-dextran coupling)

Table 1. Activity of synthetic oxytocin analogues in isolated rat uterus assay

Analogues were synthesized and tested as described in the text.

Oxytocin	Activity (units/ μ mol)
Oxytocin	500
[Lys ⁸]oxytocin	100 $\pm$ 6
ϵ -Carbamoyl-[Lys ⁸]oxytocin	450 $\pm$ 15
ϵ -3-Carboxypropionyl-[Lys ⁸]oxytocin	55 $\pm$ 4
ϵ -3-Carboxybutyryl-[Lys ⁸]oxytocin	50 $\pm$ 3.5

in 0.1% (v/v) *N*-ethylmorpholine (see Fig. 2). The α -Boc group was removed from the coupled hormone by treatment with trifluoroacetic acid (1 ml) for 30 min. The deprotected material was isolated by gel filtration on Sephadex G-50 (column dimensions 50 cm $\times$ 1 cm) in 0.1% *N*-ethylmorpholine. The excluded peak, containing 90% of the total radioactivity, was combined and assayed. The concentration of hormone coupled to the macromolecule was obtained by measuring the [3 H]isoleucine in the peptide after solubilization of a sample with Protosol and addition of scintillation fluid, as described in the

Fig. 2. Chromatography of coupling reaction mixture of 3-carboxypropionylated gelatin and α -Boc-[8-lysine]-oxytocin on Sephadex G-50

The column (50 cm $\times$ 1 cm) was eluted with 0.1% *N*-ethylmorpholine: fractions (1 ml) were collected. O, A_{277} ; Δ , 3 H c.p.m. A portion (0.1 ml) from each tube was digested with 0.5 ml of Protosol (New England Nuclear, Boston, MA, U.S.A.); 10 ml of toluene containing 2,5-diphenyloxazole (6 g/l) was added and 3 H radioactivity was counted in a Beckman liquid-scintillation counter.

Table 2. Activity of macromolecule-coupled oxytocins on isolated rat uterus

The number of mol of hormone per mol of macromolecule was calculated by using the tritium incorporation to measure hormone and the absorbance at 230 nm to measure macromolecule. Details of the macromolecular hormone preparation and bioassay are given in the text.

Macromolecule	Mol.wt.*	No. of mol of hormone/mol of macromolecule	Activity (units/ μ mol of hormone)
Gelatin	40000	2.8	0.93 $\pm$ 0.1
T500 dextran	500000	5.2	2.4 $\pm$ 0.5
T400 dextran	40000	1.3	0.24 $\pm$ 0.1

* Nominal values.

legend to Fig. 2. It was notable that the radioactivity in the deprotected macromolecular hormones on rechromatography on Sephadex G-50 emerged at the exclusion volume and there was no detectable radioactivity in the position of the uncoupled hormone. The macromolecular hormones were assayed for oxytocic activity on the isolated rat uterus (Table 2).

Discussion

The specific activity of a hormone coupled to a macromolecule would be expected to be influenced by the molecular weight of the carrier; the decreased mobility may be reflected by decrease in hormone activity. The specific activity also depends on the molar ratio of hormone to macromolecule, higher substitution of the carrier giving rise to higher activity, owing to the greater local concentration of hormone at the receptor. In addition, the topography of the macromolecule in the region of the coupled hormone will determine the compatibility of the macromolecule with the environment at the hormone receptor and thus affect the hormone activity.

[8-Lysine]oxytocin was synthesized and the specific activity was 100 ± 6 units/ μ mol, which is significantly greater than the values previously reported (Boissonas & Huguenin, 1960, 20 units/ μ mol; Kimbrough & du Vigneaud, 1961, 78 units/ μ mol). The presence of lysine at position 8 in this analogue makes possible the introduction of a peptide bond between the ϵ -amino group and the carboxyl groups of a carrier molecule.

Before coupling to a macromolecule, the effect of modification of the ϵ -amino group of [8-lysine]-oxytocin was examined. The ϵ -carbamoyl derivative was found to exhibit almost the full activity of oxytocin (Table 1), in agreement with the value given by Bodanszky & Birkhimer (1962). Replacement of the positive charge on the lysine side chain by a negative charge, however, as in the ϵ -3-carboxypropionyl or ϵ -3-carboxybutyryl derivative, lowered the activity. The results suggested that the region of the receptor adjacent to this side chain of residue 8 can effectively accommodate hydrophobic groups, whereas polar side chains led to less-favourable receptor interactions. The ϵ -amino group of [8-lysine]oxytocin therefore appeared to be a suitable site for macromolecule coupling, and the linkage area on the macromolecule should be non-polar for maximal activity of the coupled hormone.

[8-Lysine]oxytocin coupled to 3-carboxypropionyl-gelatin and carboxymethylated dextrans retained significant oxytocic activity (Table 2). The activity was shown by the Sephadex chromatography to be an intrinsic property of the macromolecular oxytocin and not the result of contamination with non-covalently bound hormone. The specific activities seem to reflect the number of hormone molecules

attached to each macromolecule and are relatively independent of the molecular weight and nature of the carrier. It is notable that the activities of these macromolecular oxytocins linked at position 8 are much higher than those reported by Ferrier & Branda (1975) for 1-deamino[Glu⁴]oxytocin coupled to agarose (0.06 unit/ μ mol), in which the attachment to macromolecule was through the side chain at position 4. The activity observed for [8-lysine]oxytocin coupled to macromolecules is unlikely to be produced by enzymic cleavage of the hormone from the macromolecule, as the onset and offset of contraction in the assay are not slow. That the conjugated oxytocins retain considerable activity implies that the myometrial receptors are accessible to large molecules and are on the surface of the plasma membrane. [8-Lysine]oxytocin coupled through the ϵ -amino group to suitable carboxylated gels should prove valuable in the isolation of the uterine oxytocin receptor.

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