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Photoaffinity labelling of the oxytocin receptor in plasma membranes from rat mammary gland

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Plasma membranes from rat mammary gland containing a high concentration of [^{3}H]oxytocin binding sites (2.8 pmol/mg protein) were used for photoaffinity labelling experiments. Competitive binding experiments show that these receptors bind with high affinity the specific oxytocin agonist [Thr^4, Sar^7]oxytocin and the analogue of 1-deamino-[8-lysine]vasopressin containing a photoreactive azidobenzoyl group (Abz) at the side chain of lysine. The tritium-labelled (50 Ci/mol) photoreactive analogue incorporated into a membrane protein with an apparent relative molecular mass of 65000 ± 3000 Da ($n = 16$). The labelling of this protein was completely suppressed by an excess of oxytocin.

Mammary gland; Oxytocin receptor; Photoaffinity labeling

1. INTRODUCTION

The neurohypophysial nonapeptide oxytocin stimulates the contraction of mammary myoepithelial cells. Previous studies have shown the existence of specific oxygen receptors which bind oxytocin with high affinity (K_D 1–5 nM) [1,2]. A good correlation between the ability of analogues to inhibit [^{3}H]oxytocin binding to these receptors and their milk ejecting potencies has been demonstrated [2].

The number of receptors in the rat mammary gland increases about 100-fold from the first day of pregnancy to late lactation [3]. Mammary membranes containing a high concentration of oxytocin receptors have been used to study the molecular properties of oxytocin receptors. Gel filtration analysis of the detergent solubilized membranes indicated that the solubilized [^{3}H]oxytocin-binding component was present in multiple molecular mass forms [4]: molecular mass values ranged from about 40 to >200 kDa. To determine the functional size of the oxytocin receptor in rat mammary gland membranes, radiation inactivation of [^{3}H]oxytocin binding sites was employed [5]. By this method the molecular mass of the mammary receptor was estimated to be 57.5 ± 3.8 kDa. For further studies and for the isolation of the mammary oxytocin receptor, identification of the receptor protein would be helpful. In this study we used photoaffinity labelling of the membrane bound mammary oxytocin receptor and SDS gel analysis to identify the oxytocin receptor protein and to determine its apparent molecular mass.

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Abbreviations: AVP, [8-arginine]vasopressin; [Mpa^1,Lys(Abz)^8]vasopressin, [1-(3-mercaptopropionic acid), 8-lysine (N^6-azidobenzoyl)]vasopressin; OT, oxytocin

2. MATERIALS AND METHODS

2.1. *Materials*

The photoreactive ligand [^{3}H][Mpa^1,Lys(Abz)^8]vasopressin was prepared by reaction of [Mpa^1,Lys^8]vasopressin with succinimidyl 4-azidobenzoate (50 Ci/mmol, NEN) as described [6]. [^{3}H]Oxytocin (20 Ci/mmol) and [Arg][^{3}H]vasopressin ([^{3}H]AVP) (18 Ci/mmol) were from Amersham. The oxytocin analogue [Thr^4,Sar^7]oxytocin was obtained from Dr Z. Grzonka (University of Gdansk, Poland).

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2.2. *Preparation of mammary membrane fractions*

Rat mammary membranes were prepared from involuted mammary tissue after weaning as described [4] and stored frozen at −80°C.

2.3. *Receptor binding assays*

Mammary gland membranes (80–100 μg protein) were incubated with [^{3}H]OT or with [^{3}H]AVP at 30°C. The binding assay and the analysis of data were performed as has been described for experiments with myometrial membranes [7].

2.4. *Photoaffinity labelling experiments*

Plasma membranes (1 mg) from rat mammary gland containing 2.8 pmol [^{3}H]OT-binding sites/mg protein were incubated at 30°C for 30 min with 7 nM [^{3}H][Mpa^1,Lys(Abz)^8]vasopressin in 350 μl binding buffer (50 mM Hepes, pH 7.6, 10 mM $MnCl_2$). After dilution with 3 ml ice-cold binding buffer containing 5 mM *p*-aminophenyl-alanine as scavenger, the mixture was exposed in a quartz tube to three 1 ms flashes produced in an apparatus for high energy ultraviolet irradiation [8]. After irradiation, plasma membranes were collected by centrifugation at 20000 × *g* for 30 min and the photolabelling was repeated with additional ligand following the same protocol. The photolabelled membrane proteins were separated by SDS gel electrophoresis and the radioactivity was determined as described [7].

3. RESULTS

Fig.1 shows the dose dependent specific binding of [^{3}H]oxytocin to a plasma membrane preparation from rat mammary gland. The data were analyzed by curve fitting procedures (fig.1A) and by Scatchard analysis (fig.1B). The apparent dissociation constant K_D for [^{3}H]oxytocin binding was calculated to be 4.9 ± 0.2 nM (n = 3), the maximal binding capacity B_{max} was estimated to be 2.8 pmol/mg membrane protein. The maximal binding capacity of these membranes for [Arg^8][^{3}H]vasopressin was roughly the same as for [^{3}H]oxytocin. The receptor affinity for vasopressin was about 5-fold lower as compared to oxytocin; the K_D for [^{3}H]vasopressin binding was calculated to be 26 ± 2 nM (n = 3).

The ligand specificity of the oxytocin binding site was examined (fig.2). The specific oxytocin agonist [Thr^4,Sar^7]oxytocin exhibits a high selectivity for oxytocin-like relative to vasopressin-like activities [9]; furthermore, its activity in the rat milk ejection assay is higher (731 ± 57 U/mg) than that of oxytocin (486 ± 15 U/mg). This analogue was more potent in displacing [^{3}H]oxytocin from its binding site in mammary membranes than unlabelled oxytocin (fig.2). In competition experiments with [^{3}H]AVP and unlabelled peptides,

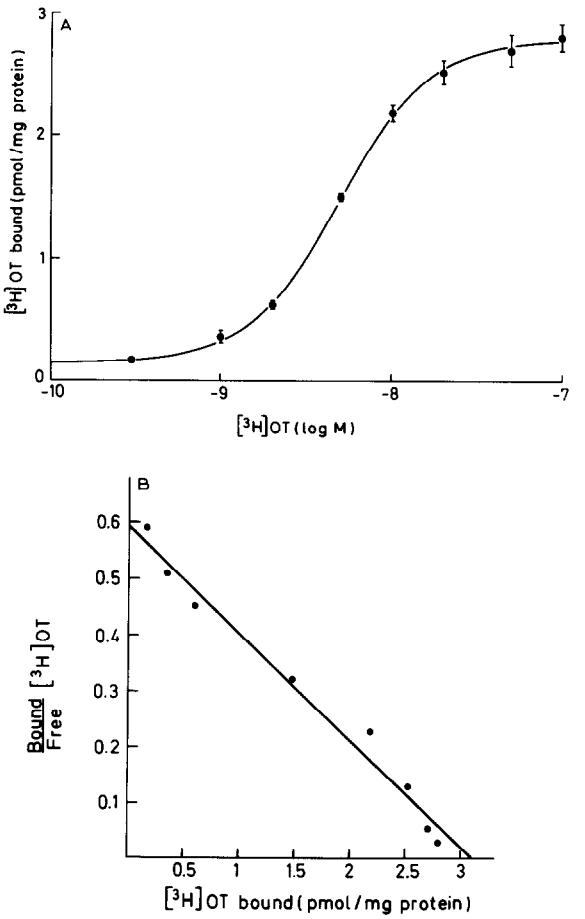

Fig.1. Binding of [^{3}H]oxytocin to mammary membranes. Membranes (80–100 μg per test) were incubated at 30°C for 30 min with various concentrations of [^{3}H]OT. Nonspecific binding was determined in the presence of a 100-fold excess of oxytocin and was subtracted from the total binding. Each point represents the mean of three experimental values. The binding curve was fitted as described in section 2 (A) and the data were also analysed and represented as Scatchard plot (B).

oxytocin and [Thr^4,Sar^7]oxytocin were more potent in displacing [^{3}H]AVP from membranes than unlabelled vasopressin (fig.3). [Thr^4,Sar^7]oxytocin (1 μM) displaced [^{3}H]AVP neither from the renal V_2 nor from the hepatic V_1 receptor [7]. These results indicate that membranes from rat mammary gland contain high affinity oxytocin receptors which bind with lower affinity vasopressin.

The photoreactive analogue of 1-deamino-[Lys^8]vasopressin containing an azidobenzoyl (Abz) group at the side chain of lysine, [Mpa^1,Lys(Abz)^8]vasopressin, retains a high af-

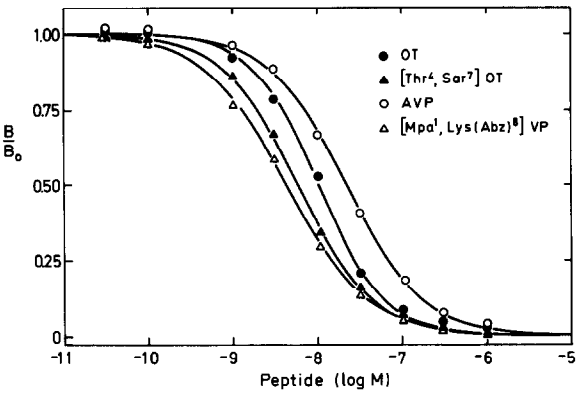

Fig.2. Competitive binding of [^{3}H]oxytocin and different peptides to mammary membranes. Plasma membranes (30–100 μg) were equilibrated at 30°C with a fixed concentration of [^{3}H]OT (10 nM) and varying concentrations of nonlabelled peptides for 30 min. The reduction in binding of [^{3}H]OT produced in two identical experiments by various concentrations of unlabelled peptides is shown. B_0 is specific binding of [^{3}H]OT in the absence of competing ligand and B is specific binding of [^{3}H]OT in the presence of competing ligand.

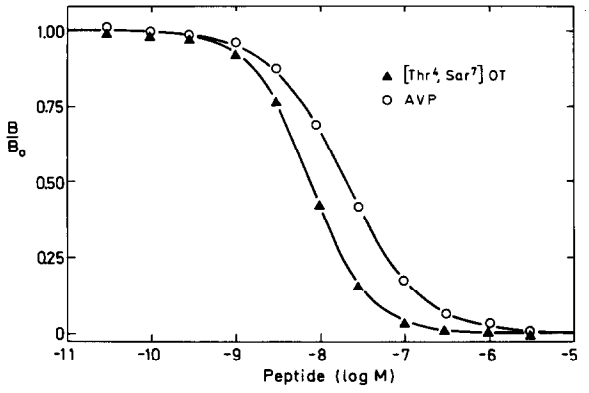

Fig.3. Competitive binding of [^{3}H]AVP, AVP and [Thr^4,Sar^7]OT to mammary membranes. Membranes were equilibrated with [^{3}H]AVP (10 nm). Other experimental conditions as described for fig.2.

finity for the oxytocin receptor in mammary membranes. We found a higher affinity in competition experiments with [^{3}H]oxytocin of this analogue than of either vasopressin or oxytocin (fig.2).

In photoaffinity labelling experiments the photoreactive ligand with tritium incorporated in the benzene ring of the azidobenzoyl group (50 Ci/mmol) was used [6]. Membranes were incubated with the photoreactive ligand at concen-

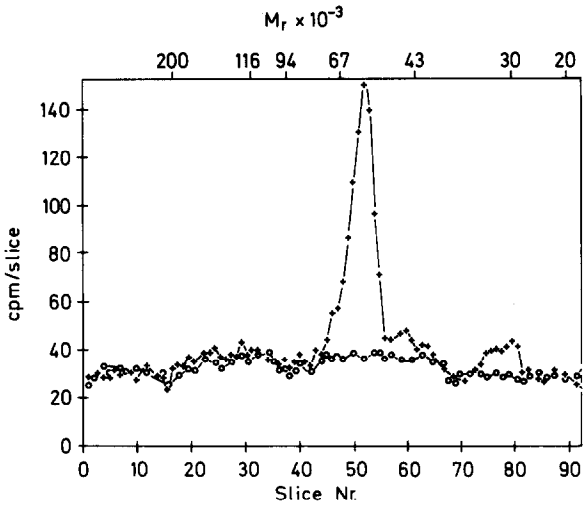

Fig.4. Effect of oxytocin on photoaffinity labelling. Mammary membranes were incubated with 7 nM photoreactive ^{3}H-labelled ligand in the absence of oxytocin and in the presence of 3.5 μM oxytocin (○). After incubation the suspension was diluted and the ligand photoactivated. This procedure was repeated and the membrane proteins separated on gradient SDS gels.

trations of 4–7 nM (3–5 times the K_D value of the ligand). To increase the specificity of the labelling, the concentration of the free ligand was reduced after incubation by dilution. Gel electrophoretic analysis (fig.4) showed the preferential labelling of a membrane protein with an apparent M_r of 65000 ± 3000 (n = 16). The labelling of this protein was completely suppressed by a 500-fold excess of oxytocin. In most experiments a second protein with a molecular mass of 28000 ± 2000 (n = 11) was labelled by the ligand with lower yield as compared to the 65 kDa protein.

4. DISCUSSION

The oxytocin receptor on membranes from mammary gland retains a high affinity for an analogue of vasopressin, containing a photoreactive group at the side chain of Lys^8. The amino acid in position 8 of the oxytocin sequence is the hydrophobic leucine residue. Apparently, the introduction of a hydrophobic aryl azido group in the side chain of the Lys^8 residue increases the affinity of the resulting analogue as compared to vasopressin for the oxytocin binding site. The photoaffinity labelling experiments provide

evidence for an oxytocin receptor protein with an apparent M_r of 65000 ± 3000. The labelling of this protein was completely inhibited by an excess of oxytocin. The labelled protein with an M_r of roughly 28000 could either be a cleavage product of the oxytocin receptor protein or it could be associated with the receptor. One has to take into account the experimental errors in the M_r values for the mammary oxytocin receptor determined by SDS-gel electrophoretic analysis (M_r = 65000 ± 3000) and by radiation inactivation (M_r = 57500 ± 3800). The results from photoaffinity labelling and radiation inactivation provide evidence that a protein with an M_r between 58000 and 65000 represents a functional mammary oxytocin receptor. Purification of the receptor and reconstitution studies will be necessary to support this conclusion.

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