Vol. 112, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1983 Pages 717-722

OXYTOCIN BINDING BY MYOEPITHELIAL CELL MEMBRANES FROM INVOLUTED MAMMARY TISSUE

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Received March 18, 1983

Oxytocin binding activity of myoepithelial cell membranes from mammary tissue was measured under a variety of different experimental conditions. Mammary tissue from non-lactating rats bound oxytocin with a K_d of 9.2 ± 1.6 nM (±S.E.) and indicates that receptors are retained by the myoepithelial cells in a non-lactating state. Ovariectomy of non-lactating rats did not depress the binding activity of the membranes. Administration of the estrogenic compounds estradiol-17β and diethylstibestrol at doses which affect uterine weight and are known to increase uterine oxytocin binding did not influence the binding activity of the myoepithelial cells. This indicates that the oxytocin receptors of the mammary gland are not under the same endocrine control as the uterine receptors.

Oxytocin is the posterior pituitary hormone responsible for stimulating uterine contractions and the ejection of milk from the lactating mammary gland. Myoepithelial cells of the mammary gland surround the ducts and form a basket-like network around alveolar secretory cells. Binding of oxytocin to specific receptors located on the plasma membrane of the cells causes the cells to contract. The contractile system of the myoepithelial cells has not been extensively studied but appears to bear some resemblance to that of smooth muscle (1). We have shown that the 20,000 M_r light chain of myosin (LC_{20}) is phosphorylated within seconds after the addition of nanomolar concentrations of oxytocin (2).

There is little information on the regulation of oxytocin receptors in the mammary gland. Some studies suggest that the

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0006-291X/83 $1.50
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steroid hormones estrogen and progesterone affect the number of uterine oxytocin receptors and their affinity for binding oxytocin (3,4). It has been reported that in rabbits, rats, and sheep, estrogen increases myometrial sensitivity to oxytocin and progesterone decreases it (4,5,6). Thus, the increased responsiveness of the uterus to oxytocin at term may be due to changes in steroid hormone levels resulting in a change in affinity and/or number of oxytocin receptors.

We wished to determine whether the oxytocin receptors of the mammary gland were regulated by steroid hormones in a similar way to those of the uterus. Our results indicate that the receptors of the mammary gland are not under the same endocrine control as in the uterus.

MATERIALS AND METHODS

Preparation of Mammary Cell Membranes - Lactating rats (Holtzman) were ovariectomized 14 days prior to use. Rats which were not ovariectomized were used six days after weaning their litters. Animals were injected subcutaneously with solutions of 1 mg of diethylstilbestrol (DES), estradiol-17β and progesterone dissolved in 0.2 ml corn oil at 12 and 24 hours before the animals were sacrificed. The abdominal mammary glands were removed, weighed and placed in ice cold Tyrode's buffer pH 7.6. The mammary tissue was then minced into 0.4 mm^2 pieces with a McIlwain tissue chopper. The tissue was then homogenized with a Polytron (PT20ST generator, Brinkmann Instruments) with three 15 sec maximum speed bursts. Between bursts the homogenate was cooled for 1 min on ice. After centrifugation at 1000 x g for 10 min, the supernatant was filtered through 2 layers of cheesecloth that was pre-wetted with Tyrodes buffer. The pellet obtained after centrifugation of the filtrate for 50 min at 98,000 x g was homogenized with a glass-teflon homogenizer in a volume of Tyrodes buffer equal to half the weight of the original tissue. Binding measurements were done immediately because freezing reduced the specific oxytocin binding of the membranes.

Purification of [^{3}H]oxytocin - The product of a catalytic tritiation of oxytocin was a gift from Dr. M. S. Soloff (Medical College of Ohio, Toledo, Ohio) and was further purified by high performance liquid chromatography (HPLC). The [^{3}H]oxytocin was fractionated on a (0.5 cm x 30 cm) C-18 reversed-phase column (Waters Associates, Milford, MA) using a linear gradient starting from 0.1% H_3PO_4 : methanol (4:1) to final conditions of 100% methanol. Fractions were collected every 30 sec and assayed for binding activity. Fig. 1 shows the chromatographic profile obtained. Biological activity was tested by its ability to stimulate the phosphorylation of the 20,000 M_r myosin light chain in isolated mammary myoepithelial cells (2). In this test the labelled peptide was as active as our reference standard. The specific activity of the eluted fractions was 13.8 Ci/mmole. Results of binding assays done with a constant concentration of labelled peptide diluted by unlabelled peptide were identical to those done by increasing the concentration of peptide by adding

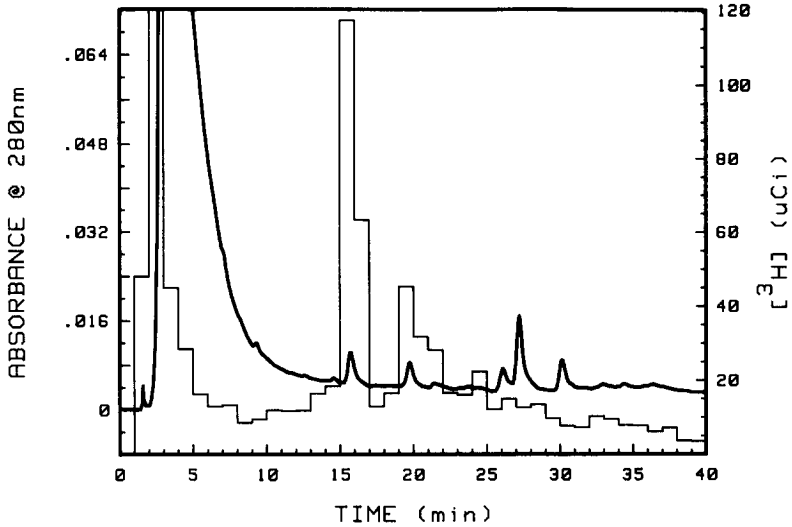

Figure 1. Reversed phase HPLC purification of [^{3}H]oxytocin from a catalytic tritiation reaction product. A two solvent system mobile phase was employed with 0.1% H_3PO_4 in H_2O as Solvent A and methanol as Solvent B. The column was pre-equilibrated with 80% A and 20% B. The sample (1 ml) was injected onto the column and a linear gradient of 20% B to 50% B was applied over a period of 30 min. At 30 min the linear gradient was accelerated to 100% B over 5 min and held at 100% B until the run was completed. The only fractions that exhibited specific binding to mammary oxytocin receptors were those associated with the peak eluting at 19.8 min which corresponded to the retention time of unlabelled oxytocin in this chromatographic system.

increasing amounts of labelled peptide. This served as an additional check on the biological activity and specific activity of the labelled oxytocin.

Oxytocin Binding Assay - Oxytocin binding measurements were done under conditions similar to those reported by Soloff and Swartz (7) and Markle et al (8). The total volume of the incubation mixture was 0.25 ml and contained the following: 1) 5 mM $MgCl_2$, 0.1% gelatin, and 50 mM Tris-HCl pH 7.6; 2) 1.2 nM [^{3}H]oxytocin; 3) unlabelled oxytocin (0-50 nM); 4) membrane particulate fraction (0.4-0.65 mg protein). The reaction mixture was incubated at room temperature for 60 min in 1.5 ml-Eppendorf microfuge tubes. Non-specific binding was determined by the addition of 10 mM EDTA to the reaction mixture.

Incubation was terminated by centrifugation in a microcentrifuge at 12,000 x g for 5 min. The supernatant was removed, and residual liquid removed from the sides of the tube with absorbant paper. Each pellet was dissolved overnight with 0.3 ml NCS (Amersham-Searle). The following day glacial acetic acid was added to neutralize the solution, then scintillation fluid was added and the samples were counted using the external standards ratio to compute the quenching in the sample. This was essential because with the low specific activity of [^{3}H]oxytocin the labelled peptide cannot be disregarded in the binding calculations.

Enzyme and Protein Assays - Alkaline phosphatase is a plasma membrane marker specific for the myoepithelial cells of the mammary gland (9). The activity of alkaline phosphatase was determined on membranes prepared as above with a kit from Sigma Chemical Co. (St. Louis, Mo.). The protein content of the membrane particulate fractions was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

RESULTS AND DISCUSSION

Secretory cells and myoepithelial cells are present in the lactating mammary gland. When the gland undergoes involution, the secretory cells regress and the myoepithelial cells remain. It has also been shown that involuted mammary tissue strips retain their sensitivity to oxytocin and contract in response to the hormone (1). In our studies, cell membranes prepared from involuted mammary glands bound oxytocin with high affinity (Fig. 2). The apparent K_d for binding was 9.2±1.6 nM. Pearlmutter and Soloff (10) reported a K_d of 2.3 nM and a value of 5 nM can be computed from their forward and reverse rate constants for the binding reaction in the presence of 5mM $MgCl_2$. These differences are small and might be accounted for by slight differences in methodology.

Maximum binding to membranes from involuted rats is about 1 pmol/mg protein. Membranes from lactating rats have been reported to bind 0.4 pmol/mg (computed from Fig. 1 of Pearlmutter and Soloff (10)). We have observed binding similar to that of Pearlmutter and Soloff in lactating

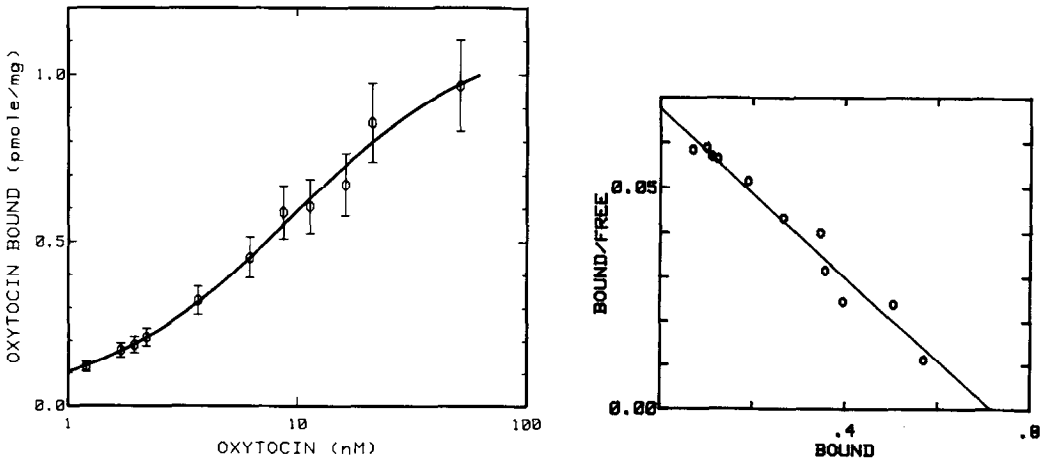

Figure 2. Binding of [^{3}H]oxytocin to membranes of myoepithelial cells from involuted mammary tissue. Each point represents the mean ± standard error of the oxytocin bound. Measurements were made on 10 groups of rats that were used as controls for the experiments summarized in Table 1. The curve is the best fit curve to the data using the non-linear regression model of Rodbard and Hutt (11). On the right is the Scatchard plot of the data. The binding units of the two plots are different. Bound and Free in the Scatchard plot are in nM whereas Bound is expressed per mg protein in the main plot.

rats (unpublished observations). These results suggest an enrichment of oxytocin receptors in mammary tissue concomitant with the involution of the secretory cells. The Hill coefficient for the data plotted in Fig. 2 is 1.03, also similar to that reported for lactating tissue (10).

A number of different hormonal regimes were tested for their potential ability to alter the number or affinity of oxytocin receptors. Table 1 summarizes these results and shows that mammary membrane particulate fractions from ovariectomized and intact rats bound approximately the same amount of oxytocin regardless of the treatment. Oxytocin binding at a concentration of 10 nM was chosen from comparative purposes because it approximates the K_d found for the control rats (Fig. 2). Statistical analysis of the complete binding curves for the different groups was done using the standard errors of the coefficients of the non-linear model which was used to fit the data (11). This analysis indicated that none of the binding curves for the different groups were statistically different from one another. Uterine weight decreased (as expected) in response to ovariectomy and returned to a value similar to the control animals after DES treatment. It has been reported that DES treatment increases both the oxytocin receptor number and affinity when compared to untreated rats (3,4). Since these effects were not apparent in the mammary gland it appears that receptors on the

Table 1. Characteristics of rats with involuted mammary glands that had been subjected to different endocrine manipulations.

TREATMENT	N	MAMMARY WEIGHT (g)	UTERINE WEIGHT (g)	ALKALINE PHOSPHATASE (umol/hr/mg)	OXYTOCIN BINDING (pmol/mg)
INTACT	6	3.2(.42)	0.77(.08)	51.3(3.6)	0.62
INTACT+P4	2	3.4(.32)	0.58(.04)	53.3(7.5)	0.78
INTACT+E2	2	2.8(.68)	0.96(.15)	58.2(9.4)	0.69
OVX	2	2.5(.05)	0.30(.01)	24.5(3.4)	0.58
OVX+DES	3	2.7(.17)	0.67(.08)	22.7(1.4)	0.59

OVX (ovariectomized), P4 (progesterone), E2 (estradiol-17β), DES (diethylstilbestrol). Injection protocols are given in the methods section. The number in parenthesis is the SEM of measurements on N groups of 3-6 rats per group. The standard error of the binding measurements can be found in Figure 2.

two target sites for oxytocin are regulated differently. The reason for the decrease in alkaline phosphatase activity after ovariectomy is not understood.

In summary, the nature of oxytocin binding to mammary membranes from non-lactating tissue was similar to the binding in membranes from lactating tissue. However, unlike the uterus, oxytocin receptors in the mammary gland appear to be unaffected by steroid hormones. The failure of a variety of endocrine manipulations to alter either the affinity or number of receptors suggest that the receptors in the two target tissues are regulated in different ways.

ACKNOWLEDGEMENTS

This work was supported by an NIH Grant (HD09764) and by the College of Agricultural and Life Sciences.

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