BINDING OF [^{3}H]OXYTOCIN TO CELLS ISOLATED FROM THE MAMMARY GLAND OF THE LACTATING RAT

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ABSTRACT

More than 90% of the cells isolated from the mammary gland of lactating rats with 0.1% collagenase were viable by dye exclusion. Myoepithelial cells comprised about one-third of the mammary cells and appeared to be morphologically intact in electron micrographs. [^{3}H]Oxytocin-binding activity was localized in an enriched myoepithelial cell fraction obtained by density gradient centrifugation of the isolated cells. The amount of [^{3}H]oxytocin bound at 20°C and pH 7.6 was proportional to the concentration of oxytocin and the number of cells, reaching a steady state by 40 min. About 0.45 fmol of oxytocin were bound per 10^6 cells. There was a single class of independent binding sites with an apparent K_d, estimated from equilibrium conditions, of 5 nM. This value agrees within experimental error with the value calculated from the ratio of reverse to forward rate constants (5.8×10^{-4} s^{-1} and 2.2×10^5 M^{-1} s^{-1}, respectively), consistent with a single-step model for the interaction of oxytocin with binding sites on the cells. Erythrocytes bound only 3.5% of the amount of oxytocin bound by an equal number of mammary cells. Oxytocin analogues competed with [^{3}H]oxytocin for binding sites in the following order: [deamino]oxytocin > [4-threonine]oxytocin > oxytocin > [*O*-methyltyrosine]oxytocin > [8-lysine]vasopressin; [lysine]-bradykinin and [4-proline]oxytocin were not inhibitory in the dose ranges tested. These results demonstrate that isolated mammary cells possess oxytocin receptors with properties comparable to those found in broken mammary cell preparations.

Milk is extruded from the mammary gland by the contraction of myoepithelial cells, which form a basketlike network surrounding the stromal surfaces of the alveolar secretory cells. This process is stimulated by oxytocin, an octapeptide hormone produced in the hypothalamus. Radioactivity from [^{3}H]oxytocin is associated with myoepithelial cells, as determined by autoradiography (25). Furthermore, particulate fractions prepared from the mammary gland of the lactating rat have specific, high affinity binding sites for [^{3}H]oxytocin (27). Although the molecular events coupling the binding of oxytocin to the contraction of myoepithelial cells are still unknown, the binding sites appear to be part of the oxytocin receptor (26). In the present studies, we have examined the binding of oxytocin to isolated mammary cells as a prelude to the study of the biochemical sequellae of oxytocin-receptor interaction in an intact, hormone-responsive system.

MATERIALS AND METHODS

Materials

PEPTIDES: [Tyrosyl-^{3}H]oxytocin (31 Ci/mmol) with full biological activity (452 IU/mg) was purchased from Schwarz/Mann Div. (Becton, Dickinson & Co., Orangeburg, N. Y.) and stored at −80°C. More than 90% of the radioactivity migrated with authentic oxytocin upon thin layer chromatography (27).

Oxytocin, [deamino]oxytocin and [8-lysine]vasopressin were gifts from Sandoz, Ltd. (Basel, Switzerland). [4-threonine]-oxytocin and [4-proline]oxytocin were donated by Dr. Maurice Manning of this Department. [2-*O*-methyltryosine]oxytocin was a gift from Dr. J. H. Cort, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague. Kallidin ([lysine]bradykinin) was purchased from Schwarz/Mann Div.

ENZYMES AND CHEMICALS: Collagenase (*Clostridium histolyticum* type I) containing both protease and peptidase activities, bovine serum albumin (fraction V), and deoxyribonuclease I from bovine pancreas were obtained from Sigma Chemical Co. (St. Louis, Mo.). Gelatin was purchased from Grand Island Biological Co. (Grand Island, N. Y.). Metrizamide [2-(3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose] was purchased from Gallard-Schlesinger Chemical Mfg. Corp. (New York, N. Y.).

Methods

PREPARATION OF MAMMARY CELLS: Mammary glands were removed from lactating primiparous rats (CFE, Carworth Farms, Inc., Portage, Mich.), 4–16 days postpartum. The tissue was trimmed of fat and fascia and minced into pieces about 1 mm^3 with a McIlwain tissue chopper (Brinkmann Instruments, Westbury, N. Y.). The minced tissue was incubated in Tyrode's solution,[1] pH 7.6, (1 g of tissue per 5 ml) containing 0.1% (wt/vol) collagenasae and 0.1% (wt/vol) bovine serum albumin, for 1 h at 37°C. The pieces were stirred vigorously with a magnetic bar in a single-arm Wheaton Celstir flask (250 ml, Wheaton Industries, Millville, N. J.). The tissue was dispersed completely after 1 h. The cell suspension was filtered through a Cellector 100-mesh tissue sieve (E-C Apparatus Corp., St. Petersburg, Fla.) three times and centrifuged at 325 *g* for 5 min. The cell pellet was resuspended and washed three times in Tyrode's solution containing 0.1% gelatin (pH 7.6, hereafter referred to as Tyrode's gelatin) and 2 × 10^{-4}% DNase to minimize cell aggregation. Some of the earlier experiments were carried out without DNase.

Cell viability of greater than 90% was determined by counting with a hemocytometer the fraction of cells excluding 0.05% erythrosine B; no change was noted between the onset and conclusion of the incubation period with oxytocin. Erythrocytes, which comprised at least 50% of the total cells, were not included in the cell count. The cell count determined with the hemocytometer agreed with the value obtained with a Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.). About 2.8 × 10^8 cells occupied a packed vol of 1 ml after centrifugation at 325 *g* for 5 min. The cell count was validated by determination of the DNA content of cell pellets. The cells were lysed by freeze-thawing twice, and DNA was measured by the Burton method (5) according to Leyva and Kelly (19). Paraldehyde was used in place of acetaldehyde (23). The content of DNA per mammary cell, 6.9 pg, was near the values of 7.2–8.5 pg reported for human fibroblasts (15, 19) and leukocytes (3). Replacement of collagenase with 0.05% lysozyme, which has been effective in preparing isolated hepatocytes (14, 21), gave few if any isolated mammary cells at the end of 2 h of incubation at 37°C. The use of 0.25% trypsin instead of collagenase resulted in cell viabilities of 50% or less.

MICROSCOPY: The ultrastructural integrity of myoepithelial cells was examined in serial sections by transmission electron microscopy. Cells were fixed for 30 min in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and washed twice with the same buffer. The cell pellet formed by centrifugation was postfixed for 30 min in 1% OsO_4 (pH 7.2), washed, dehydrated, and embedded in Epon 812. Sections of 900–1,000 Å were cut with an LKB microtome, (LKB Instruments, Inc., Rockville, Md.), stained with lead citrate (22) and examined with a Philips EM 300 electron microscope.

DENSITY GRADIENT CENTRIFUGATION: Isolated mammary cells, 1 × 10^7, were incubated with 100,000 cpm (~4.4 ng) of [^{3}H]oxytocin with and without 110 ng of nonradioactive oxytocin in 0.5 ml of Tyrode's gelatin for 30 min at 20°C. The cell suspensions were applied to 14 ml of a linear gradient of 5–30% Metrizamide layered onto 3 ml of 50% sucrose. The tubes were centrifuged at 10,000 rpm and 4°C for 1 h in a SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Fractions of 0.6 ml were assayed for radioactivity, and the cells were counted with a hemocytometer.

ASSOCIATION STUDIES: Cell suspensions, 1 vol of cells in 5 vol of Tyrode's gelatin and 2 × 10^{-4}% DNase, were incubated with 3.4 nM [^{3}H]oxytocin and increasing amounts of nonradioactive oxytocin at 20°C. Samples, 200 μl, were removed at 15- or 30-s intervals, added to 5 ml of Tyrode's solution (minus gelatin) at 4°C, and the mixture was filtered immediately through prewetted glass filters (Whatman GF/B). The filters then were rinsed with 1 ml of Tyrode's solution at 4°C, placed into scintillation vials, and incubated with 1 ml of 10 mM EDTA for 1 h or longer to dissociate the [^{3}H]oxytocin from the cells. Then 20 ml of scintillation fluid (5 g of

[1] The composition of Tyrode's solution in grams per liter is: NaCl, 8.0; KCl, 0.20; $CaCl_2$, 0.20; $MgCl_2$, 0.10; $NaHCO_3$, 1.0; NaH_2PO_4, 0.05; glucose, 1.0; adjust to pH 7.6 with 2 N HCl.

2,5-diphenyloxazole [PPO], 100 g of naphthalene, 35 ml of methanol, 135 ml of toluene, and 730 ml of dioxane) were added, and the vials were shaken overnight before counting. All of the cells were trapped in the glass filter matrix because no radioactivity was found in a 0.45-μm Millipore filter (Millipore Corp., Bedford, Mass.) placed below each glass filter. The amount of radioactivity adsorbed to the filters in the absence of cells never exceeded 6% of the amount filtered. The dissociation of radioactivity from the cells after rinsing with Tyrode's solution at 4°C was negligible as determined by the dissociation studies described below.

DISSOCIATION STUDIES: Cells, usually about 2 $\times$ 10^8 per 4 ml of Tyrode's gelatin and 2 $\times$ 10^{-4}% DNase, were equilibrated with ~4 nM [^{3}H]oxytocin for 30 min at 20°C. The dissociation rate of the oxytocin-receptor complex was studied by diluting the incubation mixture 1:50 with Tyrode's gelatin-DNase at 20°C or 4°C, with or without 1 μM nonradioactive oxytocin. Samples, 5 ml, were filtered on prewetted Whatman GF/B filters immediately after dilution to give the 100% value and at 5- or 10-min intervals to 90 min. The filters were washed twice with 500 μl of Tyrode's gelatin solution at 4°C. Radioactivity associated with the filters was determined as described above. The same results were obtained with either a Millipore manifold or an apparatus for single filtrations. The dissociation rates found upon 100-fold dilution of cell suspensions was the same as that with the 50-fold dilution.

STEADY-STATE STUDIES: The binding of increasing concentrations of cells with 1.3 nM [^{3}H]oxytocin or with a fixed number of cells with 0.65 nM [^{3}H]oxytocin and increasing concentrations of nonradioactive oxytocin was studied under steady-state conditions (incubation for 1 h at 20°C). The cells, in 250 μl of Tyrode's gelatin solution, were then centrifuged at 20,000 *g* for 10 min at 4°C. The supernates were removed and 100-μl aliquots were applied to disks of Whatman no. 40 filter paper. The pellets were washed with 500 μl of buffer at 4°C, dissolved in 0.1 ml of 2 N NaOH at 60°C and transferred to filter paper with two water rinses. The paper disks were air dried, compressed with a pill press, and combusted in a Packard tritium oxidizer to ^3H_2O as described previously (27). The amount of [^{3}H]oxytocin bound nonspecifically, determined by incubating samples with 110 nM nonradioactive oxytocin, was subtracted from the experimental results.

The binding to cells by oxytocin analogues was determined by the inhibition of [^{3}H]oxytocin binding under steady-state conditions. The tubes contained 7-11 $\times$ 10^6 cells, 0.65 nM [^{3}H]oxytocin and increasing concentrations of nonradioactive peptide in 250 μl of Tyrode's gelatin solution. The data were analyzed as 6-point, parallel-line assays according to Finney (11).

RESULTS

At least 90% of the isolated cells appeared to be viable as judged by their ability to exclude erythrosine B. Myoepithelial cells were easily recognized in electron micrographs by their typical morphological characteristics, such as myofilaments (16) (Fig. 1) and numerous cytoplasmic processes (Fig. 2 A). In all serial sections examined the cell membrane was intact and the isolated cells resembled myoepithelial cells found in mammary tissue *in situ* (Fig. 2 B). The isolated mammary cells consisted of about one-third myoepithelial cells and about two-thirds epithelial cells (Fig. 2 C). Fibroblasts and erythrocytes also were present.

Isolated cells were incubated with [^{3}H]oxytocin and subjected to centrifugation on a linear density gradient of 5-30% Metrizamide for 1 h at 4°C. The dissociation of [^{3}H]oxytocin from the cells under these conditions was not detectable. Unbound radioactivity remained at the top of the gradient (Fig. 3). Bound radioactivity was associated with two discrete cell fractions. The addition of an excess of nonradioactive oxytocin to the incubation medium resulted in a marked reduction in radioactivity in the two peaks, with a corresponding shift in radioactivity to the unbound fraction. The greatest displaceable binding activity was in the peak with a density of about 1.2 g/ml. Electron micrographs of this fraction indicated about a two-fold enrichment of myoepithelial cells over the starting material. Secretory cells with disrupted plasma membranes were abundant in this fraction. The second binding peak was composed of aggregates of epithelial and myoepithelial cells, with no significant enrichment of myoepithelial cells.

The binding of oxytocin to isolated mammary cells at 20°C and pH 7.6 was time dependent and proportional to the concentration of oxytocin (Fig. 4). Binding reached a relatively steady state by 40 min with 3.4-14 nM oxytocin (Fig. 4). The experimental points agreed with theoretical curves plotted for each concentration of hormone by assuming values for the association and dissociation rate constants of 3 $\times$ 10^5 M^{-1} s^{-1} and 1.5 $\times$ 10^{-3} s^{-1}, respectively, and a receptor concentration of 2.35 nM as determined by Scatchard analysis of the amount of oxytocin bound at 40 min (Fig. 4). The experiments were conducted with 3.4 nM [^{3}H]oxytocin alone and in combination with 5.3 and 10.6 nM nonradioactive oxytocin. Because the binding of oxytocin was proportional to the amount of total oxytocin, it is apparent that the radioactive oxytocin was indistinguishable from the nonradioactive hormone.

The specific binding of oxytocin also was proportional to the concentration of mammary cells

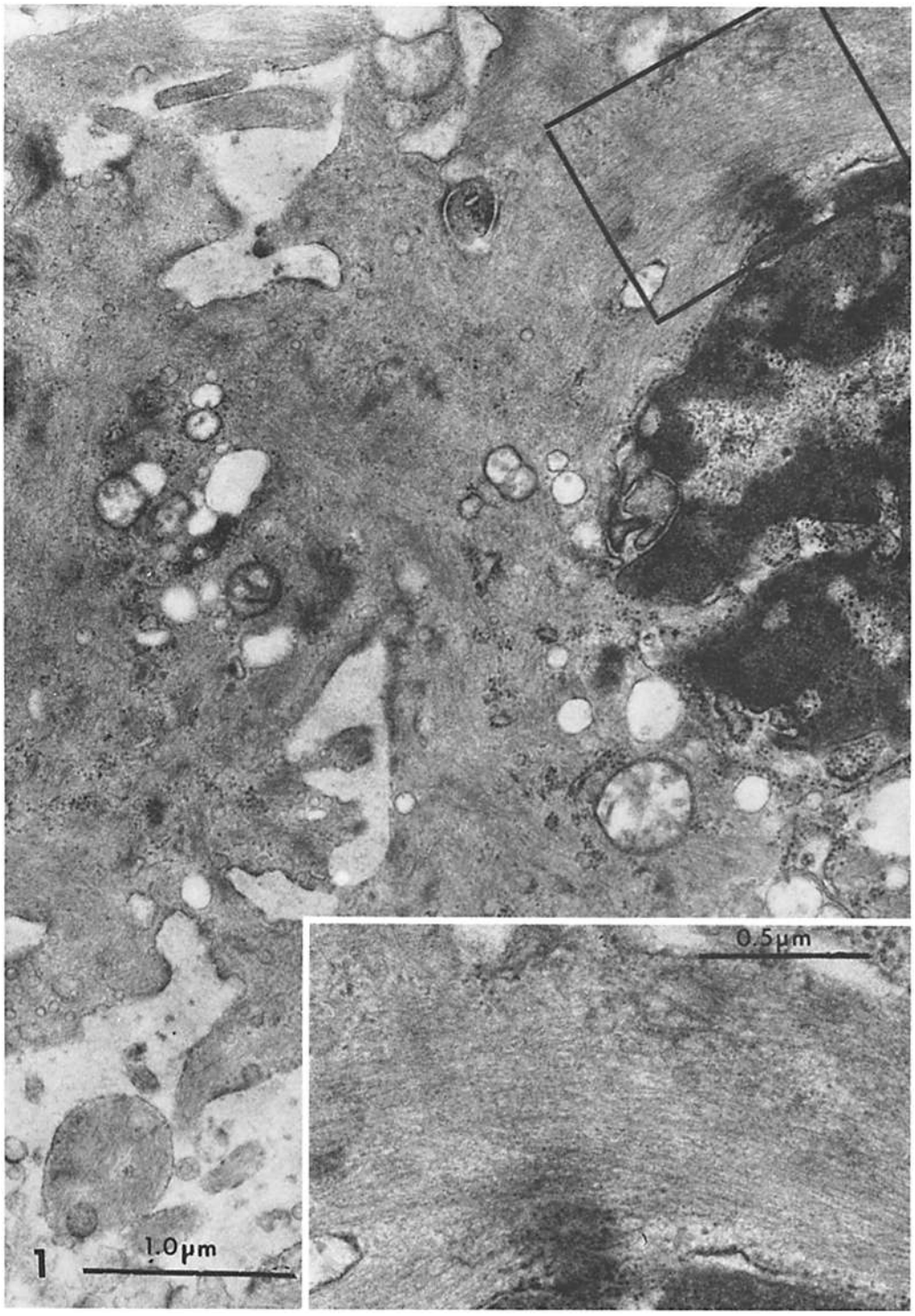

FIGURE 1 Portion of an isolated myoepithelial cell. × 30,800. Myofilaments are seen at higher magnification (*inset*). × 53,400.

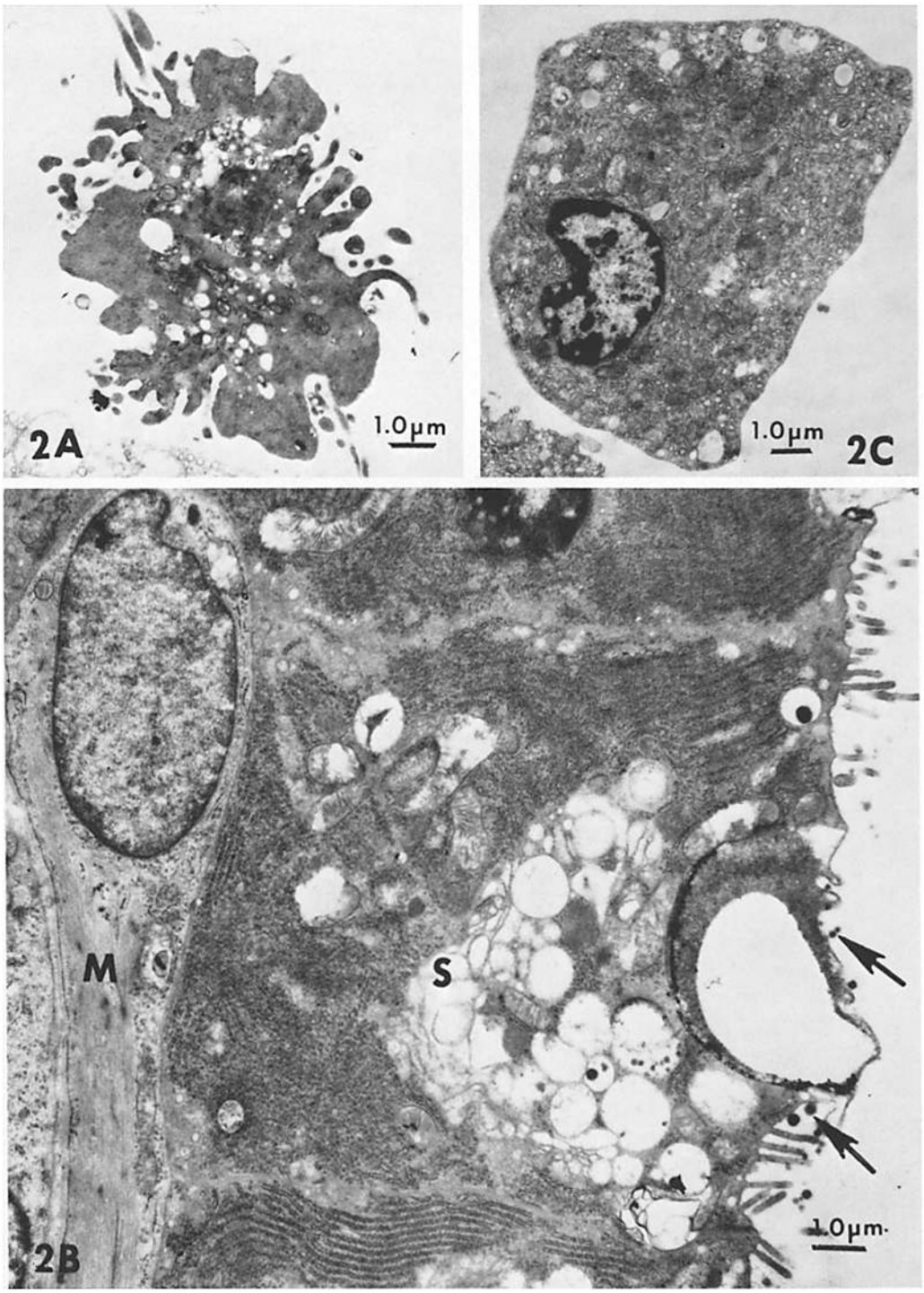

FIGURE 2 (*A*) Normal appearance of isolated, intact myoepithelial cells with cell processes. × 6,800. (*B*) Myoepithelial cell (*M*) in intact mammary tissue. Secretory products (arrows) emanating from secretory cells (*S*) are shown. × 7,800. (*C*) Isolated epithelial cell. × 6,000.

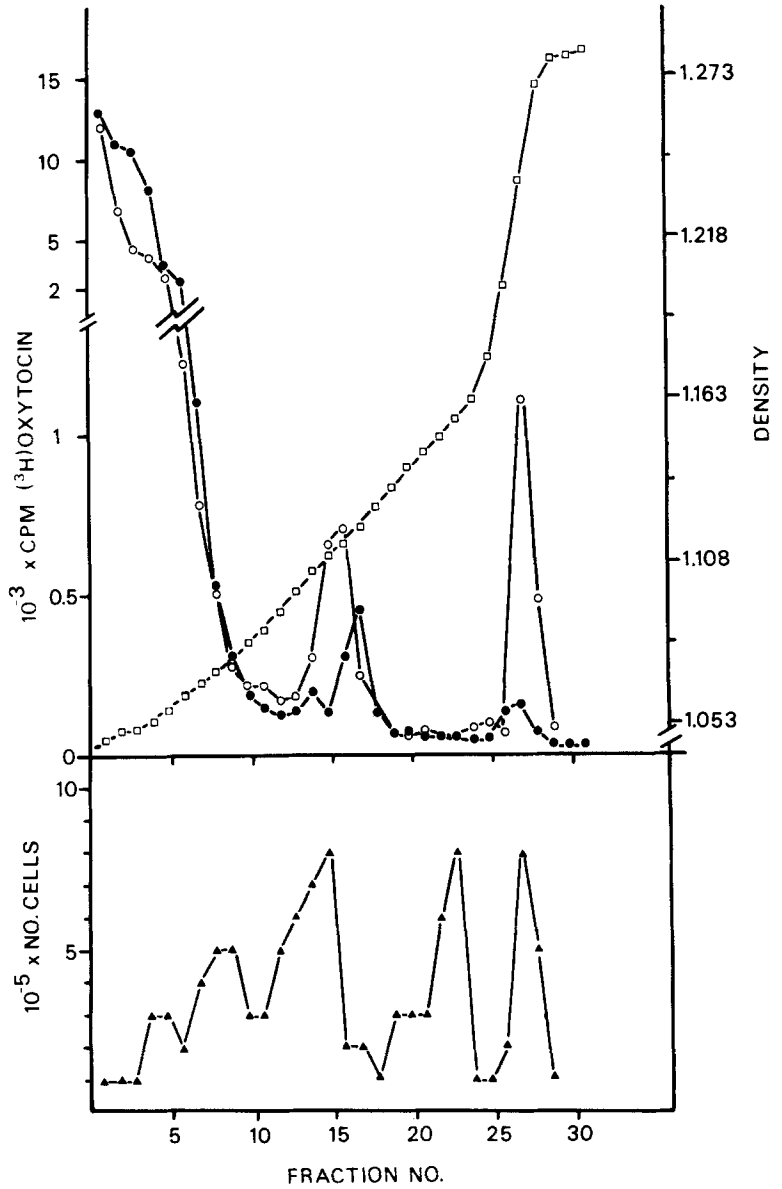

FIGURE 3 Distribution of isolated mammary cells after centrifugation in a linear gradient (5–30%) of Metrizamide. The cells, 1×10^7, were incubated with 100,000 cpm (4.4 ng) with and without 110 ng of nonradioactive oxytocin in a final vol of 0.5 ml for 30 min at 20°C and applied to 14 ml of gradient upon 3 ml of 50% sucrose. The tubes were centrifuged at 10,000 rpm for 1 h at 4°C in a SW 27 rotor. Fractions of 0.6 ml were taken and assayed for radioactivity and cell count. [^{3}H]Oxytocin (○—○); [^{3}H]oxytocin + nonradioactive oxytocin (●—●); Metrizamide density (□—□); and number of cells per fraction (▲—▲).

when 1.3 nM [^{3}H]oxytocin was incubated with increasing concentrations of cells for 1 h (steady-state conditions) (Fig. 5). The results obtained with up to 25×10^6 cells per sample agreed with a theoretical curve which was calculated by assuming a K_d of 5 nM. A binding capacity of 3.3×10^{-20} mol of oxytocin per cell was estimated from the curve. Concentrations of cells greater than 25×10^6 per 250 μl bound less [^{3}H]oxytocin than expected (Fig. 5). These results may be due to a

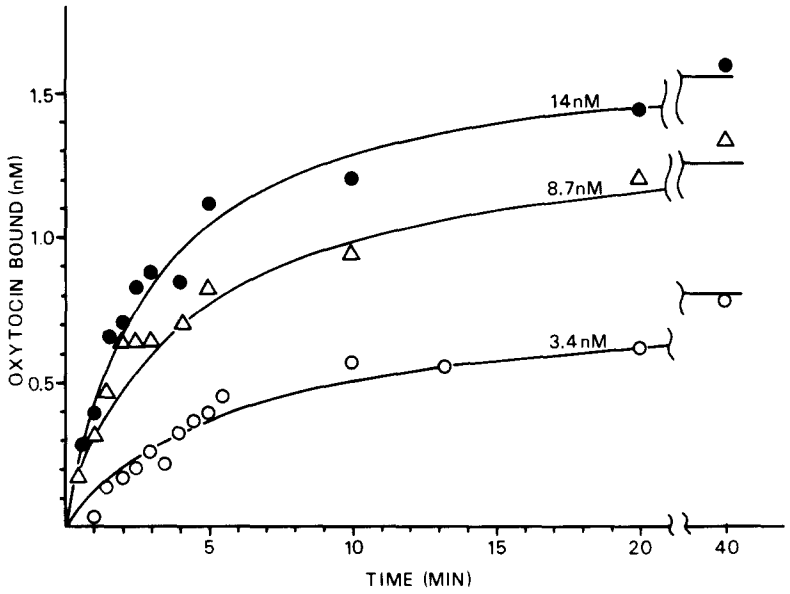

FIGURE 4 Effect of the concentration of oxytocin on binding of isolated mammary cells with increasing time. Cells were isolated from the mammary glands of four rats, two on day 16 and two on day 15 of lactation. The isolated cells were incubated with 3.4 nM [^{3}H]oxytocin (○—○), 3.4 nM [^{3}H]oxytocin + 3.5 nM nonradioactive oxytocin (△—△), and 3.4 nM [^{3}H]oxytocin + 10.5 nM nonradioactive oxytocin (●—●) at 20°C. Incubation was terminated by filtering 200 μl of sample. Each point is the mean of duplicate determinations. The lines are theoretical curves based on an estimate of the forward and reverse rate constants of 3×10^5 M^{-1} s^{-1} and 1.5×10^{-3} s^{-1}, respectively (K_d = 5 nM). The total receptor concentration was 2.35 nM, as estimated by Scatchard analysis of the amount of oxytocin bound at 40 min.

reduction in accessible receptor sites because of the increased aggregation of cells when present in higher concentrations. All of our other studies were carried out with cell concentrations below 25×10^6 per tube. In addition, cell aggregation was eliminated in most experiments by the addition of 2×10^{-4}% DNase.

Isolated cells were incubated under steady-state conditions at 20°C with 0.65 nM [^{3}H]oxytocin and increasing concentrations of nonradioactive oxytocin up to 1×10^{-7} M. The data are shown as a saturation curve and Scatchard plot, corrected for nonspecific binding (Fig. 6). The binding capacity of 2.8×10^6 cells per 250 μl was about 0.5 nM oxytocin, corresponding to about 4.5×10^{-20} mol of oxytocin bound per cell (Fig. 6). This value is similar to the binding capacity estimated from the data in Fig. 5. Assuming that oxytocin is bound with high affinity only to myoepithelial cells and that myoepithelial cells comprise about one-third of all the cells, about 80,000 molecules of oxytocin would be bound per myoepithelial cell. The Scatchard plot of the data in Fig. 6 (*inset*) was linear throughout the entire concentration range of oxytocin, indicating a single class of independent binding sites. Oxytocin was bound with an apparent K_d of 5 ± 0.7 (SE, n = 7) nM, which agrees with the value obtained either by kinetic measurements (Fig. 4) or by varying the concentration of cells (Fig. 5).

The results of the preceding experiments suggest that the binding of oxytocin to cells is a second-order process, dependent on the concentrations of oxytocin and of cells. The reaction may be represented as:

$$R + O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RO, \qquad (1)$$

where R, O, and RO are the concentrations of free receptor, free oxytocin, and oxytocin-receptor complex, respectively, and k_1 and k_{-1} are the rate constants for association and dissociation, respectively.

The rate of association, as expressed by the second order equation

$$v = k_1 [R][O] - k_{-1} [RO] \qquad (2)$$

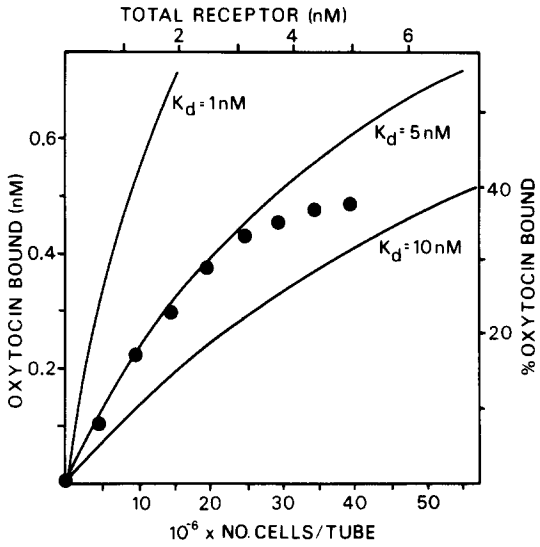

FIGURE 5 Effect of the concentration of isolated mammary cells on the binding of [^{3}H]oxytocin. [^{3}H]Oxytocin, 1.3 nM, was incubated at 20°C with increasing concentrations of mammary cells isolated from 5-day lactating rats. Incubation was terminated after 1 h by centrifugation. The three curves were calculated from apparent dissociation constants of 1, 5, and 10 nM. Each experimental point is the mean of triplicate determinations.

was simplified to

$$v = k_1 [R][O] \tag{3}$$

when v was studied at early times because $[RO]$ was small as compared to $[R]$ and $[O]$.

Integration of Eq. 3 (12) gives

$$\ln \frac{[O]_0 - [RO]}{[R]_0 - [RO]} = ([O]_0 - [R]_0)\, k_1 t + \ln \frac{[O]_0}{[R]_0}. \tag{4}$$

The subscript 0 denotes concentration at time zero. A plot of ln Y vs. t, where $Y = \ln \{([O]_0 - [RO])/([R]_0 - [RO])\}/([O]_0 - [R]_0)$ gave a value of k_1 (slope) of 2.2×10^5 M^{-1} s^{-1} with four concentrations of $[O]_0$ over a range of 3.9–16 nM (Fig. 7). The relationship of ln Y to t was linear during the first few minutes of association (Fig. 7), indicating a simple second-order association process and an insignificant reverse reaction. These studies were patterned after those of Chicheportiche et al. on the interaction of snake neurotoxins with acetylcholine receptor (8).

The dissociation rate was studied by allowing

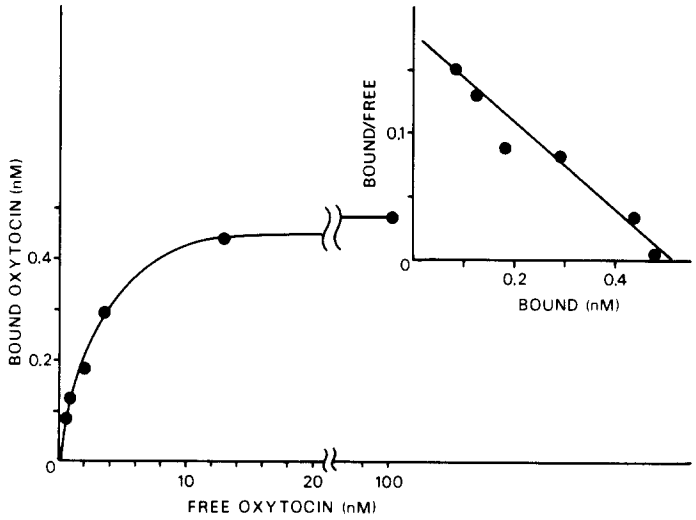

FIGURE 6 Binding of oxytocin to isolated mammary cells. The cells, about 2.8×10^6 per 250 μl of Tyrode's-gelatin solution, were isolated from rats 9 days postpartum and were incubated at 20°C with 0.65 nM [^{3}H]oxytocin (about 11,000 dpm) and increasing concentrations of nonradioactive oxytocin. After equilibration for 1 h, free and bound oxytocin were separated from each other by centrifugation, and the amount of radioactivity in the pellets and supernates was determined. The amount of oxytocin bound nonspecifically, determined by incubating cells with 0.65 nM [^{3}H]oxytocin and 110 nM nonradioactive oxytocin, was subtracted from the experimental results to give the values shown. The data are also expressed in terms of a Scatchard plot (*inset*). Each point is the mean of duplicate determinations.

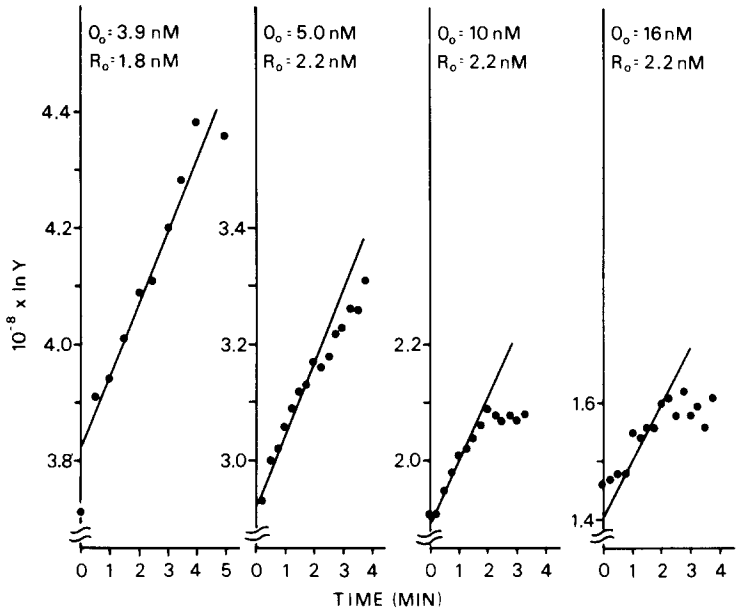

FIGURE 7 Estimation of the association rate constant for oxytocin binding by isolated mammary cells. Y refers to the term $\{([O]_0 - [RO])/([R]_0 - [RO])\}/([O]_0 - [R]_0)$, where $[O]_0$ and $[R]_0$ are the initial concentrations of oxytocin and receptor, respectively; $[O]$, $[R]$, and $[RO]$ are the concentration of oxytocin, receptor, and oxytocin-receptor complex at time t, respectively.

the binding system to equilibrate for 30 min at 20°C and by measuring $[RO]$ at various times after diluting the mixture 1:50, either in the presence or absence of 1 μM oxytocin. Plots of log percent bound vs. time were linear and the $t_{\frac{1}{2}}$ was independent of $[RO]_0$ over a sixfold range in cell concentration (2.5×10^7–1.5×10^8 cells/milliliter. These results suggest that the dissociation of the hormone-receptor complex is a first-order process.

The $t_{\frac{1}{2}}$ for the dissociation of oxytocin from cells obtained from rats lactating for 15 days was about 20 min, regardless of whether or not oxytocin was present in the diluent (Fig. 8 A). Comparable results were seen with mammary cells from rats lactating for 6–16 days. On the other hand, there was a marked effect of 1 μM oxytocin on the rate of dissociation of oxytocin from cells obtained from 26-day lactating rats. The half-time of dissociation was 16 min in the presence of 1 μM oxytocin and about 40 min in the absence of oxytocin (Fig. 8 B). An average k_{-1} of 5.8×10^{-4} s^{-1}, corresponding to $t_{\frac{1}{2}}$ of 20 min, was calculated on the basis of $k_{-1} = \ln 2/t_{\frac{1}{2}}$. This dissociation rate constant was unchanged when $[RO]$ was diluted 1:100 instead of 1:50. The dissociation constant calculated from the kinetic data, k_{-1}/k_1, was 2.6 nM compared to 5 nM, the apparent K_d estimated from measurements made at equilibrium. As mentioned earlier, no detectable [^{3}H]oxytocin was dissociated from the cells after 1 h at 4°C.

The ligand specificity of oxytocin binding was measured by the ability of several oxytocin analogues to compete with [^{3}H]oxytocin for binding sites. Each peptide was compared to nonradioactive oxytocin in a 6-point assay. As shown in Fig. 9, [^{3}H]oxytocin binding was inhibited by increasing concentrations of all the peptides except kallidin ([lysine]bradykinin), which is structurally unrelated to oxytocin and [4-proline]oxytocin, which is virtually inactive biologically (20). The regressions were parallel, indicating a common set of binding sites for the analogues. The inhibitory potencies and 95% confidence limits of each peptide, relative to oxytocin, were: oxytocin, 1; [deamino]oxytocin, 2.0 (1.6 → 2.6); [4-threonine]oxytocin, 1.3 (0.90 → 2.1); [2-*O*-methyltyrosine]oxytocin, 0.36 (0.26 → 0.58); and [lysine]vasopressin, 0.16 (0.10 → 0.24). If the peptides act by competing with oxytocin for a common binding site, the relative inhibitory potency of each compound provides a measure of its relative apparent K_d.

The binding of oxytocin was specific for mammary cells. Erythrocytes, prepared from rat blood

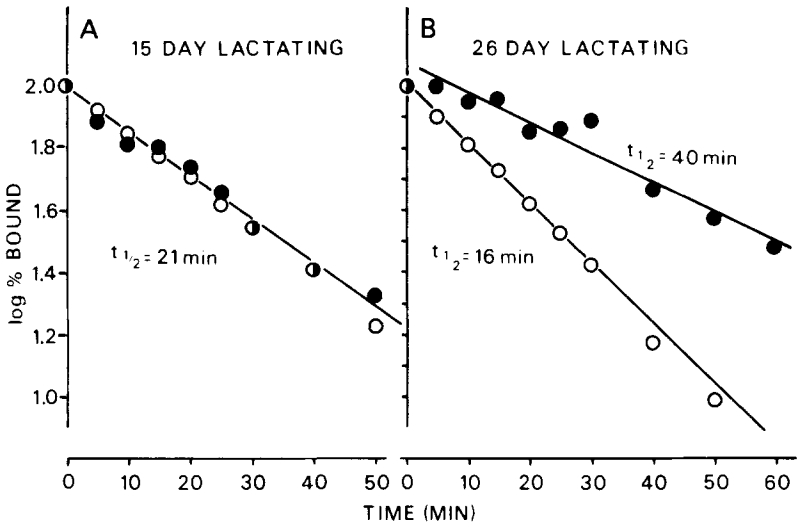

FIGURE 8 Rate of dissociation of oxytocin-receptor complexes with isolated cells from rats lactating for 15 (A) and 26 (B) days. The oxytocin-receptor complexes were formed by incubating 5.6×10^7 cells/ml with 4 nM [^{3}H]oxytocin for 30 min. Dissociation was initiated with a 1:50 dilution of the cell suspension containing either no (●—●) or 1 μM nonradioactive oxytocin (○—○).

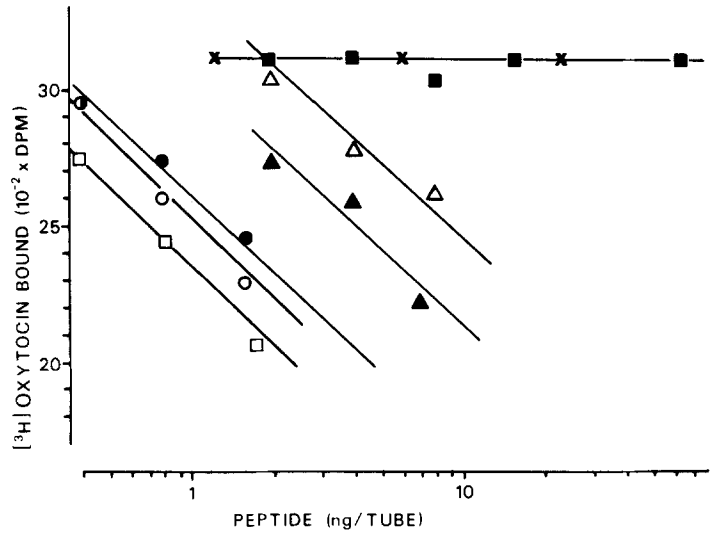

FIGURE 9 Ligand specificity of isolated mammary cells. Cells were incubated with 0.65 nM [^{3}H]oxytocin and increasing concentrations of nonradioactive peptides in 250 μl Tyrode's solution for 1 h at 20°C. Oxytocin (●—●); [desamino]oxytocin (□—□); [4-threonine]oxytocin (○—○); [2-*O*-methyltyrosine]oxytocin (▲—▲); [8-lysine]vasopressin (△—△); [lysine]bradykinin (■—■); and [4-proline]oxytocin (×—×).

and treated in the same manner described for the washing and assay of mammary cells, bound only 3.5% of the [^{3}H]oxytocin bound by an equal number of mammary cells. Furthermore, the amount of [^{3}H]oxytocin bound by the erythrocytes was not reduced by the presence of an 100-fold excess of nonradioactive oxytocin.

DISCUSSION

Isolated mammary cells, which appear to be viable

because of their ability to exclude dye and by their ultrastructural appearance, bind [^{3}H]oxytocin. Previous studies have shown that radioactivity from oxytocin was localized in the region of mammary tissue occupied by myoepithelial cells (25). We therefore assume that the oxytocin is bound by the myoepithelial cells in the mixture of mammary cells. The assumption is supported by the observed localization of radioactivity from [^{3}H]oxytocin in fractions enriched in myoepithelial cells by Metrizamide gradient centrifugation.

The results obtained in these studies are in good agreement with our previous studies on broken cell preparations from the mammary gland of the lactating rat (27). The apparent K_d for oxytocin binding to the isolated cells was about five times greater than the value obtained with mammary particulate fractions (27). There may be several reasons for this difference. The Tyrode's solution used to maintain viable cells contained 1 mM Mg^{2+}, whereas maximal binding with the mammary particles was obtained with concentrations of Mg^{2+} greater than 5 mM. Another factor contributing to the reduced affinity of the cells may have been the presence of proteolytic activity in the collagenase preparations. Protease has been shown to destroy oxytocin binding to mammary particles (27).

Binding was highly specific for the structure of oxytocin. [Deamino]oxytocin, which lacks the N-terminal amino group of oxytocin, was bound with about twice the affinity of oxytocin by the mammary receptor sites. Although [deamino]oxytocin has appreciably greater activity than oxytocin on the rat isolated uterus (7, 10), it appears to be less active than oxytocin in stimulating milk-ejection in lactating rats in vivo (1). The apparent discrepancy between the relative affinity of mammary binding sites for [deamino]oxytocin and the relative biological activity of the peptide may be due to the assay conditions. For example, the activities of many oxytocin analogues depend upon whether the assay is carried out in vivo or in vitro (1). In view of these differences, the affinity for [deamino]oxytocin should be related to its biological potency on isolated myoepithelial cells; but these data are not available. The affinities for [4-threonine]oxytocin, [8-lysine]vasopressin and [4-proline]oxytocin corresponded to the affinities found with broken mammary cell preparations (27). [*O*-methyltyrosine]oxytocin, which is an antagonist/partial agonist in the isolated uterus assay (1), was bound by mammary cells with 0.36 times the affinity of oxytocin. The potency of [*O*-methyltyrosine]oxytocin in the rat isolated mammary gland is about 0.15 that of oxytocin (1). Kallidin ([lysine]bradykinin), which is structurally unrelated to oxytocin, did not appear to bind to the oxytocin receptor in the dose range studied.

The apparent K_d of oxytocin-receptor interaction estimated under steady-state conditions was internally consistent, within experimental error, with the K_d estimated from the rate constants. The binding reaction, therefore, appears to be the single-step process shown in Eq. 1. The K_d values estimated from steady-state and kinetic analyses of a multi-step reaction would also agree, however, if the intermediates had lifetimes of shorter duration than the oxytocin-receptor complex being measured. For example, in the reaction $O + R \rightleftharpoons R'O \rightleftharpoons RO$, if $[R'O] \ll [RO]$ the binding data would be indistinguishable from those obtained with the reaction shown in Eq. 1. Our results, therefore, indicate only the minimum number of steps in the oxytocin-receptor reaction sequence.

The Scatchard analysis, showing a linear relationship between the ratio of bound:free oxytocin to the concentration of hormone bound, indicates that there is a single class of non-interacting binding sites of high affinity for oxytocin. These results are mirrored by the lack of effect of the concentration of oxytocin on the dissociation rate of the oxytocin-receptor complex on mammary cells from rats lactating from 6 to 16 days. The uniformity of the first-order dissociation rate constants found with a range of hormone-receptor concentrations is compatible with a one-step, monomolecular dissociation process involving a homogeneous phase. On the other hand, the dissociation of hormone from mammary cells prepared from the 26-day lactating rat appears to occur in a different manner.

The acceleration of dissociation rates resulting from isotopic dilution of hormone, as opposed to chemical dilution, may be explained by negative cooperativity (9). However, similar results may be obtained if the hormone-receptor complex and free hormone are in a heterogeneous, two-phase system (4, 24, 28). The mammary gland has undergone considerable involution by the 26th day of lactation because the pups suckle less frequently. Regardless of the mechanism of the altered dissociation rate, the twofold decrease in the dissociation rate constant by day 26 suggests that the myoepithelial cells may be particularly sensitive to low concentrations of oxytocin during invo-

lution of the mammary gland.

The kinetic analysis of binding illustrates inconsistencies between the rate of oxytocin action and the rate of oxytocin binding. Milk ejection or contraction of mammary strips in vitro occurs within seconds after the introduction of oxytocin. Oxytocin binding to isolated cells, however, continued to increase up to 40 min, suggesting that contraction of myoepithelial cells can occur when only a fraction of the receptor sites are occupied. Comparable findings have been made with a number of hormone-receptor complexes (2, 6, 13, 17, 18). In these systems, the concentration of hormone giving a half-maximal response was always found to be less than the concentration occupying half of the receptor sites (apparent K_d). If we assume that 10% or fewer of the receptor sites are occupied by oxytocin 1 min after the addition of hormone and that concentration has taken place by this time, then fewer than 10,000 molecules of oxytocin are able to elicit contraction of a myoepithelial cell. These estimates dramatize the amplification mechanism involved in oxytocin action. The availability of intact cells which respond to oxytocin provides a model system to determine the sequence of events involved in the amplification of oxytocin-receptor interaction.

We thank M. Morrison for technical assistance, Dr. A. F. Pearlmutter for helpful discussions, Drs. M. Manning, J. Cort, and Sandoz, Ltd. for the oxytocin analogues and C. Licata for the typescript.

This research was supported by grant HD08406 from the National Institutes of Health and by General Research Support grant RR05700 to the Medical College of Ohio.

Received for publication 20 September 1976, and in revised form 21 March 1977.

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