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Progesterone Inhibition of Glucocorticoid Binding to Mammary Tissue from Lactating and Nonlactating Cows (40883)[1]

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Abstract. Mammary tissue cytosols (105,000*g* supernatants) were prepared from lactating or from nonlactating, nonpregnant cows, then incubated with [^{3}H]dexamethasone alone (3×10^{-8} *M*) or in combination with various unlabeled hormones (1.9×10^{-7} or 1.9×10^{-6} *M*). Cortisol, corticosterone, and progesterone inhibited binding of [^{3}H]dexamethasone, whereas testosterone, cortisone, and estradiol-17 β did not. Binding affinities of mammary cytosols from lactating and nonlactating cows for [^{3}H]dexamethasone (0.5 to 100 n*M*) were virtually identical averaging 1.4 ($\pm$0.3) and 2.0 ($\pm$1.2) $\times 10^{-9}$ *M*, respectively. However, binding capacities of mammary tissue slices (fmole dexamethasone/μg DNA) were greater in lactating (2.3) than in nonlactating (1.5) cows. Specific cytosolic binding sites sedimented at 8 and 4 S on sucrose gradients. Whole mammary slices were incubated with 3×10^{-9} *M* [^{3}H]dexamethasone alone or in combination with 10^{-13} to $10^{-5} \times 3.8$ *M* progesterone or 3×10^{-7} *M* dexamethasone, and cytoplasmic and nuclear fractions (1000*g* supernatants and pellets, respectively) were subsequently isolated. Specific cytoplasmic binding of [^{3}H]dexamethasone in these slices was competitively reduced at concentrations in excess of 10^{-8} *M* progesterone in nonlactating tissue and at concentrations in excess of 10^{-7} *M* in lactating tissue. In contrast, when isolated cytosols were incubated directly, concentrations of progesterone needed to inhibit glucocorticoid binding were identical regardless of the physiological state of the mammary tissue. These data support the hypothesis that progesterone may be sequestered in milk fat and unavailable for competitive binding at the glucocorticoid binding site in lactating tissue but not in nonlactating tissue or in cytosol fractions which are relatively free of milk fat.

Progesterone concentrations are elevated throughout pregnancy and decline at parturition when copious milk secretion begins. While progesterone inhibits onset of lactation (1–3), it does not inhibit an established lactation (4, 5). In addition, progesterone competes with glucocorticoids for binding sites in mammary tissue of a number of species (6–9). Since glucocorticoids are involved in initiation and maintenance of lactation (10–12), progesterone inhibition of glucocorticoid binding may be a mechanism whereby progesterone exerts its inhibitory effect on lactogenesis. We (13) determined previously that much greater doses of progesterone were required to reduce total [^{3}H]cortisol uptake, uncorrected for nonspecific binding, in mammary tissue from lactating than from nonlactating cows. The objective of the present study was to characterize specific cytosolic glucocorticoid binding and its sensitivity to progesterone inhibition in mammary tissue from lactating and nonlactating cows.

Materials and methods. Chemicals and reagents. We obtained 6,7-[^{3}H]dexamethasone (35–50 Ci/mmole) and unlabeled 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione (R5020) from New England Nuclear Corp. (Boston, Mass.). Other steroids, bovine serum albumin fraction V, yeast alcohol dehydrogenase, dextran-T 70, reduced glutathione, Trizma acid, and Trizma base were obtained from Sigma Chemical Co. (St. Louis, Mo.). Media were from Difco Laboratories (Detroit, Mich.). Other chemicals were reagent grade and obtained from Fisher Scientific Co. (Pittsburgh, Pa.).

Animals. Animals were Holstein cows from the Michigan State University dairy herd. Studies to characterize dexametha-

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sone binding in cytosol utilized two nonlactating and four lactating, nonpregnant cows. In addition, four lactating, nonpregnant cows and four nonlactating, nonpregnant cows were used to study progesterone inhibition of dexamethasone uptake by mammary tissue slices and progesterone inhibition of dexamethasone uptake by mammary tissue slices and progesterone inhibition of dexamethasone binding by isolated cytosol. Lactating cows were in mid-to-late lactation and were milked approximately 4 hr before slaughter. Nonlactating cows had not been milked for a minimum of 2 months.

Mammary tissue was obtained within 30 min of slaughter. Tissue was immediately placed in ice-cold Tris–sucrose buffer (30 m*M* Trizma–HCl, 6.8 m*M* Trizma base, 0.3 *M* sucrose, 1.0 m*M* Na_2EDTA, and 1.0 m*M* reduced glutathione, pH 7.4). These samples were sliced or homogenized and centrifuged at 105,000*g* then processed for hormone uptake and binding, respectively.

Characterization of [^3H]dexamethasone binding in isolated mammary cytosol. The following procedures were performed at 4°. Mammary tissue (4 g) was minced with scissors, washed with Tris–sucrose buffer, then homogenized in Tris–sucrose buffer (1:4, W/v) with a Brinkman Polytron. Homogenates were centrifuged at 1000*g* for 10 min and the supernatant fluid recentrifuged at 105,000*g* for 60 min. The final supernatant (cytosol) was drawn from beneath the lipid layer with a Pasteur pipet.

Preliminary experiments indicated that maximal specific binding of [^{3}H]dexamethasone to cytosols was obtained within 60 min at 4°. Routinely, reactions were incubated for 1 hr at 4°. To titrate the number and affinity of glucocorticoid binding sites, mammary cytosol (0.2 ml) from each of two nonlactating and four lactating cows was incubated with 0.5 to 100 n*M* [^{3}H]dexamethasone. Each dose was tested in quadruplicate. After incubation, 50 μl of dextran–charcoal (Tris–sucrose buffer containing 10% Norit and 0.1% dextran-T 70) was added, the tubes vortexted for 10 sec, then centrifuged for 10 min at 1000*g*. One-tenth milliliter of supernatant was removed and radioactivity was quantified by liquid scintillation spectrometry. Data were arranged in a Scatchard plot (14), and specific binding determined (15).

Other samples of mammary cytosol from each of two lactating and two nonlactating cows were incubated with 30 n*M* [^{3}H]dexamethasone alone or in combination with 1.9×10^{-7} *M* or 1.9×10^{-6} *M* unlabeled, competing steroid in order to test specificity of dexamethasone binding sites. Unlabeled hormones used were dexamethasone, cortisol, corticosterone, cortisone, testosterone, estradiol-17β, and progesterone. Each combination was tested in quadruplicate. After incubation, steroids were adsorbed with dextran–charcoal, radioactivity was quantified, and data were expressed as a percentage of the inhibition of specific binding obtained with 1.9×10^{-6} *M* dexamethasone.

In addition, aliquots of mammary cytosol (0.2 ml) from each of two lactating cows were incubated with 0.8 to 115 n*M* [^{3}H]dexamethasone with or without 2 μ*M* unlabeled progesterone. Binding of [^{3}H]dexamethasone was determined by the dextran–charcoal technique. Double reciprocal plots of these binding data were prepared and lines of best fit determined by linear regression.

Finally, cytosols from each of two lactating cows, prepared in 10 m*M* Tris buffer, were incubated with 15 n*M* [^{3}H]dexamethasone alone or in combination with 1.5 μ*M* unlabeled dexamethasone or 1.5 μ*M* R5020. Linear gradients (4.2 ml) of 5 to 15% sucrose containing 10% (v/v) glycerol in 10 m*M* Tris buffer (10 m*M* Trizma HCl, 1.5 m*M* Na_2EDTA, 1.0 m*M* glutathione, pH 7.4) were prepared. Samples of labeled cytosol (0.2 ml) were then layered on the gradients and centrifuged for 16 hr at 200,000*g*. Following centrifugation, six-drop fractions of the gradient were collected into tubes containing 0.5 ml of 10 m*M* Tris buffer. Samples were extracted with dextran–charcoal and radioactivity quantified. Migration of bovine serum albumin and yeast alcohol dehydrogenase standards were estimated from optical density determinations at 280 nm.

Progesterone inhibition of [^3H]dexamethasone uptake in whole mammary slices. Mammary tissue was sliced using a

Stadie–Riggs hand microtome. These slices were placed in ice-cold Tris–sucrose buffer and cut into 1–2-mm^2 fragments. Tissue fragments (approximately 25 mg) were then added to tubes containing 1 ml of medium 199 at pH 7.4 and 3×10^{-9} *M* [^{3}H]dexamethasone alone or in combination with 10^{-13} to $10^{-5} \times 3.8$ *M* unlabeled progesterone or 3×10^{-7} *M* unlabeled dexamethasone. Each treatment was performed in quadruplicate. Tissue slices were incubated with hormones for 1 hr at 37°, a time which gave maximal incorporation, then washed three times with 3 ml of ice-cold Tris–sucrose buffer, and homogenized. The homogenate was centrifuged at 1000*g* for 15 min at 4°, and the supernatant ("cytoplasm") decanted into scintillation vials to which 14 ml of scintillation fluid was added (6). The 1000*g* precipitate ("nuclei") was then washed with 2 ml of Tris–sucrose buffer and extracted twice with 2 ml of ethanol. The extract was added to 14 ml of scintillation fluid and radioactivity quantified. Deoxyribonucleic acid (DNA) content of 1000*g* precipitate was quantified (6) and disintegrations per minute per microgram of DNA determined. Data were expressed as percentage inhibition of specific dexamethasone uptake, i.e., as a percentage of inhibition of [^{3}H]dexamethasone uptake obtained with a 100-fold excess of unlabeled dexamethasone.

Progesterone inhibition of [^{3}H]dexamethasone binding in isolated mammary cytosol. Cytosol was prepared as previously described, and then 0.2 ml was incubated for 1 hr at 4° with 3×10^{-9} *M* [^{3}H]dexamethasone alone or in combination with 10^{-12} to $10^{-6} \times 4.8$ *M* unlabeled progesterone or 1.5×10^{-5} *M* unlabeled dexamethasone. Each combination was tested in quadruplicate. Free steroid was adsorbed with dextran–charcoal and data were expressed as a percentage of inhibition of binding obtained with 1.5×10^{-5} *M* unlabeled dexamethasone.

Statistical analysis. Progesterone inhibition of [^{3}H]dexamethasone uptake by mammary slices and progesterone inhibition of [^{3}H]dexamethasone binding in mammary cytosol were examined by analysis of variance of split-plots with repeat measure (16). Differences among individual treatment means were tested for significance using the Bonferroni *t* statistic (17).

Results. Characterization of [^{3}H]dexamethasone binding in isolated mammary cytosol. Data from a representative lactating cow, plotted according to Scatchard (14), are in Fig. 1. A single high-affinity, low-capacity binding component and a low-affinity, high-capacity ("nonspecific") binding component were observed. Correcting the data for nonspecific binding, according to the method of Chamness and McGuire (15), yielded a straight line with an apparent dissociation constant (K_d) of 0.63 $\times 10^{-9}$ *M* and binding capacity of 90 fmole/mg protein. Separate analyses of mammary cytosol from four lactating cows yielded a mean K_d of 1.4 ($\pm$ 0.3) $\times 10^{-9}$ *M* with a binding capacity of 80 $\pm$ 11 fmole/mg protein. Analyses of cytosol from two nonlactating cows similarly yielded a mean K_d of 2.0 ($\pm$ 0.2) $\times 10^{-9}$ *M* and a binding capacity of 122 $\pm$ 12 fmole/mg protein.

Effects of various unlabeled steroids on [^{3}H]dexamethasone binding in isolated

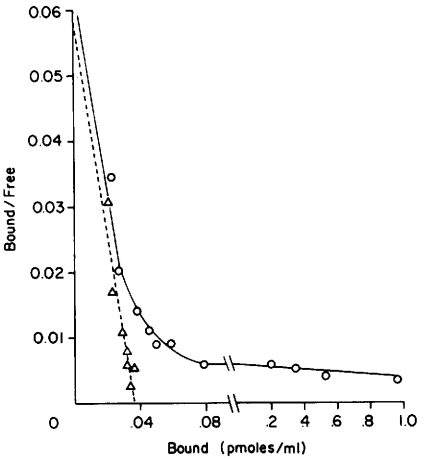

FIG. 1. Scatchard (14) plot of dexamethasone binding in mammary cytosol obtained from a representative (of four) lactating cow. Aliquots of cytosol (0.2 ml) were incubated with various concentrations of [^{3}H]-dexamethasone (0.5–100 n*M*) and total binding determined by the dextran–charcoal method. Total binding (○) was corrected for nonspecific binding according to Chamness and McGuire (15) to yield "specific" binding (△).

TABLE I. LIGAND SPECIFICITY OF [^{3}H]DEXAMETHASONE BINDING IN MAMMARY CYTOSOL[a]

Competitor	Percentage inhibition of [^{3}H]dexamethasone binding	
	1.9×10^{-7} *M*	1.9×10^{-6} *M*
None	0	0
Dexamethasone	63 ± 8	100
Cortisol	39 ± 14	81 ± 8
Corticosterone	61 ± 9	92 ± 6
Cortisone	0 ± 0	19 ± 10
Testosterone	12 ± 6	40 ± 7
Estradiol-17β	5 ± 3	15 ± 9
Progesterone	60 ± 11	102 ± 7

[a] Mammary cytosol, containing approximately 2 mg protein/ml, was incubated with 3×10^{-8} *M* [^{3}H]dexamethasone with or without unlabeled steroids and binding determined by the dextran–charcoal method. Data are expressed as a percentage of the inhibition of binding obtained with 1.9×10^{-6} M dexamethasone. Values are means ± SE for two lactating and two nonlactating cows. Cytosol from nonlactating and lactating animals, specifically bound 150 ± 10 and 100 ± 25 fmole dexamethasone/mg cytosolic protein, respectively.

mammary cytosol are in Table I. Results using cytosol from lactating and nonlactating cows were similar, and therefore data were combined. Cortisol, corticosterone, and progesterone competed well for specific [^{3}H]dexamethasone binding sites. Testosterone competed poorly, while cortisone and estradiol-17β demonstrated virtually no inhibition of binding at the concentrations tested.

Double reciprocal plots of [^{3}H]dexamethasone binding in isolated mammary cytosol showed a common intercept on the ordinate in the absence or presence of unlabeled progesterone (Fig. 2). These data are indicative of a competitive type of inhibition of [^{3}H]dexamethasone binding by progesterone.

Binding components of 8 and 4 S were observed in sucrose gradient analyses of [^{3}H]dexamethasone binding in lactating mammary cytosol (Fig. 3). Binding to both components was inhibited by a 100-fold excess of unlabeled dexamethasone. Binding was also inhibited by a 100-fold molar excess of the synthetic progestin, R5020. However, dexamethasone did not compete for R5020 binding sites (unpublished data).

Progesterone inhibition of [^{3}H]dexamethasone uptake in whole mammary slices. Progesterone inhibited specific cytoplasmic uptake of dexamethasone more effectively in mammary tissue slices from nonlactating than from lactating cows (Fig. 4). For example, progesterone at 3.8×10^{-7} *M* significantly inhibited cytoplasmic uptake of [^{3}H]dexamethasone by nonlactating tissue; whereas 3.8×10^{-6} *M* was the lowest progesterone concentration to inhibit significantly ($P < 0.05$) uptake in lactating tissue. No significant difference was detected in progesterone reduction of [^{3}H]dexamethasone uptake between nonlactating

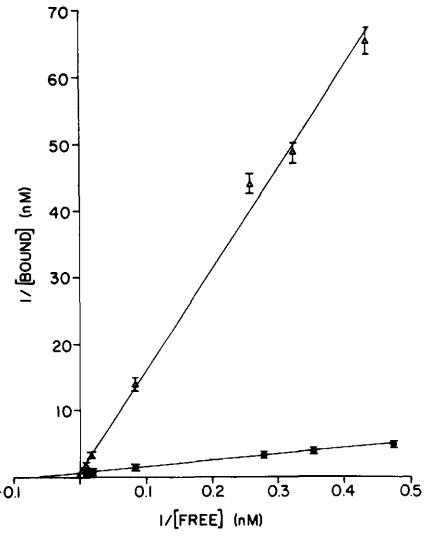

FIG. 2. Double reciprocal plot of bound versus free dexamethasone in mammary cytosol from one of two lactating cows. Aliquots of cytosol were incubated with increasing concentrations of [^{3}H]dexamethasone without (●) or with 2 μ*M* unlabeled progesterone (△). Lines of best fit were determined by linear regression analysis.

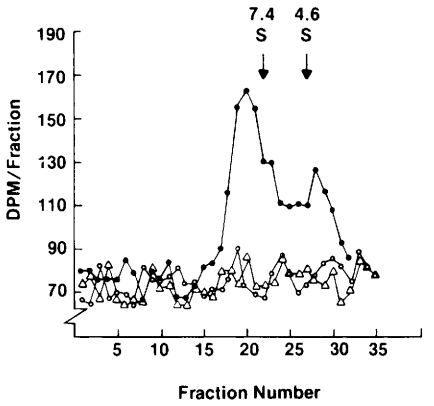

FIG. 3. Sedimentation profile in sucrose density gradients of cytosolic [^{3}H]dexamethasone binding site complexes isolated from a representative lactating cow. Cytosol was incubated with 15 nM [^{3}H]dexamethasone in the absence of unlabeled steroids (●) or presence of 1.5 μM unlabeled dexamethasone (○) or R5020 (△). Sedimentation markers of 7.4 and 4.6 S were yeast alcohol dehydrogenase and bovine serum albumin, respectively.

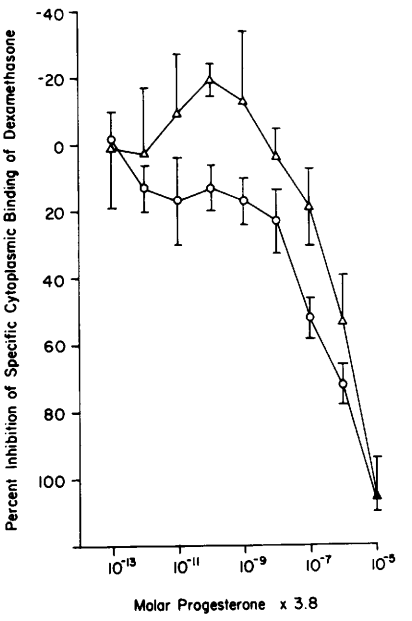

FIG. 4. Progesterone inhibition of dexamethasone binding in cytoplasm (1000g supernatant) subsequently isolated from whole mammary slices of four lactating (△) and four nonlactating (○) cows. Mammary slices were incubated with 3×10^{-9} M [^{3}H]dexamethasone with or without unlabeled dexamethasone or varying concentrations of progesterone. Data are expressed as percentage inhibition of specifically bound dexamethasone. Binding in the presence of 3×10^{-7} M unlabeled dexamethasone was taken as 100% inhibition. Values are means ± SE. Cytoplasm from nonlactating and lactating animals specifically bound 0.7 ± 0.1 and 1.1 ± 0.2 fmole dexamethasone/μg DNA, respectively.

and lactating mammary tissue in the nuclear pellet (Fig. 5). Total binding sites in nonlactating and lactating mammary tissue were 1.5 ± 0.2 and 2.3 ± 0.5 fmole/μg DNA, respectively. In the absence of progesterone, approximately 50% of the radioactivity was bound to nuclei after 1 hr incubation of either nonlactating or lactating tissue.

Progesterone inhibition of [^3H]dexamethasone binding in isolated mammary cytosol. In lactating and in nonlactating cows 4.8×10^{-8} M was the lowest concentration of progesterone which significantly inhibited ($P < 0.05$) specific binding of [^{3}H]dexamethasone in the isolated cytosol (Table II). There were no significant differences ($P > 0.05$) between isolated mammary cytosol from lactating or nonlactating cows in the ability of any of the doses of progesterone to inhibit [^{3}H]dexamethasone binding.

Discussion. As in other systems (6–9), we observed that isolated bovine mammary cytosol bound dexamethasone with high affinity and low capacity (Fig. 1), and with typical (6–9) glucocorticoid specificity (Table I). The dissociation constants of glucocorticoid binding sites in cytosol from lactating and nonlactating tissues were equivalent. However, binding capacities differed. Cytosol from lactating and nonlactating cows specifically bound 90 and 140 fmole of dexamethasone per milligram of cytosolic protein, respectively (Fig. 1, Tables I, II). Tissue slices from lactating and nonlactating cows specifically bound 2.3 and 1.5 fmole of dexamethasone per microgram DNA (Figs. 4, 5). These data indicate that lactating tissue contains more glucocorticoid binding sites per cell than nonlactating tissue which agrees with our previous results (18). Because of the greatly increased concentrations of intracellular and extracellular proteins during lactation, the number of binding sites per milligram of

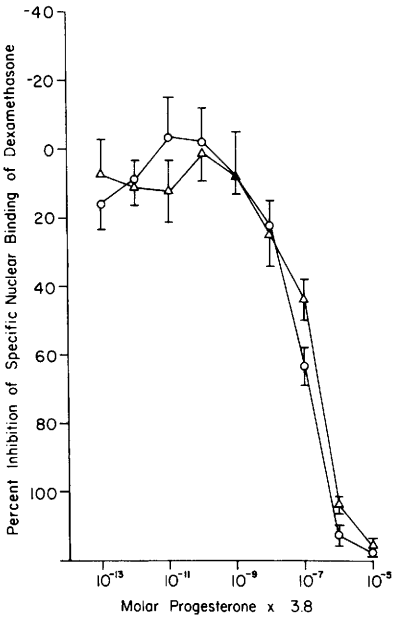

FIG. 5. Progesterone inhibition of dexamethasone binding in nuclei (1000*g* pellet) subsequently isolated from whole mammary slices of four lactating (△) and four nonlactating (○) cows. Nuclear pellets from nonlactating and lactating animals specifically bound 0.8 ± 0.1 and 1.2 ± 0.4 fmole dexamethasone/μg DNA, respectively.

cytosolic protein is lower in lactating than nonlactating tissue (9). Although only two lactating and two nonlactating cows were examined, specificity of glucocorticoid binding was identical in both physiological states. Moreover, the progesterone–dexamethasone interaction in freshly isolated bovine mammary cytosol was competitive (Fig. 2), which is in agreement with our previous studies using cultured mammary cells (6).

Sedimentation profiles in sucrose density gradients of cytosolic dexamethasone binding site complexes (Fig. 3) were similar to those reported for mammary tissue of other species (19). Nonlactating tissue was not characterized, although in other systems the sedimentation profiles are identical to those of lactating tissue (9). In addition, we observed that unlabeled dexamethasone or the synthetic progestin, R5020, each inhibited binding of [^{3}H]dexamethasone to the 8 and 4 S components. Use of [^{3}H]dexamethasone rather than naturally occurring glucocorticoids eliminates the problem of contamination of tissues by corticosteroid binding globulin, since dexamethasone does not bind to this molecule (20). Furthermore, R5020 does not bind to corticosteroid-binding globulin (21).

In pregnant, nonlactating animals progesterone inhibits lactogenesis (1–3), whereas glucocorticoids induce lactogenesis (22–24). Progesterone, however, does not affect an established lactation (4, 5). Thus, if progesterone inhibition of glucocorticoid binding is part of the mecha-

TABLE II. PROGESTERONE INHIBITION OF SPECIFIC [^{3}H]DEXAMETHASONE BINDING IN MAMMARY CYTOSOL[a]

Competitor	Percentage inhibition of [^{3}H]dexamethasone binding	
	Nonlactating	Lactating
Dexamethasone 1.5×10^{-5} *M*	100	100
Progesterone		
4.8×10^{-12} *M*	12 ± 4	22 ± 7
4.8×10^{-11} *M*	7 ± 3	2 ± 11
4.8×10^{-10} *M*	7 ± 6	1 ± 10
4.8×10^{-9} *M*	18 ± 5	13 ± 16
4.8×10^{-8} *M*	37 ± 3	57 ± 15
4.8×10^{-7} *M*	74 ± 2	71 ± 10
4.8×10^{-6} *M*	96 ± 1	101 ± 5

[a] Isolated mammary cytosol, containing approximately 2 mg protein/ml, was incubated with 3×10^{-8} *M* [^{3}H]-dexamethasone with or without unlabeled dexamethasone or varying concentrations of progesterone and data expressed as a percentage of the inhibition of binding obtained with 1.5×10^{-5} *M* unlabeled dexamethasone. Binding was determined by the dextran–charcoal method. Values are means ± SE for four lactating and four nonlactating cows. Cytosol from nonlactating and lactating animals specifically bound 140 ± 17 and 90 ± 10 fmole dexamethasone/mg cytosolic protein, respectively.

nism whereby progesterone inhibits lactogenesis, then progesterone displacement of glucocorticoids from their binding sites should be much greater in mammary tissue from nonlactating than from lactating cows. In fact, Collier and Tucker (13) reported that progesterone inhibited total uptake (uncorrected for nonspecific binding) of cortisol to a greater extent in whole mammary tissue slices from nonlactating than from lactating cows. We have shown in the present study that specific cytoplasmic uptake of dexamethasone by nonlactating mammary tissue slices was reduced at concentrations of 3.8×10^{-7} *M*, while in lactating mammary tissue dexamethasone specific uptake was not significantly inhibited until a progesterone concentration of 3.8×10^{-6} *M* was used. Nuclear binding of dexamethasone appeared more sensitive to inhibition by progesterone than was cytoplasmic binding (Figs. 4, 5) indicating that progesterone not only inhibited cytoplasmic binding of dexamethasone but also inhibited nuclear translocation. No attempt, however, was made to characterize nuclear binding components.

Mammary epithelial cells are present in much greater numbers in lactating than in nonlactating mammary tissue. Thus, the difference between lactating and nonlactating mammary tissue in their sensitivity to progesterone inhibition of glucocorticoid binding might be explained on the basis of an alteration in cell types or characteristics of the glucocorticoid binding sites. In addition, insensitivity of lactating tissue to progesterone inhibition could be caused by high-endogenous glucocorticoids or low-endogenous progesterone concentrations so that the effective progesterone/glucocorticoid ratio is lower in lactating than in nonlactating tissue. However, these factors are probably not involved because the affinity and specificity of glucocorticoid binding sites were the same in cytosols isolated from mammary tissue of lactating or nonlactating cows (Table II). Thus, the difference in sensitivity of lactating and nonlactating mammary tissue to progesterone inhibition of glucocorticoid binding apparently requires cellular integrity (Fig. 4). This difference in sensitivity may reflect differences in permeability and compartmentalization of progesterone and dexamethasone in lactating and nonlactating cells. Such compartmentalization may be brought about by membrane binders of hormones (25). Alternatively, because of its lipophilic nature (26), Collier and Tucker (13) postulated that progesterone may be sequestered in the milk fat present in the mammary cytoplasm of lactating cows and transported to the alveolar lumen, whereas in nonlactating tissue milk fat is relatively absent and progesterone is available to compete with the glucocorticoids at their binding sites. Data of the present study (Fig. 4 and Table II) support this concept. We suggest that the reason differences in progesterone inhibition of dexamethasone binding were not apparent in isolated cytosol from lactating and nonlactating cows is because these cytosols were essentially fat free.

In addition to the hypothesis supported by the present study, that progesterone exerts an inhibitory effect on lactogenesis by interfering with glucocorticoid action, progesterone may inhibit lactogenesis in a mechanism mediated by a progesterone binding site. The presence of progesterone binding sites in nonlactating mammary tissue may enable progesterone to inhibit lactogenesis, while a lack of progesterone binding sites in lactating tissue may preclude an inhibitory effect of progesterone on an established lactation. In support of this hypothesis, Shyamala and McBlain (27) were unable to detect progesterone binding sites in mammary cytosol of lactating Balb/c mice, and they cite unpublished data indicating the presence of progesterone binding sites in nonlactating mammary tissue. In contrast, Markland and Hutchens (28) characterized progesterone binding sites in lactating caprine mammary tissue, and unpublished data from our laboratory indicates the presence of progesterone binding sites in lactating bovine mammary tissue. Consequently, the role of progesterone binding sites per se remains to be ascertained, particularly as it pertains to ruminants.

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