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# Prolactin Receptors in Organ Culture of Rabbit Mammary Gland: Effect of Cycloheximide and Prolactin (40678)

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The central role of prolactin in mammary gland physiology has been extensively studied both *in vivo* and *in vitro* (1) and prolactin receptors have been identified and purified in rabbit mammary gland (2–4). However, the essential steps involved in the mechanism of prolactin action, subsequent to prolactin binding to the plasma membrane, are unknown.

It is well established that the mammary gland can be maintained in organ culture and responds well to hormones (5, 6). In addition, mammary explants can be used as an experimental model to study the steps involved in the mechanisms of hormone action (7). The aim of this study was to verify the maintenance of prolactin receptors in mammary glands in organ culture, to assess the apparent turnover of receptors, and to describe the effect of large doses of prolactin on the levels of its own receptor.

*Materials and methods. Organ culture.* Pseudopregnancy was induced in New Zealand rabbits, 6 months of age, by mating with vasectomized males. Animals were killed by cervical dislocation at 12–14 days of pseudopregnancy. Mammary glands were removed and placed in a sterile petri dish. Under a laminar flow hood, 5- to 10-g portion of tissue was selected from the periphery of the gland, rinsed in medium 199 (Gibco), and placed in an Erlenmeyer flask containing 10 ml of medium 199. Approximately 500 mg of tissue was removed and dissected from fat and conjunctive tissue under a binocular microscope. Epithelial fragments were cut into small fragments of 1 to 2 $mm^3$. Explants were placed on stainless-steel grids (approximately 20 explants per grid) and five grids were placed in 10 × 35-mm petri dishes. Medium 199 supplemented with amino acids (four times the usual concentration of amino acids in the medium) and bicarbonate (2.2 g/liter) were added to the dishes to cover the grids taking care not to entirely cover the explants with medium. The petri dishes were placed in an incubator at 37° under a controlled atmosphere of 57% $O_2$, 5% $CO_2$, 38% $N_2$ (8). Explants were cultured up to 48 hr in the presence of insulin (5 μg/ml, Sigma), cycloheximide (1 μg/ml, Sigma), or ovine prolactin (5 μg/ml, NIH-P-S12, 35 IU/mg). At the end of the culture, the grids were placed on filter paper and the explants removed, weighed and frozen at −20°.

*Membrane preparations.* Approximately 500 mg tissue was homogenized in 4 ml of 0.3 *M* sucrose in a Turax homogenizer for 30 sec at medium speed and rehomogenized in a glass–teflon homogenizer. The homogenate was centrifuged at 12,000*g* for 15 min and the supernatant was recentrifuged at 105,000*g* for 90 min and the pellet resuspended in Tris buffer (25 m*M* Tris–HCl, pH 7.5, 10 m*M* $MgCl_2$) as described previously (9). Protein was determined by the Lowry procedure (10) using bovine serum albumin (BSA) as standard on membranes solubilized with 1 *N* NaOH.

Since prolactin does not appear to dissociate from its receptor during membrane preparation, for explants cultured in the presence of prolactin, it was necessary to desaturate prolactin receptors prior to their assay. This technique involves an *in vitro* incubation of crude membranes with 4 *M* $MgCl_2$ as has been described in detail for growth hormone (11) and prolactin receptors (12).

*Receptor assay.* Prolactin binding was assayed using 200 μg membrane protein incubated with approximately 100,000 cpm of $^{125}$I-labeled human growth hormone (hGH, NIH, HS2160E, 2 IU/mg) iodinated using chloramine-T (13), in the absence and presence of 1 μg of ovine prolactin. Human growth hormone has been shown to have



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lactogenic activity in the rabbit mammary gland (6) and has been used to identify prolactin receptors in a number of target organs (14). Final incubation volumes were adjusted to 0.5 ml with Tris buffer containing 0.1% BSA. Incubation proceeded for 16 hr at room temperature and was stopped by the addition of 3 ml of cold Tris buffer containing BSA. The tubes were centrifuged at 3500*g* for 15 min and the pellets counted in a Packard gamma counter.

*Results.* Figure 1A illustrates the maintenance of prolactin receptors in mammary gland explants cultured in the presence of insulin. Binding increased slightly up to 12 hr and remained constant up to 48 hr. Addition of cycloheximide (1 μg/ml) resulted in a rapid decline of binding during the first 6 hr which remained low until 48 hr. Figure 1B shows another experiment and demonstrates the reversibility of the effect of cycloheximide (11 μg/ml). In the presence of insulin only, the level of receptors was maintained up until 48 hr as shown previously. The addition of cycloheximide resulted in a rapid decrease of receptors almost maximal at 3 hr. Removal of cycloheximide from the culture medium at either 6 or 24 hr by replacement with a medium deficient in cycloheximide resulted in a return of prolactin binding to near control levels 18–24 hr later.

Figure 2 shows the effect of prolactin on the level of its own receptor in explants cultured in the presence of a concentration of prolactin classically used *in vitro* to induce secretory activity (1 to 5 μg/ml). Free and total receptors ($MgCl_2$-treated) were measured in explants cultured for 48 hr with insulin or insulin + prolactin. As expected, free receptors were approximately 80% saturated by 5 μg/ml prolactin. Surprisingly, total receptors were also markedly reduced from 12.34 ± 1.33 to 4.4 ± 0.78%, suggesting a down-regulation of prolactin receptor by prolactin. To exclude the possibility that $MgCl_2$ was not effective at this level of saturation, membranes from explants cultured in the presence of prolactin were completely saturated *in vitro* after which they were exposed to 4 *M* $MgCl_2$. The dissociation of prolactin was equally effective under these conditions, indicating that the loss of total receptors observed in Fig. 2 was not due to an artifact of the dissociation technique.

The time dependence of prolactin saturation and down-regulation of prolactin receptors in culture is shown in Fig. 3. Saturation was incomplete at 1 hr and was maximal at 24–48 hr. The pattern of the reduction of total prolactin receptors is different from that of free receptors with a maximal effect observed in explants cultured in the presence of prolactin for 48 hr.

*Discussion.* These studies demonstrate that mammary gland explants are capable of maintaining prolactin receptors up to 48 hr and that the maintainance is not a static phenomenon, but represents an equilibrium

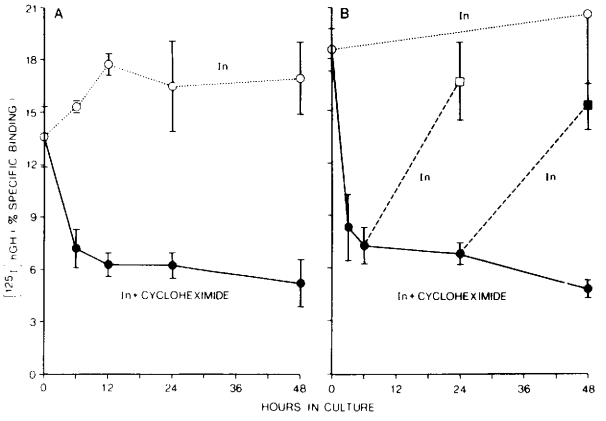


FIG. 1. Maintenance of prolactin receptors and the effect of cycloheximide on prolactin receptors in mammary gland explants. (A) Mammary explants were cultured at different times in the presence of insulin (In) or In + cycloheximide. (B) Mammary explants cultured in the presence of In or In + cycloheximide after which medium was changed at 6 or 24 hr and the cycloheximide was removed. Values are means ± SEM of three cultures.

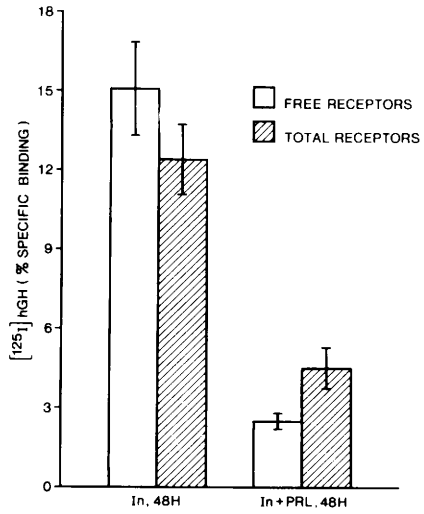


FIG. 2. The effect of prolactin (PRL) on the free and total level of its own receptor in organ culture of rabbit mammary gland. Explants were cultured in the presence of insulin (In, 5 μg/ml) or In + PRL (5 μg/ml) for 48 hr. Values are means ± SEM of seven or eight cultures.

between degradation and synthesis of receptors. Addition of cycloheximide to the explants revealed a marked loss of receptors, possibly indicating a rapid turnover. In rat liver, prolactin binding sites have been shown to be reduced by 90% within 2 hr after a single injection of 200 μg of cycloheximide with a return to normal levels 24–48 hr after injection (15).

*In vitro*, the massive doses of prolactin required to stimulate casein synthesis resulted in a 80% occupation of receptors. These receptors not saturated may represent sites inaccessible to the hormone. Magnesium chloride treatment of membranes from explants cultured in the presence of insulin alone in most cases led to a slight reduction of prolactin binding, most probably due to the loss of protein following treatment with the dissociating agent (12). For example, correction of total receptor levels shown in Fig. 2 for a 30% loss of protein results in 16.04 ± 1.73% binding, a value slightly higher than that observed for free receptors. In contrast, when explants were cultured with prolactin, treatment with $MgCl_2$ resulted in higher binding than in nontreated membranes. However, exposure to prolactin over a longer period of time (24–48 hr) led to a progressive reduction in binding corresponding to a relative reduction in total receptor levels. This down-regulation of prolactin receptors by prolactin itself is in contrast to the established stimulatory effect of prolactin on its receptor in mammary gland (16) and liver (17, 18) observed in chronically treated animals. We have observed a similar down-regulation of prolactin receptors *in vivo* in rabbit mammary gland and rat livers in animals which was maximal 6 hr after a single intravenous injection of prolactin (19). A decrease of prolactin binding following injection of increasing concentrations of prolactin has been reported in rat testis (20). This decline could however at last partially be explained by an occupation of free receptors, since total prolactin receptors (following dissociation) were not measured.

Down-regulation could reflect a compartmentalization of receptors which are progressively excluded from the crude membrane preparation we utilize. For other hormones such as LH (21) which induces a down-regulation of its own receptor, binding of the hormone to the plasma membrane is followed by an endocytosis of the hormone–receptor complex. In addition, injection of $^{125}$I-labeled ovine prolactin into rats results in a rapid concentration of intact prolactin inside the cell within Golgi vesicles (22). This could occur in mammary gland also, especially in view of the fact that prolactin has been local-

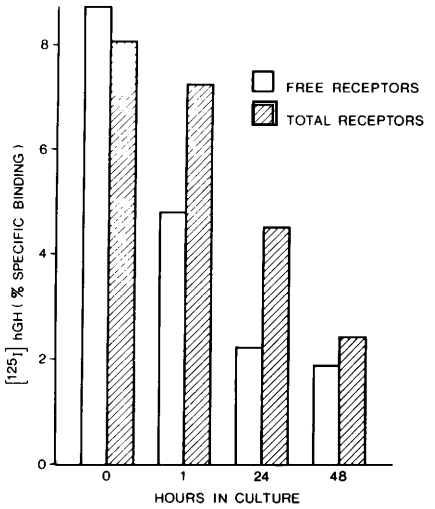


FIG. 3. Time course of the occupation of prolactin (PRL) receptors and decline in total ($MgCl_2$-treated) receptors in mammary explants cultured in the presence of insulin (In) and PRL (5 μg/ml) for the indicated times.

ized within mammary epithelial cells by immunohistochemical techniques (23). It is possible that down-regulation of prolactin receptors is more difficult to observe due to the apparent rapid turnover of receptors suggested *in vivo* for liver (15) and by the present studies *in vitro* for the mammary gland.

*Summary.* Prolactin receptors were maintained in organ cultures of rabbit mammary gland and this maintenance was related to an active synthesis since the addition of cycloheximide lead to a rapid decline in receptor levels. Inclusion of prolactin (5 μg/ml) in the culture medium resulted in an occupation of binding sites, followed by a reduction in the total receptor levels measured following dissociation of the bound prolactin from its receptor in crude membrane preparations. This apparent down-regulation of prolactin receptors may result from an endocytosis or compartmentalization of the hormone–receptor complex and may be involved in the mechanism of action of prolactin.

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