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pH DEPENDENCE OF DISSOCIATION OF THE OVINE PROLACTIN RABBIT MAMMARY RECEPTOR COMPLEX

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Ovine prolactin specifically bound to rabbit mammary membrane prolactin receptor was rapidly dissociated in a pH dependent manner with 0.1M ammonium acetate. Up to 75% of the bound hormone was dissociated at pH 4.0 or lower in less than 5 minutes. The pK of the dissociation was 4.7, implicating one or more critical carboxyl groups. Exposure of the membrane bound receptor to the dissociating buffer for up to one hour did not reduce its ability to bind hormone. The dissociated hormone was characterized as intact ovine prolactin by Bio-Gel P-150 gel chromatography and by its ability to bind to fresh rabbit prolactin receptor with the same binding affinity as native hormone.

Ovine prolactin binds to a membrane bound prolactin receptor in the rabbit mammary gland in a specific manner with a dissociation constant on the order of 10^{-10}M (1). This tight binding is essentially irreversible. Incubation at room temperature for 45 to 48 hours resulted in only 15 to 50% dissociation (1-3). Numerous attempts have been made to dissociate prolactin from its receptor, and 5M $MgCl_2$, though poor, has been the most successful dissociating agent (4-6).

Recently, Pastan (7, 8), in describing a "receptosome model" for hormone-receptor internalization, has suggested that lowered pH might serve to dissociate hormones from their receptors within the cell. Indeed, a lowered pH has been effective in dissociating human chorionic gonadotropin from Leydig tumor cells (9) and epidermal growth factor from 3T3 cells (8). Although the effect of pH on the binding of prolactin to its receptor had been examined (1), the effect of pH on dissociation had not been thoroughly studied.

The pH dependence studies we report indicate that dissociation is pH dependent and that lowered pH can rapidly and effectively dissociate prolactin from the receptor without inactivating the receptor.

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MATERIALS AND METHODS

Materials - Purified ovine prolactin (NIAMDD-oPrl-14) was provided by the National Institute of Arthritis, Metabolism, and Digestive Diseases through Dr. A. F. Parlow of the Pituitary Hormones and Antisera Center, Torrance, California. Bromocriptine (CB-154) was a gift of Sandoz Pharmaceuticals, E. Hanover, N.J. New Zealand white rabbits were obtained from Small Stock, Inc., Pea Ridge, AR, during their first pregnancy. Na (^{125}I) was from Amersham, Arlington Heights, IL. Lactoperoxidase was obtained from Boehringer Mannheim, West Germany. A 30% solution of H_2O_2 was obtained from Mallinckrodt, Paris, Kentucky. Bovine serum albumin was from Sigma, St. Louis, MO. Bio-Rad, Richmond, CA, was the source of Bio-Gel P-6, Bio-Gel P-150 and the Bio-Rad protein assay reagent. All other chemicals were of reagent grade.

Membrane preparation - Rabbit mammary gland plasma membranes were prepared from 6- to 7-day postpartum rabbits by a modification of the method of Shiu et al. (10, 11). The rabbits were injected 36, 24 and 12 hours prior to sacrifice with 1 ml of a 2 mg/ml solution of bromocriptine (CB-154) in order to suppress prolactin secretion (5). The mammary glands, weighing approximately 100 grams, were removed, cut into 1 cm^3 pieces, washed by decantation three times with 200 ml of cold 0.3 M sucrose, and homogenized in 3 volumes of 0.3 M sucrose in a Sorvall Omnimixer at a setting of 6 for a total of 5 minutes. The tissue was then filtered through 4 layers of cheese cloth and centrifuged at 15,000 x g for 20 minutes. The supernatant was then centrifuged at 100,000 x g for 90 minutes in a Beckman L5-65 centrifuge using a 42.1 rotor at 4°C. The resulting pellet was then resuspended in 50 mM tris, 10 mM $MgCl_2$, pH 7.5 at a protein concentration of 4-6 mg/ml as determined by the Bradford method (12) using Bio-Rad protein assay reagent and ovine prolactin for a standard. The resuspended membranes were stored at -20°C.

Iodination - (^{125}I) ovine prolactin was prepared by the method of Walker (13), which is a modification of previous procedures (14, 15). The specific activity of the labeled hormone was from 30-80 μCi/μg.

Radioreceptor assay - The radioreceptor assays were performed by a modification of the method of Shiu et al. (10). 40 μl of thawed and homogenized receptor preparation were incubated with 10 μl of (^{125}I) ovine prolactin equivalent to approximately 100,000 counts (unless otherwise indicated) per minute in a total volume of 500 μl with binding buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 0.1% BSA, pH 7.5) at room temperature for 18-20 hours. At the end of the incubation free ovine prolactin was separated from the bound hormone by centrifugation in a Beckman Model TJ-6R centrifuge for 10 minutes at 1400 x g at 4°C, removing the supernatant, washing the pellet with 1 ml of the cold binding buffer, centrifuging for an additional 5 minutes and removing the wash buffer. The amount of bound ovine prolactin was determined by counting the pellet in a Beckman Biogamma counter. To determine nonspecific binding 50 μl of a 1 mg/ml solution of ovine prolactin in 20 mM sodium phosphate, pH 8.0 were added to parallel incubation samples. Under these conditions nonspecific binding represented 20% or less of the total binding. Specific binding was determined from the counts bound in the absence and presence of competing excess unlabeled ovine prolactin by the proportional method of Brooks et al. (16) using a computer program written for a TRS-80 Model II microcomputer by Bill Roberts of this laboratory. All assays were performed in duplicate.

Dissociation of bound ovine prolactin - Pellets containing bound hormone were resuspended, using a glass pipet, in one ml of either binding buffer or 0.1 M ammonium acetate at pH's ranging from 3 to 7 and for 5 to 60 minutes as indicated. Then the membranes were centrifuged as before and the supernatant was removed before counting the pellets in the Beckman Biogamma counter.

Rebinding of dissociated receptor with ovine prolactin - Pellets which contained receptor bound with ovine prolactin and then dissociated with the various buffers were washed twice with 1 ml aliquots of the binding buffer and then resuspended in a total assay volume of 500 μl with fresh (^{125}I) ovine prolactin in the same procedure used for the original binding. Other membrane aliquots which were pretreated for one hour with either binding buffer or the dissociation buffers were treated in the same manner in

order to detect inactivation of the receptor by the buffers. Alternatively, 40 µl of particulate membrane bound receptor was incubated with (^{125}I) ovine prolactin in greater than tenfold excess (2.5-3.0 x 10^6 counts per minute) of the amount of labeled hormone that could be specifically bound (1.1 to 1.7 x 10^6 counts per minute) for 24 to 48 hours, dissociated with either 1 ml of 0.1 M ammonium acetate, pH 4.0 or binding buffer, pH 7.5 (control) and then reincubated under conditions identical to the initial incubation.

Characterization of acid dissociated ovine prolactin - (^{125}I) ovine prolactin bound to the particulate rabbit mammary receptor as previously indicated was dissociated with 0.1 M ammonium acetate, pH 4.0 and the supernatant, containing the dissociated hormone, was pooled. Two ml of labeled dissociated hormone was then combined with 1.0 mg of unlabeled native ovine prolactin and applied to a Bio-Gel P-150 column and eluted with 20 mM sodium phosphate, 100 mM sodium chloride, .02% sodium azide, pH 8.0, in 1 ml fractions. The fractions were then counted in the Beckman Biogamma counter and their absorbance at 280 nm was measured. Twelve mg of bovine serum albumin was added to another 12 ml of the supernatant containing acid dissociated labeled hormone and the solution was dialyzed extensively with the binding buffer for the radioreceptor assay. 4 hundred µl aliquots of the dialyzed solution were combined with 40 µl of particulate rabbit mammary receptor preparation and varying concentrations of unlabeled ovine prolactin in a radioreceptor assay.

RESULTS

Incubation of (^{125}I) ovine prolactin equivalent to 158,000 counts per minute, with particulate, membrane-bound rabbit mammary receptor for 18-20 hr at room temperature, resulted in approximately 39,000 counts per minute of specific binding and less than 5,000 counts per minute of nonspecific binding. The bound hormone was rapidly dissociated from the rabbit mammary membrane receptor in a pH dependent manner using 0.1 M ammonium acetate adjusted to the desired pH with either ammonia or acetic acid (Figure 1). Over 75% of the specifically bound hormone was removed by exposure to pH 4.0 or less. Since the pK of the dissociation was 4.7, this implicates one or more critical carboxyl groups involved in binding. The time dependence of the dissociation at pH 4.0 (data not shown) resulted in no increase in the amount of prolactin dissociated after five minutes and up to one hour, indicating that the dissociation is very rapid.

In order to determine if treatment with acidic buffers decreased the ability of the membrane mammary receptor to bind hormone two types of experiments were performed. In the first experiment, membranes were pretreated by incubating for 30 minutes at room temperature with either binding buffer or 0.1 M ammonium acetate, pH 4.0. After collecting the membranes by centrifugation and thoroughly washing them with binding buffer to remove any traces of the pretreatment buffers, the membranes were bound with (^{125}I) ovine prolactin in the standard radioreceptor assay (Figure 2). The binding affinity of the membrane bound receptor was not affected by exposure to the pH 4.0 buffer as compared to the control exposed to binding buffer (25 mM Tris-HCl, 10 mM

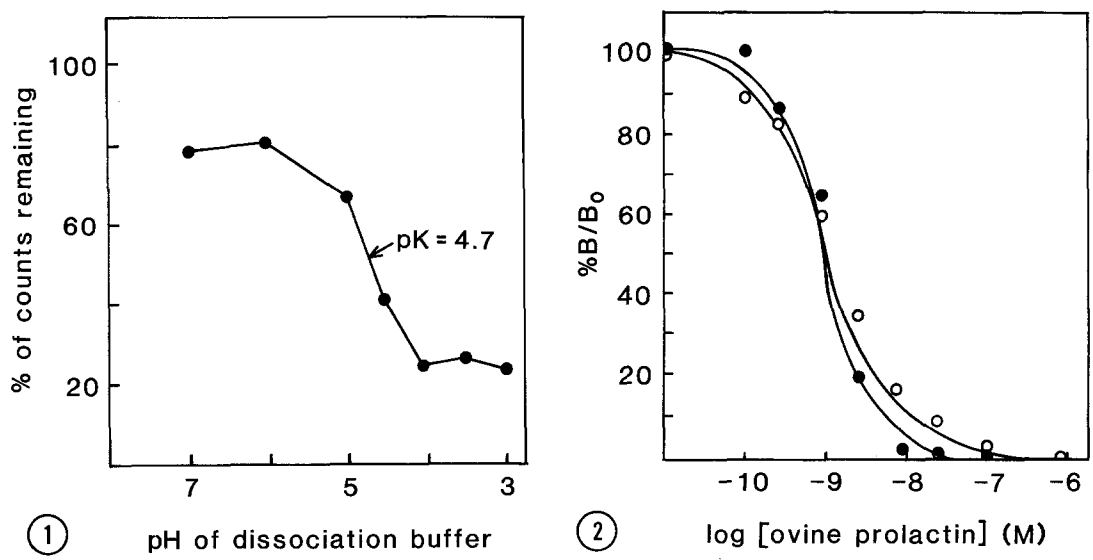

FIG. 1 pH dependence of dissociation of (^{125}I) ovine prolactin from the rabbit mammary prolactin receptor. 50 µl aliquots of particulate membrane receptor were bound with (^{125}I) oPrl for 20 hrs at 23°C in a radioreceptor assay. The bound hormone was then dissociated from the receptor by a 30 minute exposure to 0.1 M ammonium acetate at the pH indicated.

FIG. 2. Effect of exposure of the membrane bound receptor to pH 4.0 buffer on binding to ovine prolactin. 50 µl of particulate rabbit mammary membrane receptor was incubated for 30 minutes at room temperature with 0.1 M ammonium acetate, pH 4 (o o) or binding buffer (o o). The receptor was then washed twice with binding buffer and resuspended in a radioreceptor assay (Materials and Methods) for 20 hrs at room temperature with 113,900 counts per minute of (^{125}I) oPrl. The percentage ratio of the specific counts bound at various concentrations of unlabeled hormone (B) to the specific counts bound in the absence of competing unlabeled hormone (B_o) is plotted versus the log molar concentration of unlabeled hormone. The specific counts bound in the absence of unlabeled hormone (B_o) were 20735 and 21775 for the pH 4.0 and pH 7.5 pretreatments, respectively.

$MgCl_2$, 0.1% BSA, pH 7.5). The concentration of unlabeled hormone required to displace 50% of the labeled ovine prolactin, EC_{50}, was (1.24 ± .07) x 10^{-9} M and (1.27 ± .25) x 10^{-9} M for the receptor exposed to pH 4.0 buffer and the control, respectively. The total amount of specific binding in the absence of unlabeled hormone also remained unchanged although the pellet obtained from the pH 4.0 pretreatment was difficult to resuspend for the radioreceptor assay. In the second experiment, membranes were bound with ovine prolactin, dissociated with buffers of varying pH, ranging from 3 to 7.4, and then after washing with binding buffer and resuspending, rebound with fresh (^{125}I) ovine prolactin for 18-20 hours at room temperature (Table I). Rebinding with fresh hormone resulted in additional binding regardless of the amount of dissociation of the originally bound hormone from the receptor. The amount of fresh hormone newly bound was constant for all

TABLE I

The Effect of pH on Dissociation and Rebinding

pH of the Dissociation Buffer	Initial Binding	After Dissociation	Additional Upon Rebinding+
7.0	39,827	31.138	23,158
6.0	40,839	32,519	21,280
5.0	40,040	26,795	21,522
4.5	41,001	17,486	23,464
4.0	41,010	10,691	26,988
3.5	40,746	11,295	24,098
3.0	39,398	9,837	15,776

50 µl aliquots of rabbit mammary gland membranes were initially bound with (^{125}I) ovine prolactin (158,000 counts per minute) at room temperature for 20 hours in the standard radioreceptor assay. Dissociation by exposure to 1 ml of the various pH buffers for 30 minutes is described in Materials and Methods. The dissociated membranes were washed twice by resuspending in 1 ml of binding buffer (20 mM Tris-HCl, 10 mM $Mgcl_2$, 1.0% BSA, pH 7.4) before resuspending in a total of 500 µl in a second radioreceptor assay containing 158,000 counts per minute of fresh (^{125}I) ovine prolactin.

+Refers to new counts bound in addition to the counts remaining after dissociation.

the membrane receptors treated with buffers at pH 3.5 or greater. Treatment with pH 3.0 did result in a small decrease in the amount of new hormone bound. Since under these conditions the radioreceptor assay is an equilibrium assay with equilibrium reached at 23° in 5-20 hrs (1, 3, 17), pellets which contained receptor still bound to labeled prolactin showed a corresponding increase in the total amount of labeled hormone bound. Other control experiments ruled out the possibility that the labeled prolactin in the original radioreceptor assay was limited in its binding to its receptor because of aging, aggregation or degradation, as was also found by other investigators (3, 18). When the experiment was repeated under maximum binding conditions rather than equilibrium conditions (i.e., a greater than 10-fold excess of labeled ovine prolactin was used for both the initial binding and rebinding after dissociation) the results were somewhat different (Table 2) in that the total amount of hormone bound to the acid dissociated receptor upon rebinding was equal to the total amount of prolactin bound to the control, binding buffer treated receptor, confirming that the difference previously seen between the samples treated with acidic and neutral buffers was due to the equilibrium nature of the binding, coupled with an extremely slow dissociation rate (1, 3, 17). The pretreatment experiments described above, as well as experiments recently reported by Costlow and Hample

TABLE II

The Effect of pH 4.0 Dissociation on the Ability of Receptor to Rebind Hormone Under Maximum Binding Conditions

	Initial Binding	After Dissociation	Total After Rebinding
pH 4.0 treated	114,416	61,464	158,717
pH 7.5 control	114,844	114,518	142,957

40 µl aliquots of rabbit mammary gland membrane were initially bound with (^{125}I) ovine prolactin (2.6 x 10^6 counts per minute) at room temperature for 48 hours in the standard radioreceptor assay. The pellet was washed twice with 1 ml of cold binding buffer before counting each time. Dissociation was by exposure to 1 ml of either 0.1 M ammonium acetate pH 4.0 or binding buffer pH 7.5 (control). Rebinding was by resuspension of the membrane in a second radioreceptor assay also containing 2.6 x 10^6 counts per minute of fresh (^{125}I) ovine prolactin.

(19) in which rat mammary tumor cells were treated with a pH 3.0 buffer without damage to prolactin or the prolactin receptor, further confirm that the receptor is not adversely affected by these dissociation conditions.

The acid dissociated labeled hormone was characterized by Bio-Gel P-150 chromatography and in the radioreceptor assay (data not shown). The dissociated material coeluted with native ovine prolactin from the P-150 column and bound to the rabbit mammary membrane prolactin receptor with an EC_{50} of (9.06 ± .30) x 10^{-10} M, indicating that the dissociated (^{125}I) labeled material is intact ovine prolactin.

DISCUSSION

The rapid dissociation of the hormone-receptor complex at lowered pH provides some insight into the nature of the binding interaction. From the pK of the dissociation it appears that one or more carboxyl groups are involved either in the receptor and/or in prolactin. Chemical modification of the carboxyl groups of ovine prolactin has implicated their involvement in binding to receptor (20).

The dissociation of the prolactin-receptor complex at low pH also provides a possible mechanism for the dissociation of the complex within the cell which is required for models involving receptor recycling. The pH inside the lysozome (21) and the receptosome (7) is in the acidic range capable of dissociating ovine prolactin from its receptor. Low pH is effective in dissociating two other protein hormones, human chorionic gonadotropin (9) and epidermal growth factor (10), from their cellular receptors, indicating that low pH may be responsible for the dissociation of protein-hormone and other

protein receptor complexes. A low pH may also play an important role in the dissociation of ovine prolactin from its receptor by 5 M $MgCl_2$. Previous studies have utilized 5 M $MgCl_2$ dissolved in deionized, distilled water (5) or in 0.5% 3-{(3-cholamidopropyl)dimethylammonio -1-propane sulfonate (CHAPS) detergent (22). The apparent pH of a 5 M $MgCl_2$ solution prepared in distilled, deionized water is 3.9 ± 0.1 at 23°. When 5 M $MgCl_2$ was prepared by dissolving in 25 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.6 as described by Shiu and Friesen (4), the apparent pH was 5.0 ± 0.1. Under these conditions, it is quite probable that the low pH of 5 M $MgCl_2$ solutions contributes to the dissociation of the prolactin-receptor complex.

A significant difficulty in effectively using a prolactin affinity column for the purification of prolactin receptor has been the inability to readily dissociate the receptor from the column. The utilization of a low pH buffer has proven to be an effective method of eluting solubilized rabbit mammary receptor from an ovine prolactin-Sepharose affinity column (23).

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