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Water-Soluble Prolactin Receptors from Porcine Mammary Gland (42483)

PATRICIA BERTHON,*,[1] PAUL A. KELLY,† AND JEAN DJIANE*

**Laboratoire de Physiologie de la Lactation, Institut National de la Recherche Agronomique, 78350 Jouy-en-Josas, France, and †Laboratory of Molecular Endocrinology, Royal Victoria Hospital, McGill University, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1*

Abstract. Two types of prolactin receptors were identified in sow mammary gland. When light membranes were prepared on a discontinuous sucrose gradient (0.3 and 1.7 *M*) and then diluted and washed with 0.3 *M* sucrose solution, a large amount (about 50%) of receptors were released from membranes and appeared in the supernatant fraction. These two forms (hydrophobic and water-soluble) of receptors were characterized as having the same binding specificity for lactogenic hormones and a similar affinity constant for ovine prolactin ($K_a \sim 10\text{–}12 \times 10^9 M^{-1}$). Polyclonal antibodies and one monoclonal (mAb M110) antibody, obtained against partially purified prolactin receptors from rabbit mammary gland, cross-reacted effectively with sow mammary receptors. They completely inhibited the specific binding of [^{125}I]oPRL to membrane and water-soluble receptors. The present studies indicate that the two types of sow prolactin receptors could represent the same molecular entity and confirm that prolactin receptors from rabbit and sow mammary gland exhibit numerous antigenic similarities.

Receptors for prolactin have been characterized in many tissues and several species (1). As for all polypeptide hormones, these receptors are considered as hydrophobic glycoproteins located in membranes of target cells. Prolactin receptors are usually measured in the microsomal pellets sedimented at 100,000*g* after homogenization and low-speed centrifugation (10,000*g*) of tissue homogenates (2–4). Several groups (5–7) have purified prolactin receptors from rabbit mammary gland by affinity chromatography, after solubilization of membranes with detergents (Triton X-100 or CHAPS). We have recently characterized prolactin receptors from porcine mammary gland (8) and observed that an important part of the receptors could be released from membranes, when they were washed with an aqueous buffer. The same phenomenon has been reported for several peptide hormone receptors, such as FSH (9), hCG (10, 11), hGH (12, 13), and insulin (14) receptors. This water-soluble receptor could represent a distinct form of receptor which could have a specific role in the transduction of the prolactin signal inside the mammary cell or during the transport of the prolactin through the cell, from blood to milk. It is possible that this form of receptor could be more easily purified than the classical hydrophobic form, since solubilization with detergents can induce various technical problems.

The aim of the present study was to analyze this water-soluble form of the prolactin receptor, determine its specificity in binding of lactogenic hormones, and to compare, by using polyclonal and monoclonal antibodies, the immunological properties of these two forms (water-soluble and hydrophobic) of receptors.

Materials and Methods. *Animals.* Sows (Large White breed) were utilized at 15–21 days of their first lactation. They were treated with bromocriptine (CB 154, Sandoz; 10 mg/day, im) for 2 days before being slaughtered. Bromocriptine treatment greatly increases prolactin receptor level in the mammary gland, as it has been demonstrated in the rabbit (15). Mammary glands were removed and, after freezing for 2–4 hr at −80°C, were stored at −20°C until further use.

Membrane and water-soluble prolactin receptor preparations. After thawing, mammary glands were homogenized in 0.3 *M* sucrose (5 ml/g of tissue) using a Polytron homogenizer at medium speed for 2 min. The homogenate was twice filtered through four layers of cheesecloth and layered on a cushion of 1.7

[1] Present address: Laboratoire de Pathologie Porcine, Institut National de la Recherche Agronomique, 37380 Nouzilly, France.

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M sucrose solution (d = 1.24). After centrifugation at 100,000g for 18 hr at 4°C, membranes located at the interface (0.3/1.7 *M* sucrose) were delicately removed, diluted with 0.3 *M* sucrose, and centrifuged again at 100,000g for 1 hr. After this last centrifugation, membrane pellets, which have been demonstrated to contain mainly smooth membranes particularly rich in prolactin receptors (16), were resuspended in 25 m*M* Tris/HCl (pH 7.4)/1 m*M* phenylmethylsulfonyl fluoride (PMSF) buffer and stored at −20°C. The supernatant fraction of this centrifugation step represents the water-soluble prolactin receptor preparation. It also was stored at −20°C. In some experiments, this preparation was submitted to another centrifugation at 200,000g for 90 min, followed by a filtration through 0.22-μm Millipore filters (17). The concentrations of proteins were measured by the method of Lowry (18) with bovine serum albumin as standard.

Hormones and iodination. Ovine prolactin (oPRL; NIAMDD-oPRL-14; 31 IU/mg) was iodinated using [^{125}I]Na (CEA, France) and a low concentration of chloramine-T as described previously (19, 20). The following unlabeled hormones were utilized: human growth hormone (hGH; NIH-GH-MS 13940; 2.6 IU/mg), rabbit prolactin (rbPRL; AFP 1974C), porcine prolactin (pPRL; NIAMDD-pPRL-B1; AFP 5000; 111 IU/mg), bovine growth hormone (bGH; NIAMDD-bGH-B1; AFP 5200; 1.4 IU/mg), ovine follicle-stimulating hormone (oFSH; NIAMDD-oFSH-14; AFP 4493C; 9 IU/mg), human chorionic gonadotropin (hCG; NIH-batch CR121; 13.45 IU/mg), bovine insulin (INS, Sigma; 25.6 IU/mg), and ovine placental lactogen (oPL; a gift of Dr. J. Martal, INRA, Jouy-en-Josas, France).

Binding studies. Binding assays were carried out in 25 m*M* Tris/HCl (pH 7.4) buffer containing 10 m*M* $MgCl_2$ and 0.1% (w/v) bovine serum albumin. The incubation medium (final volume adjusted to 0.5 ml) contained a known amount of protein from membrane (200 μg) or water-soluble (500 μg) receptor preparations with ~10^5 cpm of iodinated oPRL in the presence or absence of various concentrations of unlabeled hormones. Incubation was performed at room temperature for 18 hr and, when membrane preparations were utilized, was stopped by the addition of 3 ml of cold buffer followed by centrifugation at 3000g, 4°C, for 15 min. For water-soluble preparations, "hormone-receptor" complexes were precipitated by adding 0.5 ml of cold 0.1% (w/v) bovine γ-globulin solution, followed by 1 ml of 25% (w/v) polyethylene glycol solution (PEG 6000; 12.5% PEG final concentration). Tubes were vortexed and centrifuged at 3000g, 4°C, for 25 min. Specific binding was calculated as the difference between the cpm bound in the absence and presence of an excess of unlabeled oPRL (1 μg/tube) and expressed as a percentage of the total counts added. In some experiments, the number of binding sites and the affinity constant were determined by Scatchard analysis (21).

Antibodies. Polyclonal antibodies obtained against partially purified preparation of rabbit mammary gland prolactin receptors were utilized. It has been shown that these antibodies are able to completely inhibit the binding of prolactin in several species including the pig (6, 8). We recently obtained monoclonal antibodies against rabbit prolactin receptor (22) and they were utilized in the present study to analyze in detail their cross-reactivity with porcine prolactin receptors.

Results. *Distribution of prolactin receptors.* Figure 1 shows the levels of prolactin receptors expressed as percentages of [^{125}I]oPRL specifically bound per 200 μg protein for the two types of receptor-containing preparations. The recovery of protein per gram of tissue is also indicated. The classical microsomal preparations (2–4) obtained after centrifugation at 100,000g resulted in an enrichment of prolactin receptors by approximately fourfold from the crude homogenate. The supernatant of these microsomal preparations contained very low levels of PEG precipitable prolactin receptors, which is in complete agreement with previous studies utilizing the same procedure for prolactin receptor preparations (2–7). However, when the mammary homogenate was layered on a 1.7 *M* sucrose cushion and a light membrane preparation was obtained as described under Materials and Methods, it can be seen that this membrane fraction became particularly rich in prolactin receptors (twofold enrichment compared to classical microsomes). The remarkable observation was that the supernatant of this centrifugation also

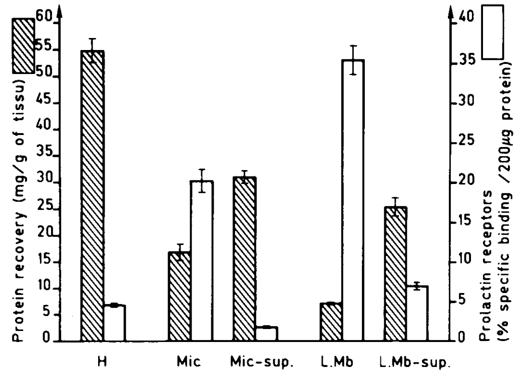

FIG. 1. Prolactin receptor levels and protein recovery in different types of membrane and soluble preparations. H, homogenate (n = 38); Mic, microsomes (prepared by centrifugation of the supernatant of a first centrifugation of the homogenate at 500g at 100,000g for 90 min; n = 13); Mic. Sup., supernatant of the microsomal preparation (n = 11); L. Mb., light membrane preparation (obtained as described under Materials and Methods) by ultracentrifugation of the homogenate on a 1.7 M sucrose cushion (n = 15); Sup. L. Mb., supernatant of light membrane preparation or water-soluble preparation (n = 12). Prolactin receptors were measured as described under Materials and Methods using 200 µg of protein for each assay. Specific binding was expressed as % of total cpm of [^{125}I]oPRL added.

contained appreciable amounts of PEG precipitable prolactin receptors. The concentration of receptors in this soluble fraction was one-third that of the microsomal preparation. These receptors appeared loosely bound to membranes since they were released after washing the light membranes with 0.3 M sucrose and appeared in the supernatant of the last centrifugation step. Many other proteins were also present in this supernatant, and if the relative concentration of prolactin receptors is only moderate, the total amount of receptors present in this fraction is relatively high. Table I illustrates this quantitative aspect. The number and affinity constant of prolactin receptors were determined in several light membrane or water-soluble preparations. It appeared that if the concentration of receptors were lower in water-soluble fractions than in light membrane preparations, the total number of binding sites per gram of fresh tissue was equivalent in the two types of preparations. Also interesting is the fact that the binding affinity was the same in water-soluble or membrane preparations, although it has been shown that detergent solubilization of prolactin receptors induced a dramatic fivefold increase in the apparent affinity for prolactin (5). In order to confirm the real soluble nature of these receptors, we submitted several water-soluble preparations to a subsequent centrifugation at 200,000g for 90 min, followed by an ultrafiltration through 0.22-µm Millipore filters. This treatment only slightly decreased the binding activity (71% of the binding sites still remained in a soluble form).

Hormonal specificity. The hormonal specificity of the binding of lactogenic hormones to the water-soluble fraction was analyzed and compared to membrane receptors. The results are shown in Figs. 2A and B. With the two types of receptors, the binding of iodinated ovine prolactin was inhibited by several lactogenic hormones (oPRL, hGH, and oPL). Half inhibition of binding was observed for prolactin concentrations between 2 and 4 ng/ml which are lower concentrations than those reported to be necessary to inhibit ovine prolactin binding to rabbit mammary gland receptors (3), suggesting a higher affinity of porcine receptor. This was confirmed by Scatchard analysis of several inhibition curves, which revealed an affinity constant (K_a) of 7.22 × 10^9 M^{-1} (n = 19), which corresponds to a value approximately threefold higher than that for rabbit receptor.

As expected, nonlactogenic hormones (bGH, INS, hCG, and FSH) had no effect on the binding of [^{125}I]oPRL. A most intriguing

TABLE I. PROLACTIN RECEPTOR LEVEL CONCENTRATIONS AND AFFINITY CONSTANTS IN THE LIGHT MEMBRANE AND WATER-SOLUBLE FRACTIONS

	Light membranes	Supernatants
PRL binding sites		
fmole/mg protein	203.5 ± 38	62.9 ± 13.4
fmole/g of tissue	1390 ± 250	1350 ± 400
Affinity constant ($\times 10^9$ M^{-1})	10.4 ± 3	12.7 ± 4.6

Note. Membrane (200 µg) or supernatant preparations (500 µg protein) were incubated with [^{125}I]oPRL (50,000 cpm) for 18 hr at 20°C in the presence of increasing concentrations of unlabeled prolactin. Numbers of binding sites and affinity constants were determined by Scatchard analysis (mean ± SEM, n = 3).

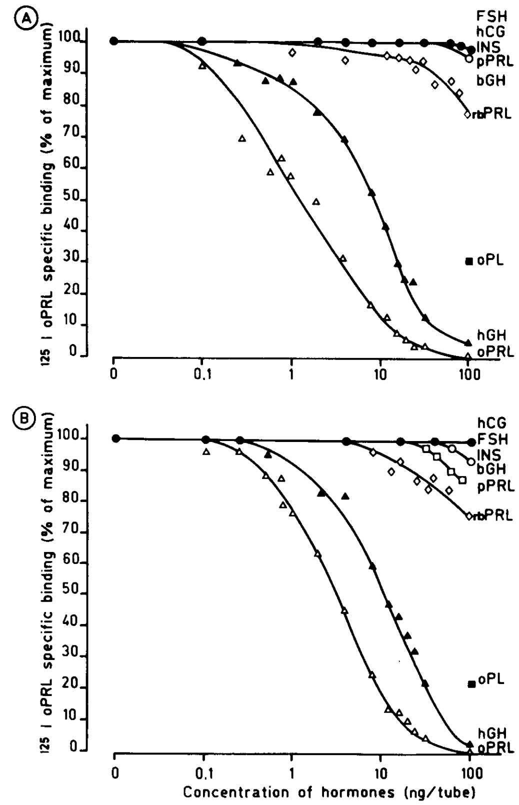

FIG. 2. Inhibition of specific binding of [^{125}I]oPRL by various polypeptide hormones. (A) Light membranes (200 μg protein); (B) water-soluble preparations (500 μg protein). Receptors were incubated with [^{125}I]oPRL (10^5 cpm) in the presence of various concentrations of hormones as described under Materials and Methods. Results were expressed as % of control specific binding.

result was obtained when rabbit prolactin (rbPRL) or porcine prolactin (pPRL) was utilized. These hormones, which are active in biological assays, competed only poorly for binding inhibition of [^{125}I]oPRL. This may suggest that the affinity of the receptor is much higher for heterologous hormones (ovine prolactin or human growth hormone) than for porcine or rabbit prolactin (see Discussion).

The specificity in the binding of lactogenic hormones of water-soluble prolactin receptor was identical to that of the particulate receptor.

Effects of antibodies against the receptor. We have previously shown that polyclonal antibodies, obtained against prolactin receptors from rabbit mammary gland, inhibited the binding of prolactin in several organs and species, including the pig (6, 8). In this study we have analyzed the effect of polyclonal antibodies on the binding of [^{125}I]oPRL to water-soluble preparations and hydrophobic receptors. As shown in Figs. 3A and B, these antireceptor sera competed effectively for [^{125}I]oPRL binding to the heterologous antigen. At dilutions of 1:1000 (10^{-3}) or less, they completely inhibited the specific binding of iodinated ovine prolactin to membrane and water-soluble receptors from sow mammary gland. Serum from nonimmunized animals slightly inhibited prolactin binding, only at concentrations higher than 1:100.

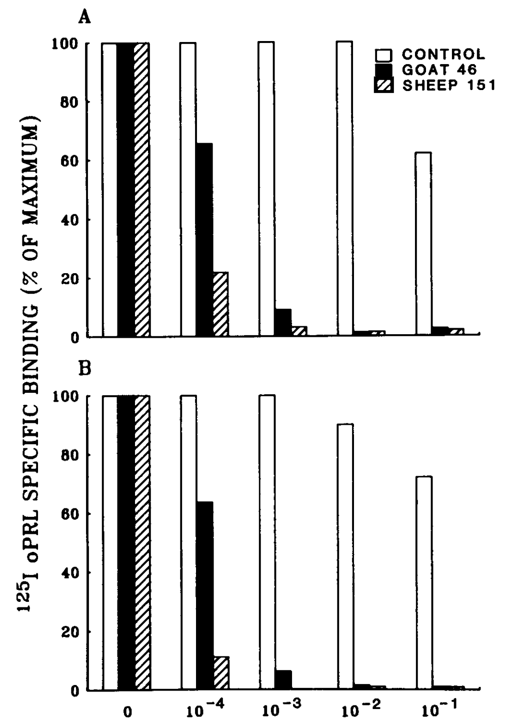

FIG. 3. Action of polyclonal antibodies to *rabbit* mammary prolactin receptor on the specific binding of iodinated prolactin to membrane and water-soluble fractions from *sow* mammary gland. (A) Light membranes (200 μg protein); (B) supernatant of light membranes (500 μg protein). Receptors were incubated with [^{125}I]oPRL (10^5 cpm) for 18 hr at 20°C in the absence or presence of various dilutions of control serum or anti-prolactin receptor serum from sheep 151 and goat 46. Results were expressed as % of the specific binding obtained when receptors were incubated with iodinated prolactin (10^5 cpm) in absence or in presence of an excess of unlabeled ovine prolactin (1 μg/tube).

Recently, monoclonal antibodies were obtained against prolactin receptors from rabbit mammary gland, namely M110 (IgG_1), A917 (IgG_2b), and A82 (IgG_1). These antibodies were characterized and the biological activity was evaluated (22, 23). Selected for their hormone-binding site specificity, these antibodies were able to completely inhibit the binding of ovine prolactin to rabbit mammary receptors. They also inhibited prolactin binding to receptor-containing preparations from several rabbit organs and from sow mammary gland (22). Therefore, the effect of these anti-rabbit receptor monoclonal antibodies on the binding of prolactin to the sow mammary hydrophobic and water-soluble receptors was investigated.

Figures 4A and B show that only the monoclonal antibody M110 was capable of completely inhibiting iodinated prolactin binding to both types of receptors. For the two other monoclonal antibodies, only partial inhibition was observed. With mAb 917, the maximal inhibition in the membrane and supernatant fractions was approximately 43%. Monoclonal antibody A82 only inhibited to a maximal value of 20%. Control γ-globulins did not affect the binding of hormone.

Discussion. The present data demonstrate the existence of water-soluble prolactin receptors from sow mammary gland, as has been reported for rat liver (24). These results confirm those obtained for other hormone receptors (9–14), generalizing the concept of water-soluble receptors for polypeptide hormones. These receptors, which do not seem to be proteolytic products generated during homogenization of the mammary tissue, appeared when light membranes were diluted and washed again with 0.3 *M* sucrose solution. Apparently spontaneously soluble, water-soluble prolactin receptors were not detected by precipitation with polyethylene–glycol in cytosol preparations from sow mammary gland using the technique described for the detection of cytosolic receptors for steroid hormones (25). This form of receptor rather seems to appear when mammary membranes come in contact with a solution with high osmotic pressure (such as 1.7 *M* sucrose) and are then diluted with isotonic buffer, therefore following osmotic shock of membrane vesicles.

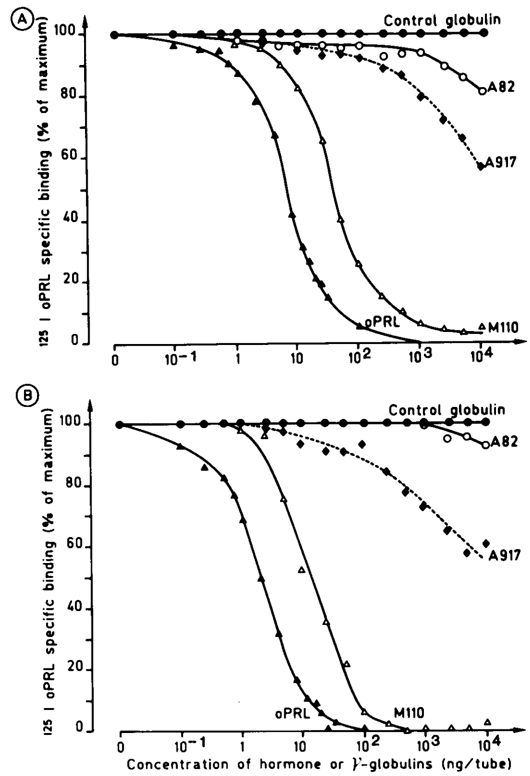

FIG. 4. Effect of anti-rabbit mammary receptor monoclonal antibodies on the specific binding of iodinated ovine prolactin to the membrane and water-soluble receptors from sow mammary gland. (A) Light membranes; (B) water-soluble receptor. Receptors were incubated with [^{125}I]oPRL (50,000 cpm) for 18 hr at 20°C in the absence or presence of various concentrations of hormone or monoclonal antibodies (γ-globulins). Results were expressed as % of the maximal specific binding obtained when receptors were incubated with iodinated prolactin (50,000 cpm) in the absence or presence of an excess of unlabeled hormone (1 μg/tube).

Although the exact nature of water-soluble receptors remains unknown, the two types of prolactin receptors from sow mammary gland have the same binding specificity and a similar or higher affinity constant for ovine prolactin ($K_a \sim 10^{10}\ M^{-1}$) when compared to that of rabbit mammary receptors (3).

During the specificity studies, we observed that, paradoxically, neither porcine prolactin nor rabbit prolactin inhibited [^{125}I]oPRL binding to either type of sow mammary receptor. This can be best explained by a difference of receptor affinity for the various prolactins tested. It can be suggested that the affinity of the receptor for porcine or rabbit prolactin is significantly lower than that observed for ovine prolactin. Thus, a complementary study revealed (data not shown) that the specific binding of iodinated porcine prolactin to receptors from homologous species was very low (<3%). A similar observation was made with rabbit prolactin binding to rabbit mammary receptors and with ovine prolactin on membrane preparations from ewe mammary gland. A heterologous system could offer the most sensitive system to measure receptor levels. However, recent studies by Haro and Talamantes (28) demonstrated that mouse prolactin receptors could be effectively measured using iodinated mouse prolactin.

Results observed with polyclonal and monoclonal antibodies to rabbit prolactin receptors suggest that prolactin receptors from rabbit and sow mammary glands have numerous antigenic similarities, although the species are relatively distant evolutionarily. Studies with monoclonal antibodies indicate that antigenic determinants shared by prolactin receptors from the two species seem to be located in the vicinity of the hormone-binding site. The use of such immunologic tools indicates that the two types of prolactin receptors from sow mammary gland (hydrophobic and water-soluble) appear to be identical. These results are in agreement with those reported by Amit *et al.* for rat liver prolactin receptors (24).

None of the results presented here exclude the possibility that the two forms of receptors correspond to the same molecular entity. The existence of two types of prolactin receptors, one firmly fixed in membranes, requiring detergent for solubilization, and the other more loosely bound, being solubilized during the production of a light membrane fraction involving an osmotic shock, could correspond to different receptor molecular states that may appear during biosynthesis or degradation. Prolactin receptors could be inserted to various degrees into membranes and thus become more soluble at certain stages of biosynthesis on the endoplasmic reticulum, during its transport to the plasma membrane before its insertion or after internalization of the hormone–receptor complex before degradation in lysosomes.

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