Characterization of prolactin receptors in pig mammary gland

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Prolactin receptors present in the particulate fraction of lactating pig mammary gland were solubilized by 7.5 mM-3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid (Chaps) and purified by affinity chromatography on prolactin coupled to Affi-Gel 10. Nearly 30% of the particulate receptors were solubilized by the detergent and over a 1000-fold purification from homogenates was achieved. A water-soluble fraction rich in receptors was observed during the preparation of membranes, although this fraction has not yet been purified. Prolactin binding to the receptors was a time-dependent, reversible and saturable reaction in particulate, Chaps-solubilized and purified receptors. In all forms, receptors showed the same specificity to peptide hormones. Prolactin and human growth hormone bound to the same receptors, whereas bovine growth hormone, follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone and insulin failed to bind. After solubilization, the dissociation constant (K_d) for prolactin was decreased 5-fold from 9.8×10^{-11} M in the particulate receptors to 1.8×10^{-11} M in solubilized and purified receptors, being due principally to an increase in the association rate constant from $1.0 \times 10^9 \text{ M}^{-1} \cdot \text{h}^{-1}$ to $(3.9\text{--}4.6) \times 10^9 \text{ M}^{-1} \cdot \text{h}^{-1}$, respectively, with the dissociation rate constant remaining unchanged at $(1.1\text{--}1.3) \times 10^{-2} \text{ h}^{-1}$. Isoelectric focusing of the prolactin-receptor complex revealed two peaks, one at a pI of 5.5–5.6 and the other at 5.2–5.3. Microsomal receptors were covalently cross-linked to ^{125}I -labelled ovine prolactin with ethylene glycol bis(succinimidyl succinate) and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Autoradiography of the gel revealed a major subunit of M_r 28 000–35 000 and a minor one of M_r 67 000–69 000. Anti-(prolactin receptor) antibodies raised against rabbit mammary gland prolactin receptors were equally effective in inhibiting prolactin binding to particulate, solubilized and affinity-purified receptors, suggesting that purified prolactin receptors have a structure indistinguishable immunologically from particulate receptors and rabbit mammary gland prolactin receptors. The present demonstration shows that particulate prolactin receptors from a domestic animal can be solubilized and purified without losing the original properties of high affinity and binding specificity for hormones.

The characteristics of mammary gland prolactin receptors, present in membranes, have been described in considerable detail in several labora-

tory animals (Frantz *et al.*, 1974; Shiu & Friesen, 1974a; Holcomb *et al.*, 1976). In order to understand further the molecular mechanisms of

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Abbreviations used: Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonic acid; PRL, pro-

lactin; GH, growth hormone (somatotropin); TSH, thyroid-stimulating hormone (thyrotropin); LH, luteinizing hormone (lutropin); FSH, follicle-stimulating hormone (follicitropin); the prefixes o, h and b refer to the ovine, human and bovine hormones respectively; SDS, sodium dodecyl sulphate; PMSF, phenylmethane sulphonyl fluoride.

prolactin action and the interaction of prolactin with its receptors, as well as the regulation of prolactin receptors (Djiane & Durand, 1977; Djiane *et al.*, 1979; Harigaya *et al.*, 1982), the isolation and purification of prolactin receptors is important.

The solubilization of particulate prolactin receptors in mammary gland is an initial step for subsequent purification of prolactin receptors. The first attempt to solubilize and purify mammary gland prolactin receptors was carried out a decade ago (Shiu & Friesen, 1974a), using the combination of a non-ionic detergent, Triton X-100, for solubilization and affinity chromatography with hGH for purification (Shiu & Friesen, 1974b). Using similar procedures, mammary prolactin receptors from several species have already been solubilized and characterized (Waters & Friesen, 1979; Koppelman & Dufau, 1982; Haeuptle *et al.*, 1983; Katoh *et al.*, 1984).

These procedures, however, have some limitations for general applications. Even after solubilization, the addition of Triton X-100 is essential to maintain receptors in a soluble form which may be important for the functional integrity of the receptors during subsequent steps of purification and binding to lactogenic hormones. Triton X-100 at working concentrations affects the physical properties of labelled prolactin. Therefore, prolactin was not utilized as a binding ligand (Shiu & Friesen, 1974b). hGH, however, which is unaffected by Triton X-100, has both somatogenic and lactogenic properties (Cowie & Tindall, 1971; Kleinberg & Frantz, 1971), and hence binds to both non-primate GH receptors and prolactin receptors (Kelly *et al.*, 1974; Parke & Forsyth, 1975; Herington & Veith, 1977). If primate or non-primate GH receptors are present in the tissue, GH receptors may contaminate to some degree the preparation of prolactin receptors.

More recently, the zwitterionic detergent Chaps was introduced (Hjelmeland, 1980) and has been used with success for solubilization of prolactin receptors from mouse liver (Liscia *et al.*, 1982). The authors showed that Chaps did not affect the molecular characteristics of labelled prolactin and, in the presence of Chaps, prolactin binding to the soluble receptors was similar to that with particulate receptors, suggesting that prolactin could be used as a binding ligand in the presence of Chaps.

The present study on the solubilization and purification of the prolactin receptors in the lactating pig mammary gland was undertaken as an initial step toward future studies of prolactin receptor structure and of the precise mechanism of prolactin-receptor interactions. The present data demonstrate that prolactin receptors can be readily purified from a domestic animal. We also exam-

ined kinetics of prolactin binding and hormonal specificity by particulate, Chaps-solubilized and purified prolactin receptors.

Materials and methods

Hormones

The hormones used (ovine prolactin, oPRL, NIH-P-S15, 30.5 units/mg; human growth hormone, hGH, NIH-GH-AFP-2243E, 2.2 i.u./mg; ovine follicle-stimulating hormone, oFSH, NIH-FSH-S14; ovine luteinizing hormone, oLH, NIH-LH-S23; ovine thyroid-stimulating hormone, oTSH, NIH-TSH-S8; ovine growth hormone, oGH, NIH-oGH-12; human prolactin, hPRL, NIH-I-6) were gifts from the National Institute of Health, Bethesda, MD, U.S.A. Bovine insulin (25 units/mg) was purchased from Schwartz/Mann, Spring Valley, NY, U.S.A. Bovine growth hormone (bGH, bGH-I-1) was a gift from the United States Department of Agriculture.

Crude mammary microsomal preparations

Mammary glands were obtained from lactating pigs (Yorkshire) from a commercial slaughterhouse. The mammary glands were removed and stored at -20°C until use. The glands were homogenized with 4 vol. (v/w) of 0.25 M-sucrose for 30–45 s in a Polytron homogenizer fitted with a PT10 head at a speed setting of 7. The homogenate was filtered through three layers of cheesecloth and centrifuged at 8000 g for 15 min at 4°C . The supernatant was centrifuged at 100000 g for 75 min at 4°C . The pellet was suspended in 25 mM-Tris/HCl (pH 7.4)/10 mM-MgCl₂/1 mM-PMSF (Tris/Mg/PMSF). The crude microsome suspension was kept frozen at -20°C until use.

Water-soluble prolactin receptors

In a small series of studies, an enriched fraction of lighter density membranes and water-soluble prolactin receptors was prepared. The homogenate (15 ml) was layered on 8 ml of 1.7 M-sucrose and the tubes were centrifuged at 100000 g for 16 h at 4°C . The interface of the gradient was removed, diluted with an equal volume of 0.25 M-sucrose and centrifuged at 100000 g for 1 h at 4°C . The supernatant was taken as 'water-soluble' fraction and the pellet, consisting of lighter density membranes, was resuspended as described previously.

Solubilization of the crude microsomes by Chaps

The crude microsomal suspension was diluted to a final protein concentration of 10 mg/ml with Tris/Mg/PMSF buffer and adjusted to 1 mM-Chaps (Pierce Chemical Co., Rockford, IL, U.S.A.) by adding powder directly. The suspension was stirred for 30 min at room temperature and

centrifuged at 100000g for 60 min at 4°C, and the supernatant was discarded. The pellet was resuspended in Tris/Mg/PMSF buffer and treated again with Chaps at a final concentration of 7.5 mM for 30 min at room temperature. After centrifugation a turbid layer present on the top of the supernatant was carefully removed and the remaining supernatant was collected and stored at -20°C. This fraction was referred to as the 'Chaps extract'.

Preparation of affinity adsorbent

Prolactin (20 mg dissolved in 10 ml of 0.1 M-NaHCO₃, pH 8.3) was added to 90 ml of 0.1 M-NaHCO₃ containing 2 g of the *N*-hydroxy-succinimide ester of 3,3'-diaminodipropylamino-succinyl-agarose (Affi-Gel 10; Bio-Rad Laboratories, Richmond, CA, U.S.A.) and incubated at 4°C overnight under constant shaking. The coupling reaction was terminated by the addition of 6 ml of 1 M-glycine dissolved in 0.1 M-NaHCO₃. Unbound prolactin was removed by repetitive washing in 8 M-urea and 4 M-guanidine hydrochloride. Coupling efficiency, which was monitored by ¹²⁵I-prolactin included in the reaction mixture, was about 85%.

Affinity purification of prolactin receptors

Affinity purification of prolactin receptors was performed according to the method described by Shiu & Friesen (1974b). The Chaps extract was diluted with Tris/Mg/PMSF buffer to reduce the Chaps concentration to 5 mM from 7.5 mM. The extract was allowed to run through 40 ml of the Affi-Gel 10 coupled to prolactin (column size 3 cm × 30 cm) at room temperature with a flow rate of 35–40 ml/h. The Affi-Gel 10 column was washed with the following solutions at maximum flow rate: 10 bed vol. of 0.1 M-sodium borate buffer (pH 7.4) containing 1 mM-Chaps (borate/Chaps), 1 bed vol. of 4 M-urea in borate/Chaps and 4 bed vol. of borate/Chaps. Elution was achieved with 1 bed vol. of 5 M-MgCl₂ in borate/Chaps, followed by several bed vol. of borate/Chaps. The pooled MgCl₂ eluate was passed through a Sephadex G-100 column (3 cm × 85 cm) equilibrated previously with borate/Chaps. Active fractions were dialysed at 4°C against 50 vol. of 10 mM-borate (pH 7.4)/0.1 mM-Chaps buffer with a total of three changes of buffer every 12 h, and were then concentrated by lyophilization.

Iodination of hormones by chloramine-T

Prolactin or hGH (5 µg) were iodinated in the presence of 500 ng of chloramine-T and 0.75 mCi of Na¹²⁵I in a 55 µl reaction mixture for 4 min (Kelly *et al.*, 1979). The specific radioactivity of both labelled hormones was 80–100 µCi/µg. In the

presence of excess receptor, greater than 85% of the labelled hormones could be specifically bound.

Prolactin binding to receptors

For the binding assay of particulate receptors, 10⁴–10⁵ c.p.m. of ¹²⁵I-labelled hormone was added to each tube. Binding was performed in the presence or absence of excess (1 µg) unlabelled oPRL. The total volume was adjusted to 0.5 ml with 25 mM-Tris/HCl (pH 7.4)/10 mM-MgCl₂/0.1% bovine serum albumin (assay buffer). Tubes were incubated for 15–17 h at room temperature and incubation was terminated by adding 6 vol. of cold assay buffer followed by centrifugation at 2300g for 15 min. Tubes were counted in a LKB gamma spectrometer with a counting efficiency of 63%. For the soluble receptors the tubes were incubated in the same manner as for the particulate receptors except that the final Chaps concentration was maintained between 4.5 and 6 mM. Hormone-receptor complexes were precipitated by adding 0.5 ml of cold 0.1% bovine γ-globulin in 0.1 M-phosphate buffer, pH 7.4, and 1 ml of a 32% (w/v) solution of poly(ethylene glycol) (PEG-8000). Tubes were vortex-mixed for 10 s and centrifuged at 2300g for 15 min. Specific binding was calculated as the difference between the radioactivity bound in the absence and presence of excess unlabelled oPRL. Non-specific binding of ¹²⁵I-oPRL was 2.9%, 1.9–3.5%, 5.2–7.3% and 2.9–5.2% of total radioactivity added for homogenate, microsomal, solubilized and purified receptors, respectively, and that for ¹²⁵I-hGH was 2.7%, 4.8% and 4.5% of total radioactivity added for microsomal, solubilized and purified receptors, respectively. All determinations were carried out in triplicate and repeated twice. The results are all expressed as mean values. The S.D. was always less than 3%.

Treatment of the receptors with anti-(prolactin receptor) serum

Goat anti-(prolactin receptor) serum raised against rabbit mammary gland prolactin receptors was used. This antiserum recognizes the rabbit and rat prolactin receptors in the particulate and soluble fraction, as judged by inhibition of the binding of labelled prolactin to the prolactin receptors. However, it does not bind prolactin in the reaction mixture (Djiane *et al.*, 1981; Katoh *et al.*, 1984). Various concentrations of antiserum or control serum (100 µl) were included in the reaction mixture described above and prolactin receptor activity was measured in the same manner.

Isoelectric focusing

The samples were focused in a tube gel (0.6 cm × 9 cm) which consisted of 4.85% (w/v)

acrylamide, 0.15% (w/v) *NN'*-methylenebisacrylamide, 2.5% (v/v) Pharmalyte (40% solution, pH 4.0–6.5; Pharmacia, Uppsala, Sweden), 10% (v/v) glycerol and 1% (v/v) Triton X-100. Purified prolactin receptor protein (2 μ g) was incubated overnight at room temperature with 125 I-oPRL in the presence or absence of 1 μ g of unlabelled prolactin, and then was precipitated with poly(ethylene glycol). The precipitated prolactin–receptor complex was dissolved in 100 μ l of 2.5% Pharmalyte/15% (w/v) sucrose/1% (v/v) Triton X-100 and layered onto the surface of the gel, and electrofocusing was continued at 200 V for 1 h, at 600 V for 8 h, at 900 V for the last 1 h (5900 V·h). After iso-electric focusing, the gels were cut into 4 mm slices and each gel was shaken with 1 ml of water for 1 h at room temperature to wash out the Pharmalyte from the gel. The pH in the solution was measured at room temperature with a pH meter. The washed gel slices were used for measurement of radioactivity.

Affinity labelling

Crude microsomes were incubated with 125 I-oPRL as mentioned under 'Prolactin binding to receptors'. Incubation was terminated by adding 3 ml of cold 0.01 M-sodium phosphate buffer, pH 7.4, and the mixture was centrifuged at 2300 g for 20 min. This washing step was repeated twice. The resulting pellets were suspended in 0.5 ml of the same buffer and 5 μ l of 25 mM-ethylene glycol bis(succinimidyl succinate) was added and incubated for 15 min on ice. The reaction was quenched with 3 ml of 25 mM-Tris/HCl buffer, pH 7.4, and the tubes were centrifuged. Pellets were solubilized by boiling with 2% SDS in 50 mM-Tris/HCl buffer, pH 6.8, and subjected to SDS/polyacrylamide-gel electrophoresis using 5–15% linear gradient gels and the Laemmli (1970) buffer system. Gels were dried and exposed to Kodak XAR-5 films for 24–48 h at -70°C . M_r determination was made by comparing the migration distance with those of the following standards: myosin, 200 000; β -galactosidase, 116 250; phosphorylase *b*, 92 500; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrase, 31 000; soya bean trypsin inhibitor, 21 500.

Protein determination

The concentration of insoluble and soluble proteins were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Solubilization of prolactin receptors and proteins

Crude microsomes were treated with the various concentrations of Chaps. Treatment with 1 mM or

2.5 mM-Chaps solubilized approx. 20% of the microsomal proteins, but most prolactin receptors were still insoluble. At concentrations of 5 mM or greater, Chaps was effective for the solubilization of the membrane prolactin receptors. For example, at 5 mM-Chaps, 23.5% of microsomal prolactin receptors, as determined by Scatchard analysis, and 29.5% of microsomal proteins were solubilized. As the Chaps concentration increased from 7.5 to 15 mM, the proportion of protein solubilized increased from 31.5 to 36%, whereas the amount of prolactin receptors solubilized remained unchanged at around 30% (results not shown). Routinely, crude microsomes were treated with 1 mM-Chaps to remove unwanted proteins and then the pellet was exposed to 7.5 mM-Chaps.

Water-soluble prolactin receptors

Hormone receptors are generally considered to be hydrophobic proteins localized within membrane structures. However, during the preparation of lighter density membranes, a large proportion of prolactin receptors appeared in the water-soluble fraction. When expressed per g of fresh tissue, approximately equal amounts of prolactin receptors were recovered in the membrane and supernatant fraction (Fig. 1). As might be expected, the water-soluble fraction contained much less activity when expressed per mg of protein. The dissocia-

Fig. 1. Distribution of prolactin binding in the membrane and water-soluble supernatant fraction

Membrane or supernatant fraction were incubated with labelled oPRL. Bound and free hormone were separated by simple centrifugation for membranes and by poly(ethylene glycol) precipitation for the supernatant fraction. Binding capacities were calculated by Scatchard plots (see Fig. 2).

tion constant (K_d) of the water-soluble fraction, which was calculated by Scatchard plot analysis, was comparable with that of the membrane fraction (both 0.1 nM). The lighter density membrane fraction resulting from this procedure was approx. 8-fold richer in prolactin binding than was the classical microsome fraction.

Affinity purification of prolactin receptors

Prolactin receptors in the Chaps extract were further purified by affinity chromatography using prolactin coupled to Affi-Gel 10; the purification (-fold) of the prolactin receptor is summarized in Table 1. More than 95% of receptor activity was adsorbed and nearly 100% of the protein was unadsorbed during the application of the solubilized receptor to the affinity column. Washing with borate/Chaps and urea in borate/Chaps resulted in a detectable loss of receptor activity (the exact amount was not calculable due to the interference of prolactin binding by urea). Prolactin receptors were effectively concentrated by the one-step purification by affinity chromatography from 37.8 to 6715 fmol/mg of protein with a receptor recovery of about 6%. Over a 1000-fold purification from homogenates was finally achieved.

Scatchard analysis of prolactin binding to receptors

Prolactin receptors in homogenate, crude microsomes, Chaps extract, and purified fractions were analysed by Scatchard analysis (Fig. 2). All data could be resolved by straight line plots, suggesting that each fraction has only a single class of binding site. The K_d for the homogenate, 9.8×10^{-11} M, was not significantly different from that of crude microsomal receptors (9.6×10^{-11} M). After solubilization with Chaps, the K_d for prolactin binding changed significantly to $(1.8-2.1) \times 10^{-11}$ M.

Association and dissociation of prolactin

The binding of 125 I-oPRL prolactin to the microsomal, Chaps-extracted or purified prolactin

receptors was a time-dependent and saturable reaction. As can be seen in Fig. 3(a), the rapid portion of the binding of prolactin occurred during the first 5 h of incubation, and near equilibrium binding was achieved at 16 h. Dissociation of prolactin from receptors was a time-dependent reaction. However, 80% still remained bound after

Fig. 2. Scatchard plots of binding of prolactin by homogenate, microsomes, Chaps-extracted and purified prolactin receptors

Particulate receptors were incubated with 40000 c.p.m. of 125 I-oPRL in the presence of 0, 0.1, 0.2, 0.4, 0.8, 1.5, 3, 6 ng, and 1 μ g of unlabelled prolactin. Soluble receptors were incubated with 10000 c.p.m. of 125 I-labelled oPRL in the presence of 0, 0.02, 0.04, 0.08, 0.15, 0.3, 0.6, 1.2 ng and 1 μ g of unlabelled prolactin. Bound and bound/free values were plotted according to Scatchard (1949). Reaction volume was 0.5 ml. Each tube contained the following amount of protein: 500 μ g of homogenate (■), 300 μ g of microsomes (○), 120 μ g of Chaps extract (●) or 1 μ g of purified receptors (□).

Table 1. Summary of binding capacity, purification and apparent dissociation constant of prolactin receptor preparations

Prolactin receptors were isolated, solubilized and partially purified from three batches (600-800 g wet wt. of tissue/batch) of pig mammary glands as described under 'Materials and methods'. For receptor preparations of each purification step, binding capacity and dissociation constant were determined using Scatchard (1949) analysis as described in Fig. 2, and protein concentrations were measured by the method of Lowry *et al.* (1951). Values in columns 2 and 6 are means $\pm$ S.D. ($n = 3$).

Step	Prolactin binding capacity (fmol/mg of protein)	Purification (-fold)	Total yield of protein (g)	Recovery of prolactin receptors (%)	$10^{11} \times K_d$ (M)
Homogenate	6.5 ± 1.2	1	157.0	100	9.8 ± 0.3
Microsome	29.3 ± 4.9	4.5	20.6	59.1	9.6 ± 0.7
Chaps extract	37.8 ± 3.6	5.8	5.3	19.6	2.1 ± 0.1
Partially purified	6715 ± 1536	1033	1.8×10^{-3}	1.2	1.8 ± 0.2

Fig. 3. Time-course of binding of ^{125}I -oPRL by microsomes, Chaps-extracted and purified receptors (a) and determination of rate constant for association (b)

(a) ○, Crude microsomes (120 μg of protein); ●, Chaps extract (110 μg of protein); □, purified receptor (0.6 μg of protein). The receptors were incubated with 10^5 c.p.m. of ^{125}I -oPRL. Specific binding of ^{125}I -oPRL was determined by the subtraction of non-specific binding of the parallel control incubated with 1 μg of unlabelled prolactin for each determination. Specific binding at 24 h was 7130, 17070 and 20110 c.p.m. for microsomal, Chaps extract and purified receptors, respectively. (b) Association rate constant k_{+1} is calculated using the following formula for the second-order reaction:

$$k_{+1} = 1/t(A-B) \times \ln[B(A-X)/A(B-X)]$$

where A is the concentration of labelled prolactin (38.4 fmol/tube). The term B in the equation is the concentration of prolactin receptors (4 fmol/tube). X is the concentration of the prolactin receptor complex at time t (h). Prolactin receptor concentration was obtained from the data of Scatchard analysis listed in Table 1.

24 h incubation, suggesting the irreversible nature of prolactin-receptor interaction. Binding kinetics were analysed according to the method described by Williams & Lefkowitz (1978). Since the reversibility of binding has not been established, caution should be used in evaluating these values. The association and dissociation properties are consistent with a simple bimolecular process (Figs. 3 and 4). The linear portion (up to 5 h of incubation) of the association rate curve in Fig. 3(a) was transformed according to the equation:

$$k_{+1} = 1/t(A-B) \times \ln[B(A-X)/A(B-X)]$$

for the second-order reaction; values of $\ln[B(A-X)/A(B-X)]$ were plotted against time of incubation (t), k_{+1} is the association rate constant, A is the concentration of ^{125}I -oPRL, B is the concentration of prolactin receptors, and X is the

concentration of the prolactin-receptor complex at time t . Straight lines were obtained, as shown in Fig. 3(b). The dissociation rate constant (k_{-1}) was obtained from the first-order rate equation:

$$k_{-1} = 1/t \times \ln(C/X)$$

where C and X are the concentrations of prolactin-receptor complex at time 0 hour and time t , respectively. The values for k_{+1} and k_{-1} are summarized in Table 2; the k_{-1} value did not change greatly, and the major difference is that the k_{+1} value of the Chaps-extracted and purified prolactin receptors were 3.9–4.6 times greater than that of the crude microsomal receptors, resulting in about 4.6-fold smaller K_d values for prolactin binding obtained by direct calculation ($K_d = k_{-1}/k_{+1}$). The K_d values estimated from the velocity kinetics were 6.6–7.4-fold less than those estimated from

Fig. 4. Dissociation of ^{125}I -oPRL from microsomes, Chaps-extracted and purified prolactin receptors

The receptors (microsomes, $\circ$; Chaps extract, $\bullet$; purified receptors, $\square$) were allowed to react with ^{125}I -oPRL (10^5 c.p.m./tube) for 16 h at room temperature. Prolactin binding was terminated by dilution with 0.5 ml of buffer solutions with the same constituents as the corresponding reaction mixtures, containing $2\text{ }\mu\text{g}$ of unlabelled prolactin plus $500\text{ }\mu\text{g}$ of γ -globulin. Incubation was continued for 6, 12 and 24 h. At the end of incubation, 1 ml of 32% poly(ethylene glycol) or 3 ml of Tris/Mg/albumin were added and prolactin binding was separated by centrifugation, as described under 'Materials and methods'.

the equilibrium kinetics. However, the pattern between the various K_d values was unchanged between these independent studies.

Hormonal specificity of prolactin receptors

The binding specificity of hormones to prolactin receptors in the crude microsomal and purified fraction was assessed by incubating ^{125}I -oPRL in the presence of an increasing amount of peptide hormones (Figs. 5a and 5b). The binding of ^{125}I -oPRL to the membrane and soluble receptors was clearly inhibited by the presence of unlabelled prolactin and hGH in a dose-related manner, with the curves for hGH shifted slightly to the right. Non-primate GH (bGH) did not compete with prolactin binding. All other non-lactogenic hormones tested also had no effect on the binding of ^{125}I -oPRL to the receptors. Data show that both

Fig. 5. Competitive displacements of specific binding of ^{125}I -oPRL by peptide hormones: (a) crude microsomes and (b) purified prolactin receptors

The amounts of competitor hormones were 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.5, 3, 6 and 12 ng/tube. The amounts of tracer was 49000 and 13000 c.p.m. for microsomal and purified receptors, respectively. Other conditions for incubation and determination of prolactin binding have been described under 'Materials and methods'. Data were expressed as percentage binding calculated from specific binding of ^{125}I -oPRL in the presence of competitor hormones divided by that in the absence of competitor hormone. Control specific binding was 5930 and 3980 c.p.m. for microsomal and purified receptors, respectively.

fractions contained identical prolactin binding components having the same specificity for hormone binding.

The hormonal specificity of the prolactin receptors in the crude microsomes, Chaps extract or purified fraction was further analysed by using the competitive inhibition of binding of ^{125}I -oPRL and hGH by unlabelled prolactin and hGH (Fig.

Table 2. Summary of apparent association–dissociation rate constants and dissociation constant of prolactin receptor preparations

With receptor preparations of each purification step, characterized in Table 1, association and dissociation rate constants were determined from the results of time-course experiments as indicated in Figs. 3 and 4. Dissociation constants were calculated from rate constants and from Scatchard analysis as shown in Fig. 2.

Step	Rate constant		10 ¹¹ × Dissociation constant (M)	
	10 ^{−9} × Association constant, <i>k</i> ₊₁ (M ^{−1} ·h ^{−1})	10 ² × Dissociation constant, <i>k</i> _{−1} (h ^{−1})	<i>k</i> _{−1} / <i>k</i> ₊₁	Equilibrium
Microsome	1.0 (0.8–1.2)	1.3 (1.2–1.4)	1.3	9.6
Chaps extract	3.9 (3.4–4.4)	1.1 (1.0–1.2)	0.28	2.1
Partially purified receptor	4.6 (4.5–4.7)	1.3 (1.1–1.5)	0.28	1.8

Fig. 6. Comparison of binding specificity of hormones by microsomes (○), Chaps extract (●) and purified prolactin receptors (□) using ¹²⁵I-oPRL and hGH

Conditions for incubation were described under ‘Materials and methods’ and in legend to Fig. 2. As tracer, 49000 c.p.m. of ¹²⁵I-labelled hormone was used for microsomes and 13000 c.p.m. was used for Chaps extract and purified receptors. Specific binding of ¹²⁵I-oPRL in the absence of competitor was 5930, 2070 and 3980 c.p.m. and those of ¹²⁵I-hGH were 8750, 3020 and 5750 c.p.m. for microsomal, Chaps extract and purified receptors, respectively.

6). The ID₅₀ of inhibition of prolactin binding to Chaps extract was shifted to the left, compared with that of either crude microsomes or purified receptor. Almost complete inhibition of binding was obtained at the higher concentrations of unlabelled hormones. The profiles of the dose-response curves shown in Fig. 6 did not change

greatly, whether labelled prolactin or hGH was utilized.

Effect of anti-(prolactin receptor) serum on binding of prolactin

Prolactin receptors in the crude microsomes, Chaps extract and purified fractions were incubated

Fig. 7. Effect of anti-(prolactin receptor) serum on ^{125}I -oPRL binding to prolactin receptors

Goat antiserum raised against partially purified rabbit mammary prolactin receptors or normal goat serum was mixed with microsomes (○), Chaps extract (●) or partially purified (□) prolactin receptors from the pig mammary gland and then incubated with 100 000 c.p.m. of ^{125}I -oPRL for 16 h at room temperature. Control bindings (100%, without serum) were 9220, 9730 and 21430 c.p.m. for microsome, Chaps extract and partially purified receptors, respectively.

in the presence of the various concentrations of anti-(prolactin receptor) serum. Fig. 7 shows that the binding of prolactin to the receptors was clearly diminished as the concentration of antiserum was increased. At the final dilution of 1:100, the binding of prolactin by the receptors was completely abolished. Serum from a non-immunized animal at 100-fold dilution showed only a slight interference of prolactin binding. The three dose-response curves appear parallel to each other.

Isoelectric focusing of the prolactin-receptor complex

Over 85% of radioactivity applied to the gels was recovered. The prolactin-receptor complex was separated into two distinct peaks with pI values 5.9–6.0 (peak I) and 5.5–5.6 (peak II). A small shoulder (pI 5.2–5.3) was detected in two separate runs (Fig. 8). Peak I coincided exactly with the peak of radioactivity observed for non-specific binding of prolactin or for ^{125}I -oPRL electro-focused alone. Therefore, peak II and the shoulder region (pI 5.2–5.3) represent the prolactin-receptor complex.

Affinity labelling of the prolactin-receptor complex

As shown in Fig. 9, cross-linked ^{125}I -oPRL binding subunit complexes were detected by SDS/polyacrylamide-gel electrophoresis as a broad

Fig. 8. Isoelectric focusing of prolactin-receptor complexes over a pH range of 4–6.5

One sample was obtained by incubation of purified receptors with ^{125}I -oPRL along (○) and the other, representing non-specific binding (●), was from an incubation in the presence of 1 μg of unlabelled prolactin.

M_r 55 000–62 000 major band and an M_r 94 000–96 000 faint band. These two bands disappeared when incubated in the presence of lactogenic hormones (oPRL, hGH, hPRL), whereas addition of oGH, oLH or oFSH had no effect. By reducing the concentration of the reductant, dithiothreitol, the major band migrated somewhat faster to M_r 52 000–58 000, although there was no significant loss of intensity of this band and other bands did not appear in the higher- M_r region in the absence of dithiothreitol. Since ^{125}I -oPRL also migrated faster at the decreased concentration of dithiothreitol, resulting in an apparent M_r change from 27 000 to 23 000, these slight changes in migration rate are considered to be due to the oPRL molecule which has three intramolecular disulphide linkages. Assuming a 1:1 binding ratio, the M_r values of the binding subunits can be calculated as 28 000–35 000 and 67 000–69 000 for major and minor bands, respectively.

Discussion

The present results show that the pig mammary gland contains a binding site specific for prolactin

Fig. 9. Autoradiogram showing prolactin-binding subunit of microsomal receptors

(a) Microsomes (800 µg of protein) were incubated with 8×10^5 c.p.m. of ^{125}I -oPRL in the absence or presence of hormones (8 µg) as indicated. Affinity labelling was carried out as described under 'Materials and methods'; hormone-receptor complexes were treated with 2% SDS and 20 mM-dithiothreitol followed by SDS/polyacrylamide-gel electrophoresis and autoradiography. (b) Cross-linked hormone-receptor complexes were solubilized by SDS in the presence of various concentrations of dithiothreitol. Values indicated are the M_r of standard proteins (on left) and the M_r of the middle of each band.

and hGH (prolactin receptor) with a high affinity for lactogenic hormone binding. The prolactin receptor is present in the membrane fraction. The interaction between prolactin and the microsomal prolactin receptors is a time-dependent and saturable process. The properties of the pig mammary gland prolactin receptor are almost identical with the well-characterized properties of mammary gland prolactin receptors obtained from several other species (Frantz *et al.*, 1974; Shiu & Friesen, 1974a; Holcomb *et al.*, 1976). Dissociation constants (K_d) of microsomal receptors were somewhat lower than that of pregnant or early lactating rabbit mammary gland (Djiane *et al.*, 1977), but were comparable with those of late lactating rabbit mammary gland (Perry & Jacobs, 1978). Furthermore, M_r values of binding subunits of microsomal prolactin receptors, demonstrated by using affinity labelling followed by SDS/polyacrylamide-gel electrophoresis and autoradiography, were 28000–35000 for the major, and 67000–69000 for the minor, subunits. Similar values for the major subunit have been observed in other species, e.g.

32000–40000 for rabbit mammary gland (Haeptle *et al.*, 1983; Hughes *et al.*, 1983; Katoh *et al.*, 1983) and 36000 for rat liver (Borst & Sayare, 1982).

We further attempted solubilization and purification of the membrane prolactin receptors by affinity chromatography and characterized these receptors at each step. The optimum concentration of Chaps for pig mammary gland prolactin receptors was 7.5 mM with nearly 30% of the receptors being solubilized. In comparison, 40–65% of the prolactin receptor from the mouse liver has been solubilized by Chaps at comparable concentrations, as shown by Liscia & Vonderhaar (1982). Using the procedure described in the present study, we consistently obtained solubilization efficiencies of 30–40% with Chaps for rabbit mammary gland prolactin receptors (M. Katoh, J. Djiane & P. A. Kelly, unpublished work). The solubilization efficiency for the opiate receptor by Chaps was 10–20% of the total receptor activity present in the membrane before solubilization (Simonds *et al.*, 1980).

A purification of prolactin receptors of over

1000-fold was achieved by affinity chromatography on prolactin-coupled agarose. The purification and the recovery obtained were comparable with results of others (Shiu & Friesen, 1974b; Liscia & Vonderhaar, 1982; Katoh *et al.*, 1984). However, it should be pointed out that the 'purified fraction' obtained by the present procedures is, in actuality, relatively impure, based on silver staining of polyacrylamide gels, as several bands were observed (S. Sakai, M. Katoh, P. Berthon & P. A. Kelly, unpublished work).

A slight but non-significant increase in the K_d was observed in mouse and rat liver after solubilization of the particulate prolactin receptors with Triton X-100 or Chaps (Bonifacino *et al.*, 1981; Carr & Jaffe, 1982; Liscia & Vonderhaar, 1982). We observed a decrease in the K_d (the increase in the affinity constant $K_a = 1/K_d$) for prolactin binding following solubilization of prolactin receptors, consistent with the findings of Shiu & Friesen (1974b) and Haeuptle *et al.* (1983). The latter group also found no alteration of the affinity constant for hGH and bGH binding by rabbit liver prolactin and GH receptors solubilized by Chaps or Triton X-100 in parallel experiments, suggesting that the affinity change for prolactin binding in the mammary gland prolactin receptor before and after solubilization does not simply imply an effect of Chaps or Triton X-100 on the rigidity of the receptor molecule itself.

It is also unlikely that prolactin receptors with low K_d values have been selectively solubilized by the treatment with Chaps or Triton X-100. Prolactin receptors with increased affinity were not seen in pig and rabbit mammary gland homogenates and microsomes, as revealed by Scatchard plot analysis in this and other experiments (Shiu & Friesen, 1974b). These results suggest some structural difference (i.e. molecular assembly, conformation, subunit, etc.) between prolactin receptors present in the mammary gland and the liver.

The fact that it is possible to localize a major portion of prolactin receptors in a water-soluble fraction may be important in the future, facilitating purification of receptor in the absence of any detergents, which in general make classical purification procedures less effective.

The inhibitory effect of anti-(prolactin receptor) serum on prolactin binding has been demonstrated (Shiu & Friesen, 1976; Djiane *et al.*, 1981; Katoh *et al.*, 1984). Important data are obtained from the experiment involving the inhibition of prolactin binding to the receptors by anti-(prolactin receptor) serum raised against rabbit mammary gland prolactin receptors. This antiserum equally inhibited prolactin binding to pig mammary gland prolactin receptors in a dose-related manner,

regardless of whether the receptors were membrane-bound or solubilized. We speculate that the Chaps-solubilized prolactin receptor must have a similar structure, being immunologically undistinguishable from the pig as well as the rabbit mammary gland particulate prolactin receptors.

Chaps-solubilized and purified prolactin receptors maintained the original properties of the particulate prolactin receptors with the same hormonal specificity and high affinity for binding of prolactin. Following solubilization of microsomal prolactin receptors, the association rate constant of prolactin binding increased 3.9–4.6-fold, as compared with that of the microsomal receptors, with a resulting decrease in K_d of 4.6-fold. This decrease is comparable with that obtained by the equilibrium kinetics. Initial physicochemical characterization of the receptor by isoelectric focusing revealed the major pI of 5.5–5.6.

The present data demonstrate that solubilization and partial purification of prolactin receptors from a domestic animal is feasible and that the purified receptors appear to have the same characteristics as those of prolactin receptors in classic target tissues of other species. Since the supply of tissue presents no problem, further purification to homogeneity by a combination of techniques is the next step.

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