Interaction of Cell-Membrane Prolactin Receptor with its Antibody

By ROBERT P. C. SHIU* and HENRY G. FRIESEN
*Department of Physiology, University of Manitoba, Faculty of Medicine,
Winnipeg, Manitoba, Canada R3E 0W3*

(Received 23 January 1976)

Antisera against a partially purified prolactin-receptor preparation derived from pregnant-rabbit mammary glands were generated in guinea pigs. On double immunodiffusion, each antiserum produced a single precipitin line with the prolactin receptors. The anti-receptor sera also specifically inhibited the binding of ^{125}I -labelled sheep prolactin to membrane particles as well as to highly purified prolactin receptors derived from the rabbit mammary glands. The same antisera, however, had no effect on the binding of ^{125}I -labelled insulin to the same membranes. These antisera did not bind or destroy prolactin. Moreover, the binding of ^{125}I -labelled prolactin to membrane particles derived from different tissues from a number of species was also inhibited by the antisera, thus suggesting that the immunological determinants of the prolactin receptors are similar in various tissues derived from different species. The factors in the antisera that were responsible for inhibiting the binding of ^{125}I -labelled prolactin to its receptors were found to be associated with the γ -globulin fraction. In addition, ^{131}I -labelled γ -globulins derived from one antiserum were shown to bind to membrane particles derived from mammary glands, and an increase in binding of γ -globulin was accompanied by a decrease in binding of prolactin. Kinetic analyses of inhibition of ^{125}I -labelled prolactin binding by antisera by using the methods of Lineweaver & Burk [*J. Am. Chem. Soc.* (1934) 56, 658–666] and Dixon [*Biochem. J.* (1953) 55, 170–171], revealed that the mechanism is a hyperbolic competitive inhibition. The demonstration of hormone-receptor-antibody complexes further favours this mechanism. The availability of anti-receptor sera should facilitate studies on the functional role as well as other biochemical, immunological and physiological properties of the prolactin receptors.

Research on peptide-hormone receptors has largely been devoted to identification and characterization of hormone-receptor interactions, measurement of hormone-binding activities in target tissues from animals under different physiological circumstances and correlation of hormone binding with adenylate cyclase activity and other cellular functions (Roth, 1973; Cuatrecasas, 1974, 1975; Birnbaumer *et al.*, 1974). It is now apparent that more subtle approaches are required to gain a greater insight into the structure and function of these receptors. Needless to say, the availability of purified receptors may facilitate such studies. The potential importance of this approach is borne out by the fact that a number of peptide-hormone receptors, among them the prolactin receptor, have been purified to varying degrees of purity (Cuatrecasas, 1972; Giorgio *et al.*, 1974; Shiu & Friesen, 1974a; Dufau *et al.*, 1975). By using a partially purified prolactin receptor from rabbit mammary glands, we have obtained antisera to the prolactin receptor. In the present paper, detailed studies on the interaction between a protein hormone,

its receptors in target-cell membranes and antibodies to the receptors are outlined. These findings also formed the basis for applying the anti-receptor sera to elucidate the functional role (Shiu & Friesen, 1976) as well as other biological properties of these membrane receptors for prolactin.

Materials and Methods

Immunization

A prolactin-receptor preparation with a hormone-binding capacity of 40 pmol/mg prepared from rabbit mammary glands by affinity chromatography (Shiu & Friesen, 1974a) was used for immunization. A solution of 100 μg of receptor protein in 0.5 ml of 0.1 M-Tris borate buffer, pH 7.6, containing about 0.1% (v/v) Triton X-100, was mixed with an equal volume of complete Freund's adjuvant. Six adult female guinea pigs (approx. 500 g) were each injected intradermally at 15–20 sites with 100 μg of immunogen, and 14 days later the animals were injected in a similar manner. One animal died before completion of immunization. A third injection containing 50 μg of immunogen in complete Freund's

* Present address: Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014, U.S.A.

adjuvant was given over five sites subcutaneously, 1 month after the initial injection. At 1 week after the last injection the guinea pigs were bled by cardiac puncture under ether anaesthesia. Thereafter the animals were bled at monthly intervals. At intervals of 2 months, 50 μ g of immunogen was administered subcutaneously. Two animals which served as controls were immunized in the same manner with vehicle which included complete Freund's adjuvant and borate buffer containing 1% (v/v) Triton X-100. Sera obtained from these two animals are referred to as 'control sera' in subsequent experiments. Then 12 other guinea pigs were immunized in the same manner with either detergent-solubilized or particulate crude membrane fractions (1 mg of protein) derived from pregnant-rabbit mammary glands and liver as described previously (Shiu & Friesen, 1974a,b). Thus a total of 19 animals were immunized, and antisera from them were studied.

Preparation of γ -globulin fractions

(NH₄)₂SO₄ dissolved in 0.05 M-sodium phosphate buffer, pH 7.4, was added to the serum to 33% saturation. Precipitates were obtained by centrifugation at 1500g at 4°C for 10 min, and were washed twice with 33%-satd. (NH₄)₂SO₄ and then re-dissolved in the phosphate buffer. The proteins were fractionated on a Sepharose 6B (Pharmacia, Uppsala, Sweden) column (1 cm $\times$ 70 cm), and the proteins that were eluted with phosphate buffer from the column with a K_{av} value equal to that of guinea-pig γ -globulin standard (Miles Laboratories, Kankakee, IL, U.S.A.) were collected. Generally only one major protein peak was observed. Therefore in subsequent experiments the gel-filtration step was omitted, and the γ -globulins were dialysed against phosphate buffer at 4°C.

Immunodiffusion

The double-immunodiffusion method of Ouchterlony, as detailed by Clausen (1969), was used. The receptor (2 μ g in 5 μ l) was added three times to the centre well; serum (5 μ l) was added twice to the peripheral wells. Diffusion in 1% (w/v) agar gel was carried out at 4°C for 3 days. The agar slide was washed, dried and stained with 0.5% (w/v) Buffalo Black (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Preparation of ¹²⁵I-labelled hormones and ¹³¹I-labelled γ -globulins

Sheep prolactin (25 units/mg) and bovine growth hormone (somatotropin, 2 units/mg) were obtained from the Endocrine Study Section, National Institute of Arthritis, Metabolism and Digestive Diseases,

National Institutes of Health, Bethesda, MD, U.S.A. These two hormones were iodinated with Na¹²⁵I (New England Nuclear Corp., Boston, MA, U.S.A., and Amersham/Searle Corp., Arlington Heights, IL, U.S.A.) by using lactoperoxidase as described previously (Shiu & Friesen, 1974b). The specific radioactivity of these two labelled hormones varied from 80 to 120 μ Ci/ μ g. Pig insulin (24 i.u./mg; Connaught Laboratories, Toronto, Ont., Canada) and γ -globulins derived from guinea-pig anti-receptor serum were iodinated with Na¹²⁵I and Na¹³¹I respectively by the method of Hunter & Greenwood (1962). The specific radioactivity of ¹²⁵I-labelled insulin was 80 μ Ci/ μ g and that of ¹³¹I-labelled γ -globulins was 165 μ Ci/ μ g.

Preparation of hormone receptors from tissues

Essentially two types of receptor preparations were used, namely crude membrane particles and highly purified soluble receptors. Crude membrane particles were isolated from various tissues of a number of species (see Table 1) by using procedures previously described (Shiu & Friesen, 1974b; Posner *et al.*, 1974). Partially purified soluble receptors for prolactin were obtained by using affinity chromatography (Shiu & Friesen, 1974a). Highly purified prolactin receptors were obtained by subjecting the partially pure material to polyacrylamide-disc-gel electrophoresis as detailed previously (Shiu & Friesen, 1974a). On 7.5% (w/v) polyacrylamide gel containing 0.2% (w/v) cross-linker (NN'-methylenebisacrylamide) at pH 8.9, the prolactin receptor has a relative mobility (R_F) of approx. 0.12 (Shiu & Friesen, 1974a). The prolactin receptors that were found in gel segments that correspond to this R_F were eluted with 0.025 M-Tris/HCl buffer, pH 7.6, containing 0.1% (w/v) bovine serum albumin.

Hormone-binding assays

Procedures for determining specific binding of ¹²⁵I-labelled prolactin, ¹²⁵I-labelled bovine growth hormone and ¹²⁵I-labelled insulin to particulate membrane receptors and to soluble receptors, and the characteristics of binding of the three ¹²⁵I-labelled hormones to tissues, have been detailed elsewhere (Shiu & Friesen, 1974a,b; Posner *et al.*, 1974). Specific binding refers to the amount of ¹²⁵I-labelled hormone bound to a receptor preparation in the absence of unlabelled hormone in the incubation medium minus that bound in the presence of at least 1000-fold excess of unlabelled hormone. Determinations were always carried out in duplicate.

In experiments where the effects of sera on hormone binding were tested, each serum was diluted with assay buffer (0.025 M-Tris/HCl, pH 7.6, containing 10 mM-MgCl₂ and 0.1% bovine serum albumin

EXPLANATION OF PLATE I

Ouchterlony immunodiffusion pattern

Procedures for this determination are described in the Materials and Methods section. Key: P, partially purified prolactin receptors obtained by affinity chromatography; 3-7, sera from guinea pigs immunized with 'P'; 1, serum from animal immunized with vehicle alone; 9, serum from animal immunized with Triton X-100-solubilized crude membranes derived from pregnant-rabbit mammary tissues; 14, serum from animal immunized with Triton X-100-solubilized crude membranes derived from liver of pregnant rabbits.

unless otherwise noted). Serum dilution factors were indicated in the Figures and 0.1 ml of these diluted solutions was added to each assay tube just before addition of ^{125}I -labelled hormone.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard.

Results

Immunodiffusion pattern

Plate 1 shows the results of immunodiffusion studies. When sera from five guinea pigs (nos. 3–7) which were immunized with prolactin receptors were made to react with a prolactin-receptor preparation, only three sera (nos. 3, 4 and 7) produced precipitin lines with the receptor (P) in the centre well. Sera from animals immunized with vehicle alone (no. 1), crude membranes derived from rabbit mammary tissue (no. 9) and crude membranes derived from rabbit liver (no. 14) did not produce any precipitin lines.

Effect of anti-receptor sera on binding of ^{125}I -labelled prolactin to receptors

Fig. 1 shows that when a total of 19 sera, obtained after bleeding the guinea pigs for the first time after

Fig. 1. Effect of sera from guinea pigs injected with prolactin receptors on binding of ^{125}I -labelled sheep prolactin to membrane receptors of rabbit mammary tissue

Crude membranes derived from pregnant-rabbit mammary tissue were used. Determination of specific binding of ^{125}I -labelled prolactin is described in the Materials and Methods section. Specific binding of ^{125}I -labelled prolactin to 250 μg of membrane proteins was 15% of the ^{125}I -labelled prolactin added, and this value is referred to as 100% on the ordinate. $\circ$, Means $\pm$ s.d. (vertical bars) for three anti-receptor sera (nos. 3, 4 and 7). Shaded area represents the binding of ^{125}I -labelled prolactin in the presence of sera obtained from the remaining 16 animals.

immunization, were tested for their effects on the binding of ^{125}I -labelled prolactin to crude membranes derived from rabbit mammary glands, only three anti-receptor sera inhibited the binding of ^{125}I -labelled prolactin to membranes. These three sera were the same ones that formed precipitin lines on immunodiffusion. Antisera raised against the crude rabbit mammary membranes were without effect. This experiment shows that anti-receptor sera diluted 1:100 caused a 50% inhibition of binding of ^{125}I -labelled prolactin. However, after subsequent bleedings, the titre of all three anti-receptor sera increased such that they produced a 50% inhibition of binding of ^{125}I -labelled prolactin at a dilution of 1:1000 and complete inhibition at a dilution of 1:100. This represents a tenfold higher potency of the antisera compared with the first bleeding.

To demonstrate further that inhibition of prolactin binding by antisera was due to an antibody effect, a γ -globulin fraction prepared from one of the antisera (no. 3) was tested in the same manner. Fig. 2 demonstrates that γ -globulins derived from anti-receptor serum ($\circ$) but not that derived from a control

Fig. 2. Effect of γ -globulins on binding of ^{125}I -labelled prolactin to mammary membranes and purified receptors

For the determination of binding of ^{125}I -labelled prolactin to crude membranes ($\circ$, $\bullet$) of mammary tissues, 250 μg of membrane proteins was used and the specific binding of ^{125}I -labelled prolactin in the absence of γ -globulins was 16% of the added ^{125}I -labelled prolactin, and this is referred to as 100% on the ordinate. For the determination of binding of ^{125}I -labelled prolactin to highly purified receptors (Δ , $\blacktriangle$), the latter was obtained by polyacrylamide-gel electrophoresis (see the Materials and Methods section). Too little receptor protein was thus obtained to be quantified accurately, and therefore samples of receptor solution that could bind 15% of the ^{125}I -labelled prolactin were used for incubation. This value is referred to as 100% on the ordinate. Procedures for the determination of binding of ^{125}I -labelled prolactin to particulate membranes as well as to soluble purified receptors have been described in the Materials and Methods section. $\blacktriangle$, $\bullet$, Control serum (no. 1); Δ , $\circ$, anti-receptor serum (no. 3).

serum (●), were effective in inhibiting the binding of ^{125}I -labelled prolactin to crude membranes.

It was possible that the antibodies were directed against some components other than the receptor molecules, and that the binding of antibodies to these components might secondarily interfere with the binding of prolactin by the receptor molecules. To examine this possibility, partially purified prolactin receptors were subjected to disc-gel electrophoresis (see the Materials and Methods section) and the prolactin receptors, which have an R_f value of 0.12 (Shiu & Friesen, 1974a), were eluted from the gel and tested. Fig. 2 (▲, △) illustrates that anti-receptor γ -globulins were able to inhibit binding of ^{125}I -labelled prolactin to this highly purified receptor preparation, demonstrating that the antibodies were directed against the receptor molecules and not against other contaminating proteins.

The γ -globulins prepared from the anti-receptor serum was labelled with Na^{131}I , and the ^{131}I -labelled γ -globulins obtained were able to bind to membranes derived from mammary tissue. About 1–2% of ^{131}I -labelled γ -globulins tracer was bound by 300 μg of membrane proteins. Approx. 90% of the ^{131}I -labelled γ -globulins bound to the membranes was displaced by 100 μg of unlabelled γ -globulins derived from anti-receptor serum, whereas the same amount of unlabelled γ -globulins derived from control serum caused only 5% displacement. When mammary membranes were incubated in the presence of both ^{131}I -labelled anti-receptor γ -globulins and ^{125}I -labelled prolactin in the presence of increasing quantities of unlabelled anti-receptor γ -globulins, there was a sharp increase in the binding of γ -globulins and a concomitant decrease in binding of prolactin to the same membranes (Fig. 3). These observations further suggest that the binding of antibodies prevented the binding of the hormone to the receptors.

Inhibition of prolactin binding by the anti-receptor serum would also occur if the antiserum bound the hormone, preventing the latter from binding to the receptor. Fig. 4 shows that none of the three anti-receptor sera bound ^{125}I -labelled prolactin (●), whereas a rabbit anti-(sheep prolactin) serum (○) at a dilution of 1:100 bound 90% of the ^{125}I -labelled prolactin added to the incubation mixture. In addition, when ^{125}I -labelled prolactin was incubated with γ -globulins derived from anti-receptor serum and the mixture subsequently fractionated on a column of Sephadex G-100, the ^{125}I -labelled prolactin was eluted at a position corresponding to that of untreated ^{125}I -labelled prolactin (as shown by the arrow in Fig. 5) or ^{125}I -labelled prolactin that had been incubated with the same amount of γ -globulins derived from control serum. This shows, again, that ^{125}I -labelled prolactin did not bind to anti-receptor antibodies, because antibody-bound ^{125}I -labelled

Fig. 3. Relationship between binding of anti-receptor antibodies and prolactin to membrane receptors

About 1.2×10^5 c.p.m. of ^{125}I -labelled prolactin and 6×10^5 c.p.m. of ^{131}I -labelled γ -globulins derived from anti-receptor serum (no. 3) were incubated together with 250 μg of crude membranes derived from pregnant-rabbit mammary tissues. Specific binding of ^{125}I -labelled prolactin and ^{131}I -labelled γ -globulins was determined after 6 h of incubation at 23°C in the presence of an increasing concentration of unlabelled γ -globulins. Dual-isotope counting was carried out in a LKB gamma counter. Specific binding of ^{125}I -labelled prolactin and ^{131}I -labelled γ -globulins in the absence of unlabelled γ -globulins were 15% (3.45 fmol) and 1.9% of the total tracer added respectively. The absolute amount of γ -globulins bound could not be computed because the proportion of specific anti-receptor antibodies in the γ -globulin fraction was not known. By knowing the change in specific radioactivity of ^{131}I -labelled γ -globulins, the total amount of γ -globulins bound at each concentration of unlabelled γ -globulins used can be calculated and the results were expressed as binding relative to that observed in the presence of only ^{131}I -labelled γ -globulins. Molecular weights of γ -globulin and sheep prolactin were taken to be 165 000 and 23 000 respectively. ●, Relative amount of γ -globulins bound; ○, absolute amount (fmol) of prolactin bound.

prolactin would be expected to be eluted in the void volume (fractions 16–18) of the column. Moreover, the ^{125}I -labelled prolactin thus recovered was almost all immunoprecipitated by rabbit anti-(sheep prolactin) serum. This shows that the immunological properties of the ^{125}I -labelled prolactin were not altered by exposure to anti-receptor antibodies.

Hormone-, tissue- and species-specificity of the anti-receptor serum

Membranes derived from rabbit mammary glands possess not only receptors for prolactin but also receptors for other hormones such as insulin (Posner

Fig. 4. Immunoprecipitation of ^{125}I -labelled prolactin by anti-prolactin and anti-receptor sera

About 10^5 c.p.m. of ^{125}I -labelled sheep prolactin was incubated with 0.1 ml of serially diluted guinea-pig anti-receptor sera (nos. 3, 4 and 7, ●) in the presence of 0.5 ml of 0.05M-sodium phosphate buffer, pH 7.4, containing 0.5% (w/v) bovine serum albumin. Normal guinea-pig serum (0.1 ml), diluted 1:10, was added to all tubes that contained guinea-pig anti-receptor serum used at dilutions greater than 1:10, so that all tubes contained the same amount of guinea-pig serum. Control tubes contained no anti-receptor serum. Determinations were carried out in duplicate. The tubes were incubated at 23°C for 3 h and then at 4°C overnight. Rabbit anti-(guinea-pig γ -globulin) serum (0.1 ml) was then added to each tube, and incubation at 4°C was allowed to continue for 24 h. Immunoprecipitates were centrifuged at 4°C for 15 min at 1500g and the radioactivity in the pellets was determined. For comparison, rabbit anti-(sheep prolactin) serum (○) was tested similarly and the second antiserum used in this situation was sheep anti-(rabbit γ -globulin) serum.

et al., 1974). However, our anti-receptor sera (in fact, sera obtained from all 19 animals) had no effect on the binding of ^{125}I -labelled insulin to membranes (results not shown), showing that the anti-receptor serum specifically inhibited the binding of prolactin. This point is further substantiated by the observation that the binding of ^{125}I -labelled prolactin to membranes derived from rabbit liver was inhibited by anti-receptor serum (Fig. 6), whereas the binding of ^{125}I -labelled bovine growth hormone to specific growth-hormone receptors in the same membranes was unaffected (results not shown). These observations illustrate that only the binding of prolactin is inhibited by the anti-receptor sera with membranes derived from different tissues and indicate that the antisera exhibit hormone- but not tissue-specificity. The immunological similarity between prolactin receptors of different tissues from a number of species is evident from the results summarized in Table 1. Thus the anti-receptor serum inhibits the binding of prolactin to a variety of tissues (both normal and pathological) derived from both sexes of a number of species.

Fig. 5. Comparison of elution pattern from a Sephadex G-100 column and immunoprecipitability of ^{125}I -labelled prolactin after exposure to γ -globulins derived from anti-receptor serum and control serum

Approx. 2×10^5 c.p.m. of ^{125}I -labelled prolactin was incubated at 23°C for 10 h with $84 \mu\text{g}$ of γ -globulins derived either from anti-receptor serum (no. 3) or control serum (no. 1) in a final volume of 0.5 ml of 0.025M-Tris/HCl, pH 7.6, containing 10mM-MgCl₂ and 0.1% (w/v) bovine serum albumin. The mixture was then fractionated on a column (1 cm $\times$ 70 cm) of Sephadex G-100 at 4°C at a flow rate of 6 ml/h. The above buffer was used for elution (1.1 ml/fraction) and the radioactivity in these fractions was determined by counting the entire fraction in a LKB gamma counter. To fractions that contained the ^{125}I -labelled prolactin which had been exposed to γ -globulins derived from anti-receptor serum were each added 0.1 ml of rabbit anti-(sheep prolactin) serum diluted 1:500, followed by 0.1 ml of normal rabbit serum diluted 1:50. After incubation at 4°C for 24 h, 0.1 ml of sheep anti-(rabbit γ -globulin) serum diluted 1:10 was added and incubation at 4°C was continued for another 24 h. Immunoprecipitates were centrifuged at 1500g for 15 min and the radioactivity in the pellets was determined. ●, Elution pattern of ^{125}I -labelled prolactin after exposure to anti-receptor γ -globulins; ○, elution pattern of ^{125}I -labelled prolactin after exposure to γ -globulins derived from control serum; △, immunoprecipitation of ^{125}I -labelled prolactin after exposure to anti-receptor antibodies. Arrow indicates the position where untreated ^{125}I -labelled prolactin appeared. The radioactive peak at fraction 50 represents ^{125}I .

Kinetics of inhibition of prolactin binding by anti-receptor serum

To gain some insight into the mechanism by which the antibodies to receptors inhibit the binding of prolactin, we examined our data by classical kinetic analyses used for studies on enzymes. By monitoring the extent of the binding of ^{125}I -labelled prolactin (v) at different concentrations of ^{125}I -labelled prolactin (S) used in the presence of various amounts of anti-receptor serum (I , inhibitor), a Lineweaver-Burk (Lineweaver & Burk, 1934) plot was obtained (Fig.

Fig. 6. Effect of guinea-pig sera on binding of ^{125}I -labelled prolactin to rabbit liver membranes

Crude membranes from pregnant-rabbit liver were prepared by using procedures outlined in the Materials and Methods section. Specific binding of ^{125}I -labelled prolactin to 200 μg of membrane proteins was 11%, and this value is referred to as 100% on the ordinate. $\circ$, Means $\pm$ s.d. (vertical bars) for three anti-receptor sera (nos. 3, 4 and 7). Shaded area represents specific binding of ^{125}I -labelled prolactin in the presence of sera from the remaining sixteen animals.

7a). All the lines intercept at the same point on the $1/v$ axis, illustrating a classical competitive-inhibition mode. However, analysis of the same data by the method of Dixon (1953), in which $1/v$ was plotted against inhibitor (antiserum) (I) concentration at various doses of prolactin (Fig. 7b) shows that the inhibition resembles the classical hyperbolic competitive inhibition. This model would suggest the existence of hormone-receptor-antibody complexes under appropriate circumstances (Dixon & Webb, 1964; Worcel *et al.*, 1965; Mahler & Cordes, 1971). Indeed, if the receptors that were rendered soluble by Triton X-100 were pre-labelled with ^{125}I -labelled prolactin and then incubated with anti-receptor serum, followed by precipitation of the complexes by a second antiserum, i.e. rabbit anti-(guinea pig γ -globulin) serum, the resulting precipitates contained 85% of the total ^{125}I -labelled prolactin-receptor complexes (Fig. 8, Δ). By omitting receptors ($\circ$) or substituting control guinea-pig serum ($\bullet$) for anti-receptor serum, no precipitation of ^{125}I -labelled prolactin occurred. These findings demonstrate the existence of hormone-receptor-antibody complexes. It should be emphasized that if anti-receptor serum was introduced into the incubation mixture at the same moment as ^{125}I -labelled prolactin, no ^{125}I -labelled prolactin-receptor complexes would form, because the anti-receptor antibodies would have bound the

Table 1. Immunological similarity of prolactin receptors in different tissues from various species

The inhibition of binding of ^{125}I -labelled sheep prolactin to different receptor preparations by anti-receptor sera was determined by using procedures described in the Materials and Methods section. Anti-receptor serum (0.1 ml) at a dilution of 1:50 was used to inhibit more than 95% of specific binding of ^{125}I -labelled sheep prolactin to all receptor preparations listed, except in mammary explants maintained in culture, in which 10% (v/v) of anti-receptor serum was used (Shiu & Friesen, 1976). Rabbit tissues were obtained from 31-day-pregnant animals. Rabbit mammary explants for culture purposes were obtained from 12-day-pseudo-pregnant rabbits (for details, see Shiu & Friesen, 1976). Normal rat mammary glands and liver were obtained from 20-day-pregnant animals. Prostate glands were obtained from 100-day-old Sprague-Dawley rats (Aragona & Friesen, 1975). Prolactin-dependent mammary tumours were induced in female rats by 7,12-dimethylbenzanthracene as described previously (Kelly *et al.*, 1974). Spontaneous mammary tumours were obtained from C3H mice. Human mammary adenocarcinomas were obtained as biopsy specimens and ^{125}I -labelled human prolactin was used for this determination (see Holdaway & Worsley, 1975).

Species	Tissue	Preparation
Rabbit	Mammary	Explants in culture, membranes, purified receptors
	Liver	Membranes
Rat	Mammary (normal)	Membranes
	Mammary tumours	Membranes
	Liver	Membranes
	Prostate	Membranes
Mouse	Mammary tumours	Membranes
Human	Mammary carcinomas	Membranes

receptors first and prevented the binding of the hormone.

Discussion

The potential usefulness of an antiserum generated against a neurotransmitter receptor for the elucidation of the structure and function of this entity has been demonstrated for the acetylcholine receptor by Patrick & Lindstrom (1973) and Patrick *et al.* (1973). As described in the present paper, the availability of a partially purified preparation of prolactin receptor has allowed us to generate antisera against this hormone receptor. Owing to the scarcity of hormone receptors in cells (of the order of 10^{-4} – $10^{-5}\%$ of total cellular proteins; see also Cuatrecasas, 1972), we have only been able to immunize guinea pigs with a partially purified preparation of prolactin receptors. It is possible that antibodies were also generated against a number of contaminants in the preparation. However, our findings suggest that antibodies to

Fig. 7. Kinetic study of inhibition of binding of ^{125}I -labelled prolactin to membrane receptors by anti-receptor serum

(a) Lineweaver-Burk plot. Approx. $350\mu\text{g}$ of membrane proteins derived from pregnant-rabbit mammary tissues was incubated for 6h at 23°C with increasing concentrations of ^{125}I -labelled prolactin in the presence of various amounts of anti-receptor serum (no. 3); 0.1ml of anti-serum diluted 1:50, Δ ; 1:100, $\bullet$; 1:500, $\circ$; 1:1000, $\square$. The specific binding of ^{125}I -labelled prolactin (v , in fmol) at each concentration of ^{125}I -labelled prolactin used ($[S]$, in nM) for a given amount of anti-receptor serum was computed. (b) Dixon plot. Data from (a) were used for this analysis. $1/v$ was plotted against $[I]$, the concentration of anti-receptor serum (inhibitor), expressed as a dilution. The concentration (nM) of ^{125}I -labelled prolactin for each plot is: Δ , 0.049; $\bullet$, 0.093; $\circ$, 0.181; ∇ , 0.344; $\square$, 0.654.

other contaminants were present in sufficiently low titre that they did not play any significant role in our studies. Further, on immunodiffusion (Plate 1), even an antiserum (no. 9) generated against a detergent-solubilized crude mammary-membrane preparation

Fig. 8. Precipitation of ^{125}I -labelled prolactin-receptor complexes by anti-receptor serum

Partially purified soluble prolactin-receptor protein obtained by affinity chromatography (see the Materials and Methods section; 500ng) were incubated with 10^5 c.p.m. of ^{125}I -labelled prolactin at 23°C until equilibrium was reached (8h). Four tubes (two with $1\mu\text{g}$ of unlabelled prolactin added at the same time as the tracer prolactin) were set aside at 4°C for subsequent determination of specific binding of ^{125}I -labelled prolactin. Guinea-pig anti-receptor serum (Δ , 0.1ml) or control serum ($\bullet$), which were diluted serially as indicated on the abscissa, was added to the tubes. Normal guinea-pig serum (0.1ml), diluted 1:10, was added to all the tubes that contained serum used at dilutions greater than 1:10 such that all tubes contained the same amount of guinea-pig serum. Tubes were then incubated for 24h at 4°C . Rabbit anti-(guinea-pig γ -globulin) serum (0.1ml) was added to each tube and incubation at 4°C was continued for another 24h. Immunoprecipitates were centrifuged at 4°C for 15min at 1500g and the radioactivity in the pellets was determined. Specific binding of ^{125}I -labelled prolactin as determined in the four tubes that were set aside at 4°C for 48h was 24000c.p.m. Since the amount of non-specific precipitation ($\bullet$) of ^{125}I -labelled prolactin as determined in incubation tubes that contained control serum was 11000c.p.m. , the maximum amount of ^{125}I -labelled prolactin specifically precipitated by anti-receptor serum (Δ) was therefore 20500c.p.m. ($31500-11000\text{c.p.m.}$). This value represents 85% of the total ^{125}I -labelled prolactin-receptor complexes. When receptor proteins were omitted, no ^{125}I -labelled prolactin was specifically precipitated by anti-receptor serum ($\circ$).

that by itself produced strong immunoprecipitin lines against the crude membrane preparation (result not shown) failed to produce precipitin lines with the prolactin receptors. Even though this antiserum cross-reacted with other membrane components, it failed to inhibit the binding of prolactin to its receptors (Fig. 1).

Perhaps more convincing evidence for specificity of the anti-receptor serum was the demonstration that it selectively inhibited the binding of prolactin to its

receptor without affecting the binding of insulin and bovine growth hormone to their receptors in the same membrane preparation. The fact that γ -globulins derived from anti-receptor serum possessed the inhibitory activity (Figs. 2 and 3) adds further support to the view that the inhibitory factor is an antibody.

The antiserum generated against the prolactin receptors derived from rabbit mammary glands is effective in inhibiting the binding of prolactin to membrane preparations derived from several tissues and from a number of species (Table 1). These findings suggest that the immunological determinants of the receptor molecule are very similar for tissues derived from both sexes and from different species. These data also suggest that there is a phylogenetic similarity between prolactin receptors. It is evident that there is a considerable potential for the application of our anti-receptor sera to studies of prolactin receptors in species such as primates.

The fact that the anti-receptor serum prevented the binding of prolactin to a highly purified preparation of receptors obtained by disc-gel electrophoresis (Fig. 2) indicates that the antibodies were generated to the receptor molecules. Kinetic analyses of the inhibition data further substantiate this point. Our data fit the classical competitive-inhibition model as illustrated by the Lineweaver-Burk (1934) analysis (Fig. 7a). A Dixon (1953) plot (Fig. 7b), however, revealed that the mechanism belongs to a hyperbolic competitive inhibition rather than a dead-end competitive inhibition, as the two mechanisms cannot be distinguished by the Lineweaver-Burk plot (Dixon & Webb, 1964; Mahler & Cordes, 1971). This mechanism suggests that the inhibition is competitive, but with allostery involved (Worcel *et al.*, 1965; Mahler & Cordes, 1971). It is possible that the antigenic determinant on the rather large receptor molecule (approx. 220000 daltons; see Shiu & Friesen, 1974a) is not the active site, but is rather at a region distinct from, but with close proximity to, the active site. The binding of the antibody would, either by physical blocking or by steric hindrance, prevent the receptor from binding the hormone. The demonstration of the existence of hormone-receptor-antibody complexes (Fig. 8), as would be predicted from such a hyperbolic competitive-inhibition model (Dixon & Webb, 1964; Worcel *et al.*, 1965; Mahler & Cordes, 1971), further favours this mechanism.

The present study provides the basis for applying the anti-receptor sera to other studies on the prolactin receptors. In fact we have demonstrated that these anti-receptor sera are capable of blocking the biological actions of prolactin (Shiu & Friesen, 1976), thus showing unequivocally that prolactin receptors are obligatory in mediating the action of prolactin. The availability of anti-receptor sera should prove

useful for studies of other biological properties of the prolactin receptor.

We thank Dr. T. Tsushima for his helpful suggestions on immunization procedures, Mrs. C. Froese for typing the manuscript and Mr. J. Harris for the preparation of the Figures. This work was supported by grants from the Medical Research Council of Canada (MT-1862), U.S.P.H.S. Child Health and Human Development (HD 07843-02) and National Cancer Institute (ICP 43250). R. P. C. S. is a recipient of a fellowship from the University of Manitoba.

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