Immunoprecipitation of the lutropin receptor

Loss of receptor molecules during down-regulation

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Specific anti-(lutropin receptor) antibodies were produced by immunizing rabbits with lutropin receptor purified from pseudopregnant rat ovary. The anti-receptor serum at 1:100 dilution together with anti-(rabbit γ -globulin) serum immunoprecipitated 70% of ^3H -labelled, purified lutropin receptor and 42% of ^{125}I -choriogonadotropin–receptor complex. The antiserum inhibited hormone binding to rat ovarian particles. Pseudopregnant rat ovarian particles were labelled with periodate/ NaB^3H_4 and solubilized with Triton X-100. The Triton X-100 extract was subjected to immunoprecipitation using the anti-receptor serum. When the immunoprecipitate was dissolved and analysed by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate under reducing conditions followed by fluorography, a receptor polypeptide with an apparent M_r 95 000 was detected. A receptor down-regulating dose of choriogonadotropin was injected into pseudopregnant rats and their ovaries were removed and homogenized 4 days later, and analysed for immunoprecipitable receptors as above. No receptor molecules were found. Accordingly, the lutropin receptor molecules actually disappear rather than merely become masked from hormone during homologous down-regulation.

Lutropin target cells respond to this hormone and its agonist, human choriogonadotropin, via a specific plasma-membrane receptor. We have isolated this receptor from rat ovary by affinity chromatography and it comprises only one component of apparent M_r 90 000 (Metsikkö & Rajaniemi, 1982). A sensitive radiolabelling method was developed for the receptor and it appears to be a single sialoglycopolypeptide (Metsikkö, 1984). There is not, however, general agreement on the polypeptide composition of the lutropin receptor at present, since conflicting results have been reported (Ji *et al.*, 1981; Dattatreyaumurthy *et al.*, 1983).

We now report production of specific anti-(lutropin receptor) antibodies that immunoprecipitate the above receptor sialoglycopolypeptide from a Triton X-100 extract of pseudopregnant rat ovary, but not of non-target tissue. These antibodies recognize both the unoccupied and the occupied receptor. Using these antibodies we demonstrate that during down-regulation of

receptors by homologous hormone the lutropin receptor molecules actually disappear rather than merely become masked from hormone.

Methods

Induction of pseudopregnancy

Immature 26-day old female Wistar rats were induced pseudopregnant by injecting subcutaneously 30 i.u. of pregnant-mare serum gonadotropin (Sigma) at 09.00 and 2 days later 30 i.u. of choriogonadotropin (Diosynth, Oss, Holland) at 13.00. Animals were used for experiments 7 days after the choriogonadotropin injection.

Induction of receptor down-regulation in pseudopregnant rats

The pseudopregnant animals were injected intravenously with 1×10^6 c.p.m. of ^{125}I -choriogonadotropin and 30 min later with $100 \mu\text{g}$ of choriogonadotropin. Preparation of ^{125}I -choriogonadotropin has been described earlier (Markkanen *et al.*, 1980). Five animals were killed 2 h later, at which time $355\,000 \pm 25\,000$ c.p.m. (mean $\pm$ s.d.) had accumulated per ovarian pair.

Abbreviations used: PBS, 10 mM-sodium phosphate, pH 7.4, containing 140 mM-NaCl; SDS, sodium dodecyl sulphate.

Ovaries from another five animals were removed 4 days later, at which time point they contained only 352 ± 95 c.p.m. Ovaries from both time points were used for experiments.

Immunization

Lutropin receptor was purified from pseudo-pregnant rat ovaries by affinity chromatography utilizing choriogonadotropin coupled to agarose (Metsikkö & Rajaniemi, 1982). Rabbits were injected intradermally with $10 \mu\text{g}$ of purified receptor (in PBS containing 0.1% Triton X-100) suspended in complete Freund's adjuvant (1:1) at ten sites, four times at 2 week intervals. One animal out of three produced a high-titre antiserum.

Immunoprecipitation of the lutropin receptor

Pseudopregnant rat ovarian or liver particles (5.0 mg of protein) were oxidized with 5 mM- NaIO_4 and reduced with 5 mCi of NaB^3H_4 (Metsikkö, 1984). The ^3H -labelled particles were solubilized with 3 ml of PBS containing 0.5% (w/v) Triton X-100. The suspension was centrifuged at 100 000 g for 1 h at 4°C . Anti-receptor serum or preimmune serum was added to 1 ml of the supernatant (1:100 final serum dilution) and the solutions were incubated overnight at 4°C . An equivalent dose of anti-(rabbit γ -globulin) serum (Sigma) was then added to $100 \mu\text{l}$ aliquots of the above solutions and incubated for 4 h at 20°C . The precipitates were pelleted at 5000 g for 15 min and the pellets were washed twice with 0.5 ml of PBS containing 0.1% Triton X-100.

Immunoprecipitation of purified ^3H -labelled lutropin receptor and ^{125}I -choriogonadotropin-receptor complex was performed as described above. The purified ^3H -labelled receptor (10000 d.p.m.) and the ^{125}I -choriogonadotropin-receptor complex (10000 c.p.m.), solubilized with Triton X-100 (Dufau *et al.*, 1974) from ovarian particles prelabelled with ^{125}I -choriogonadotropin *in vivo*, were incubated with anti-receptor serum or preimmune serum (1:100 serum dilution in PBS containing 0.1% Triton X-100) overnight at 4°C . The incubation volume was $100 \mu\text{l}$. An equivalent dose of the second antibody was subsequently added and incubated at 20°C for 4 h. After pelleting and washing as above, the ^3H radioactive samples were counted in a toluene-based cocktail in a liquid-scintillation counter (LKB Wallac Rackbeta 2), and ^{125}I radioactive samples were counted with a gamma counter (LKB Wallac Ultrogamma).

Analytical techniques

Electrophoresis in SDS was performed according to Laemmli (1970) with 9% polyacrylamide

slab gels ($0.2 \text{ cm} \times 7 \text{ cm} \times 12 \text{ cm}$). Immuno-precipitates were dissolved by boiling for 1 min in the Laemmli sample buffer containing 4 M-urea and 1% (v/v) mercaptoethanol. Gels were treated for fluorography according to Bonner & Laskey (1974). Fluorography of dried gels was performed at -70°C with Kodak XR-2 film.

Western blotting was carried out according to the method of Towbin *et al.* (1979). Transfer of protein from polyacrylamide gel to nitrocellulose sheet was performed in 40 mM-phosphate buffer, pH 7.0, using a Trans-Blot Cell (Bio-Rad). Running time was 16 h at 100 mA. After protein transfer, the nitrocellulose paper was stained for antibody-binding polypeptides by the peroxidase-antiperoxidase complex method of Sternberger *et al.* (1970). The filter was agitated in 3% (w/v) bovine serum albumin solution for 10 min at 20°C , and then incubated overnight at 4°C in a 1:100 dilution of the anti-receptor serum followed by a 1:50 dilution of pig anti-(rabbit γ -globulin) serum (Dakopatts, Copenhagen, Denmark) for 4 h at 20°C . The antisera were diluted in 50 mM-Tris/HCl, pH 7.4, containing 5 mM-EDTA, 150 mM-NaCl and 0.01% Triton X-100. The filter was then incubated in a 1:100 dilution of peroxidase-antiperoxidase (Dakopatts) for 4 h at 20°C followed by a substrate solution containing 0.01% (w/v) diaminobenzidine and 0.03% (v/v) H_2O_2 in water. The filter was washed three times between incubations with 50 mM-Tris/HCl, pH 7.4, containing 5 mM-EDTA, 150 mM-NaCl, and 0.01% Triton X-100 over a period of 1 h.

Choriogonadotropin-binding capacity of ovarian particles was determined as described earlier (Rajaniemi *et al.*, 1977).

Protein was determined by the method of Dulley & Grieve (1975) using bovine serum albumin (Sigma, fraction V) as standard.

Results

Properties of the anti-receptor serum

The anti-receptor serum at 1:100 dilution immunoprecipitated $70 \pm 2\%$ of the ^3H -labelled, purified receptor protein, whereas preimmune serum precipitated only 3%. The anti-receptor serum precipitated $42 \pm 2\%$ of ^{125}I -choriogonadotropin-receptor complex solubilized with Triton X-100 from pseudopregnant rat ovarian particles, but it did not recognize free hormone (Table 1). Ovarian particles were agitated overnight at 4°C in PBS containing 0.1% albumin in the presence of various dilutions of the anti-receptor serum. This incubation impaired dose-dependently the choriogonadotropin-binding capacity of the ovarian particles when compared with those treated identically with preimmune serum (Fig. 1).

Table 1. Immunoprecipitation by anti-receptor serum or preimmune serum of purified ^3H -labelled lutropin receptor, ^{125}I -choriogonadotropin-receptor complex extracted from prelabelled ovarian particles, and ^{125}I -choriogonadotropin

Values are means $\pm$ S.D. of five determinations.

Serum	Precipitation (%) of:		
	^3H Receptor	^{125}I -choriogonadotropin-receptor complex	^{125}I -choriogonadotropin
Anti-receptor	70 $\pm$ 2	42 $\pm$ 2	5 $\pm$ 0.5
Preimmune	3 $\pm$ 0.5	3 $\pm$ 0.5	3 $\pm$ 0.5

Fig. 1. Inhibition of hormone binding to rat ovarian particles by the anti-(lutropin receptor) serum

Pseudopregnant rat ovary was homogenized in PBS containing 0.1% bovine serum albumin. Aliquots (200 μl , 60 μg of protein) of the homogenate were agitated with various dilutions of anti-(lutropin receptor) serum (●) or preimmune serum (○) for 3 h at 20°C. ^{125}I -Choriogonadotropin was then added (50 μl , 72000 c.p.m.) and agitated overnight at 0°C. Unbound hormone was removed by centrifugation at 5000g for 30 min followed by two washes with 0.5 ml of PBS containing 0.1% bovine serum albumin. Nonspecific binding was determined in the presence of 1 μg of human choriogonadotropin. The points represent means of triplicate determinations.

Immunoprecipitation of the lutropin receptor before and after hormone stimulation

The anti-receptor serum was used to precipitate the receptor polypeptide from the Triton X-100 extract of periodate/ NaB^3H_4 -labelled ovarian particles. An aliquot of the ovarian Triton X-100 extract (100 μl) was subjected to immunoprecipitation with anti-receptor serum. A precipitate showing radioactivity of 25000 d.p.m. was obtained, whereas the preimmune serum precipitated 8300 d.p.m. Both immunoprecipitates were subjected to electrophoresis in SDS and detected by fluorography. A single major component with an apparent M_r 95000 was observed when the anti-receptor serum was used (Fig. 3, lane 3). The pre-immune serum precipitated only material migrating with the dye front (lane 4). When the Triton X-100 extract from periodate/ NaB^3H_4 -labelled liver particles was subjected to immunoprecipitation with the anti-receptor serum, no receptor polypeptide was observed. We have stated previously that the receptor polypeptide changes into a slightly more slowly moving component upon reduction, and thereafter an apparent M_r 95000 was observed, instead of the M_r 90000 reported for the non-reduced polypeptide (Metsikkö & Rajaniemi, 1982).

A receptor-saturating dose of choriogonadotropin was administered intravenously to pseudopregnant rats, and the animals were killed 4 days later. The hormone-binding capacity of these ovaries was about 2% of that of the control pseudopregnant ovaries, and they contained only 0.1% of the ^{125}I radioactivity taken up 2 h after the administration of ^{125}I -choriogonadotropin. The down-regulated ovaries from five animals were homogenized, and a portion of the particulate fraction (5.0 mg of protein) was labelled with 5 mCi of NaB^3H_4 and then extracted with 3 ml of 0.5% (w/v) Triton X-100. An aliquot of the Triton X-100 extract (100 μl) was incubated overnight with anti-receptor serum (1:100) or preimmune serum (1:100), whereafter an equivalent dose of anti-(rabbit γ -globulin) serum was added. When the precipitates were dissolved and analysed by

A Western blot of the material used as antigen was performed using nitrocellulose filter as support. When the filter was stained with the method of Sternberger *et al.* (1970) using the anti-receptor serum, a single M_r 90000 polypeptide became visible (Fig. 2). This result suggests that the anti-serum contained antibodies against the receptor polypeptide, and the minor impurities in the purified receptor (Metsikkö & Rajaniemi, 1982) did not elicit antibodies. The antiserum was also unable to recognize free hormone by this method.

Fig. 2. Identification of the lutropin receptor by Western blotting using lutropin receptor-directed antiserum

Lutropin receptor protein was purified by affinity chromatography and subjected to electrophoresis on SDS/9% polyacrylamide-slab gels. Polypeptides were electrophoretically transferred from the gel to nitrocellulose paper, and the paper was stained for the anti-(lutropin receptor) antibody-binding bands by the peroxidase-antiperoxidase complex method of Sternberger *et al.* (1970). Lane 1, purified lutropin receptor, 5 μ g; lane 2, the same, 0.5 μ g; lane 3, choriogonadotropin, 5 μ g.

electrophoresis in SDS followed by fluorography, no receptor polypeptide was observed (Fig. 3, lanes 1 and 2).

Discussion

The anti-(lutropin receptor) antibodies produced in this study inhibited hormone binding to

Fig. 3. Immunoprecipitation of the lutropin receptor from pseudopregnant down-regulated and control ovaries

Ovarian particles from down-regulated or control pseudopregnant rats were labelled with periodate/ NaB^3H_4 and extracted with Triton X-100. The extracts were subjected to immunoprecipitation using anti-(lutropin receptor) serum or preimmune serum (1:100) and anti-(rabbit γ -globulin) serum as a second antibody. The immunoprecipitates were dissolved and analysed by slab gel electrophoresis in SDS under reducing conditions. The ^3H -labelled bands were detected by fluorography. No receptor band was seen in down-regulated ovaries whether anti-receptor serum (lane 1) or preimmune serum (lane 2) was used. A receptor polypeptide (apparent M_r 95 000) was observed in the control ovaries when anti-receptor serum (lane 3) was used but not when preimmune serum was used (lane 4). Lane 5, Triton X-100 extract of down-regulated ovaries.

particulate receptor and bound to free and occupied receptor, indicating that there are antibodies against different regions of the receptor molecule. This is in contrast with the previous antibodies we have produced (Metsikkö & Rajaniemi, 1981). The present antibodies are thus useful when the fate of the occupied receptor in the cell is followed during desensitization and down-regulation. Because the present antibodies were capable of immunoprecipitating the solubilized receptor polypeptide they can be used to study receptor biosynthesis when employing an endogenously incorporated label. However, in spite of that, the anti-receptor serum precipitated nonspecifically only a trace amount of an M_r 55 000 ^3H -labelled polypeptide (Fig. 3, lane 4); it may in fact precipitate unlabelled polypeptides, too.

The present antibodies recognized a receptor polypeptide with an M_r of approx. 90 000–95 000. We have several lines of evidence that the lutropin receptor is a single polypeptide, based on receptor purification, covalent labelling and affinity cross-linking to hormone in dilute solution (Metsikkö, 1984; Metsikkö & Rajaniemi, 1982). Three other laboratories have also reached the conclusion that the lutropin receptor comprises a single polypeptide (Rebois *et al.*, 1981; Aubry *et al.*, 1982; Rapoport *et al.*, 1984), although there exist differences between the M_r values reported from various laboratories. It has been claimed, however, that the pig ovarian lutropin receptor is composed of several polypeptides of M_r between 63 000 and 120 000 (Ji *et al.*, 1981) and a recent publication has arrived at the conclusion that the rat ovarian lutropin receptor comprises four subunits (Hwang & Menon, 1984). These results are hard to reconcile with our own. The rat gonadal receptor has turned out to be very prone to endogenous proteolysis (Kellokumpu & Rajaniemi, 1983), and our explanation is that the proteolysis produces receptor fragments that still bind hormone.

Our finding that the anti-receptor antibodies were unable to recognize receptor molecules in down-regulated ovaries suggests that during down-regulation the receptors were degraded rather than merely masked from hormone. It has been shown for the β -adrenergic receptor of the frog red blood cell that during desensitization the receptors become internalized and remain masked from hormone within small vesicles (Stadel *et al.*, 1983). We were, however, unable to demonstrate hormone-binding capacity in the 20 000g supernatant of down-regulated ovaries in spite of solubilization of microsomes with Triton X-100. The ovarian receptor concentration begins to rise 4 days after a down-regulating dose of choriogonadotropin (Conti *et al.*, 1976). It remains to be established

whether the prevailing cells resume receptor synthesis during the subsequent recruitment period of receptors or if completely new cells must differentiate and luteinize.

In conclusion, anti-receptor antibodies were produced which precipitated an M_r 95 000 ^3H -labelled polypeptide from rat ovarian detergent extract as determined by SDS/polyacrylamide-gel electrophoresis under reducing conditions. This component was not present in down-regulated ovaries or in liver.

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