Two polyclonal antisera to rat luteal LH/CG receptor with different ligand binding inhibition and immunohistochemical receptor detection capabilities

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Abstract. Polyclonal antisera to a SDS-denatured and partially renatured rat luteal 90 K LH/CG receptor were raised in rabbits, characterized, and their applicability for immunohistochemical location of the receptor examined. The LH/CG receptor was purified by hCG-affinity chromatography and subjected either to a preparative SDS-PAGE or Western blotting. Gel slices containing the SDS-denatured or nitrocellulose strips containing the renatured 90 K LH/CG receptor were used for immobilization. The antisera, termed ARS-2 and ARS-3, respectively, possessed similar antibody titres. Both antisera were able to recognize the native, SDS-denatured, and SDS-denatured and reduced forms of the LH/CG receptor on dot blots, but only ARS-3 contained antibodies to the hormone binding site or a region near to it, as it was able to inhibit the hCG binding to the membrane-bound LH/CG receptor in a dilution-dependent manner. Both antisera recognized the receptor-hCG complex, but ARS-2 stained the complex with about 50% less intensity than the free receptor. ARS-3 located the LH/CG receptor distinctly on the luteal cell surfaces in immunohistochemical staining with peroxidase antiperoxidase complex method, but ARS-2, although it possessed similar antibody titre, revealed negligible staining. Thus, the antisera readily recognize the native receptor, but differ in their capability for inhibiting hormone binding. Only ARS-3, produced against the renatured receptor, contains sufficient amounts of antibodies capable of recognizing free and occupied receptors in immunohistochemistry.

Both polyclonal and monoclonal antisera have proved to be useful probes for studying the structure, function and cellular location of various hormone and growth factor receptors. In general, the production of monoclonal antibodies does not necessarily require homogeneous antigen preparation for primary immunizations, since each hybrid cell line can be cloned and tested for antigen specificity. In order to produce a monospecific polyclonal antiserum, however, an antigen of high purity is essential. Recent methodological advances (1-3) have rendered it possible to purify the LH/CG receptor from rat gonads virtually to homogeneity and in sufficient amounts to allow the production of polyclonal antisera against it (3-5). The findings of Roseblit et al. (3) and Lakkakorpi et al. (5) suggest that the antisera seem to differ, e.g. in their ability to inhibit hormone binding depending upon whether they are produced against the native or the denatured form of the LH/CG receptor. Thus, the use of LH/CG receptors treated in different ways for immobilization may give rise to antisera with different properties that will be useful for studying the structural and functional relationships and regulation of the receptor.

In the present work we produced polyclonal antisera to SDS-denatured and partially renatured rat luteal 90 K LH/CG receptors. The affinity-purified LH/CG receptor preparation was further purified by a preparative SDS-PAGE or Western blotting and the immunizations were performed with crushed gel slices containing the SDS-denatured 90 K receptor protein or dimethylsulphoxide-dissolved nitrocellulose strips containing its partially renatured counterpart. The antisera produced to these antigens differed particularly in terms of their ability to inhibit hormone binding and their applicability for immunohistochemical use. Immunization with the Western-blotted LH/CG receptor...
provides a new way to produce a versatile polyclonal antiserum to the receptor.

**Material and Methods**

**Reagents**

Pregnant mare serum gonadotropin, PMSG (2500 IU/mg) and human chorionic gonadotropin, hCG, were purchased from Diosynth (Oss, The Netherlands). The chloramine-T method (6) was used to radioiodinate highly purified hCG (11900 IU/mg) with Na\(^{125}\)I (Amersham International, Amersham, Bucks, UK) to a specific activity of 50-70 µCi/µg (7), as determined by the self-displacement method (8). Affinity-purified goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate was the product of Bio-Rad Laboratories (Richmond, CA). Swine anti-rabbit IgG serum and horseradish peroxidase-rabbit anti-horseradish peroxidase (PAP) complex were obtained from Dako Immunoglobulins (Copenhagen, Denmark) and 3,3’-diaminobenzidine tetrahydrochloride (DAB) from Fluka Ag (Buchs, Switzerland). Freund’s adjuvants were purchased from Gibco Laboratories (Grand Island, NY) and dimethylsulphoxide (DMSO) from Merck.

**Animals and treatments**

Sprague-Dawley rats were rendered pseudopregnant with sequential injections of PMSG and hCG (9), as described in detail (5). The ovaries were removed 7 days after the hCG injection, frozen immediately with liquid nitrogen and stored at -80°C until used. Alternatively, one ovary was removed from some animals under light ether anesthesia, frozen and stored as before, the other one being removed 2 h after an iv injection of 500 IU hCG. The ovaries bearing the saturated receptors were stored as before.

**Production of the antisera**

The LH/Crone receptor was purified as described in detail (2). The hCG-affinity-purified receptor was subjected to preparative SDS-PAGE (2,5). Strips from both sides of the gel containing the mass standards and receptor sample were silver-stained (10) to locate the receptor band in the middle part of the gel and to demonstrate its homogeneity. The 90 K receptor band was then cut out from the middle part of the gel, crushed and used for immunization (ARS-2). In addition, the proteins separated out were electroblotted from SDS-polyacrylamide gels onto a nitrocellulose sheet, and the 90 K receptor band was visualized with Ponceau S, cut out, dissolved in 0.5 ml of DMSO and used for immunization (ARS-3). The rabbits received approximately 10 µg of the 90 K receptor 4 times at 2-week intervals (5). In both cases, the first two immunizations were given in complete Freund’s adjuvant and the last ones in incomplete Freund’s adjuvant.

**Immunoblotting and dot blotting**

SDS-PAGE, electroblotting and dot blotting procedures are described in detail (2,5). The pH 4 eluate from the hCG-affinity columns represents the native receptor in the experiments. The purified receptor was denatured for dot blotting experiments by incubating it for 3 min at 95°C in 2% SDS in the presence or absence of 100 mmol/l dihydrotestosterone (DHT). To determine whether the antisera recognize the receptor and receptor-hormone complex, ovaries bearing free and occupied receptors (see Animals and treatments) were homogenized separately in PBS-buffer (100 mg of tissue/ml phosphate-buffered saline (PBS) containing 5 mmol/l EDTA, 5 mmol/l N-ethylenemaleimide, NEM and 0.2 mmol/l Phenylmethylsulfonyl fluoride, PMSF). The homogenate was centrifuged for 30 min at 27 000 × g and the resulting membrane pellet was suspended in 1 ml PBS containing 1% (v/v) Triton X-100 and 20% (v/v) glycerol. The suspension was stirred for 30 min on ice and then centrifuged for 1 h at 100 000 × g. The supernatant was diluted with 2 vol of PBS containing 20% (v/v) glycerol and stored in aliquots at -80°C until used.

Western or dot blots were incubated with undiluted porcine serum overnight at 4°C followed by incubations with different receptor antiserum or pre-immune serum dilutions in the range 1:20-1:3000 (in TAG; 10 mmol/l TRIS-HCl pH 7.4, 1% BSA, 10% glycerol) for 2 h at room temperature. The blots were washed five times with 0.05% (v/v) Triton X-100 for 5 min each and twice with PBS for 5 min each. After incubating for 1 h with affinity-purified goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (dilution 1:3000 in TAG) at room temperature, the sheets were washed again and stained for 7 min at room temperature with a solution containing 3 mg DAB and 3.3 µl 30% H\(_2\)O\(_2\) in 10 ml PBS. They were then air-dried and the staining intensities measured with a computing densitometer (Model 300 A, Molecular Dynamic, Palo Alto, CA).

**Binding inhibition assay**

One ovary was homogenized in 2 ml 0.1% BSA/PBS on ice with a motor-driven glass homogenizer. The homogenate was centrifuged for 10 min at 80 × g followed by centrifugation of the supernatant for 30 min at 25 000 × g. The resulting membrane pellet was suspended in 10 ml 0.1% BSA/PBS. Aliquots of 100 µl of the suspension were mixed with 50 µl of receptor antiserum dilutions ranging from 1:20 to 1:10 000 and incubated overnight at 4°C. After the incubation, 1 ml of 0.1% BSA/PBS was added and the membranes were pelleted by centrifugation for 30 min at 15 000 × g. The washing of the pellet was repeated. The final membrane pellets were suspended in 50 µl 0.1% BSA/PBS, whereafter 50 µl \(^{125}\)I-hCG (200 000 cpm) per tube was added. After overnight incubation at 4°C, 1 ml 0.1% BSA/PBS was added to the mixture, centrifuged for 30 min at room temperature, and the was repeated. The final pellets were counted for \(^{125}\)I-radioactivity.
Fig. 1.
Antibody titre analysis of ARS-2 (○) and ARS-3 (●). The hCG-affinity-purified receptor samples (500 ng) were electroblotted from SDS-PAGE onto nitrocellulose sheets, strips of which were incubated with different antiserum dilutions and subjected to peroxidase staining as described in Materials and Methods. The staining intensity of the bands was analysed with a computing densitometer. Each point represents the mean ± s.d. of the three independent immunoblots. The inserts show representative immunoblots.

Fig. 2.
Antibody binding to Western blots of Triton X-100 extracts of pseudopregnant rat ovaries obtained prior to (Panel A) and 3 days after (Panel B) the down-regulating dose of hCG. The Triton X-100-solubilized proteins were electroblotted from SDS-polyacrylamide gels onto a nitrocellulose sheet, which was incubated with ARS-2 or ARS-3 (dilutions 1:300) followed by peroxidase staining as described in Materials and Methods. Pre-immune serum (NRS) for ARS-2 was used as a negative control (Panel C).

activity with a 1280 Ultrogramma 8-spectrometer (LKB-Wallac). For the control experiments the receptor antisera were replaced with pre-immune serum. Nonspecific hormone binding was tested in the presence of an excess of unlabelled hCG.

Immunohistochemistry
Pseudopregnant rat ovaries were fixed in Carnoy fluid for 12 h, embedded in paraffin and sectioned at 5 μm.

The PAP complex method (11) was used to visualize the receptor location, as described earlier (5).

Briefly, the sections were deparaffinized and treated with undiluted porcine serum for 30 min. All the incubations were performed in a humid chamber at room temperature unless otherwise mentioned. The sections were then incubated with different receptor antiserum or pre-immune serum dilutions in PBS containing 0.3% Triton X-100 for 1 h at 37°C, and washed several times for

Fig. 3.
Antibody binding (dilution 1:300) to a native, SDS-denatured or SDS-denatured and reduced rat luteal LH/CG receptor on dot blots. The nitrocellulose sheets were immunostained as described in Materials and Methods. The staining intensities of the dots were analysed with a computing densitometer. The columns represent the mean ± s.d. of three independent stainings. Pre-immune serum (NRS) for ARS-2 was used as a negative control.
Antibody binding (dilution 1:300) to occupied and free rat luteal LH/CG receptors on dot blots. The nitrocellulose sheets were stained as described in Materials and Methods, and the staining intensities of the dots were analysed with a computing densitometer. The columns represent the mean ± so of three independent stainings. Pre-immune serum (NRS) for ARS-2 was used as a negative control.

5 min each with 0.3% Triton X-100/PBS. After incubation for 30 min with undiluted porcine serum the sections were incubated with a 1:10 dilution of swine anti-rabbit IgG serum in 0.3% Triton X-100/PBS for 1 h at 37°C. After further washings with 0.3% Triton X-100/PBS and treatment with undiluted porcine serum for 5 min, the sections were incubated with a 1:100 dilution of PAP complex in PBS for 30 min at 37°C followed by incubation for 5 min in a solution containing 9 mg DAB and six drops of 30% H₂O₂ in 15 ml PBS.

Results

Comparison of the properties of the antisera
The antisera obtained against the SDS-denatured (ARS-2) or partially renatured (Western blotted) 90 K rat luteal LH/CG receptor (ARS-3) possessed several interesting properties. Their antibody titres, determined by analysing the staining intensities of the immunoblotted receptor bands with a computing densitometer were fairly similar (Fig. 1). Both antisera produced specific staining of a single 90 K band on the Western blot of the Triton X-100 extract of pseudopregnant rat ovaries (Fig. 2). This band was absent from the down-regulated pseudopregnant rat ovarian tissue. The sharp band stained with ARS-2 was also present on Western blot obtained with its pre-immune serum. Both antisera recognized the native, SDS-denatured and SDS-denatured and reduced forms of the receptor as detected by computer densitometer analysis of the dot blots (Fig. 3), but the staining intensity was most distinct for the native receptor with both antisera. ARS-3 recognized both the free and occupied

Fig. 5.
Analysis of the inhibition of the binding to pseudopregnant rat ovarian membranes. The membranes were incubated with different dilutions of ARS-2 (○) or ARS-3 (●) ranging from 1:20 to 1:10 000 and then with ¹²⁵I-hCG (see Materials and Methods for details). After intensive washings, the membrane pellets were counted for radioactivity. Each point represents the mean ± so of triplicate samples. Control experiments were performed by replacing the receptor antiserum with pre-immune serum (■).
forms of the receptor with almost identical intensities, but ARS-2 recognized the occupied receptor with an approximately 50% lesser intensity (Fig. 4).

ARS-3 was found to contain detectable amounts of antibodies to the hormone binding region of the receptor or near to it, as it was able to inhibit the $^{125}$I-hCG binding to the treated membranes in a dilution-dependent manner (Fig. 5). ARS-2 caused only a marginal reduction at 1:20 dilution in the $^{125}$I-hCG binding as compared with pre-immune serum.

**Applicability of the antisera for visualizing the LH/CG receptor in immunohistochemistry**

Although both antisera possessed similar antibody titres, only ARS-3 located both the free (Fig. 6 a-d) and occupied (Fig. 6 e) LH/CG receptors on the luteal cell membranes. Single luteal cells located outside the corpora lutea and surrounded by connective tissue were often unstained (Fig. 6 f). The staining distribution with ARS-3 was identical to that observed with our earlier antisera to the native receptor (4,5). ARS-2 gave only a faint diffuse staining in the pseudopregnant rat ovaries bearing either free (Fig. 7 a-d) or occupied (Fig. 7 e) receptors and was regarded as nonspecific on the basis of the stainings obtained with the pre-immune serum (Fig. 7 f).

**Discussion**

Specific polyclonal antisera to a SDS-denatured and renatured 90 K rat luteal LH/CG receptors were produced in rabbits and their properties assessed, in particular their applicability for immunohistochemical purposes. The affinity-purified receptor preparation was subjected to a preparative SDS-PAGE or Western blotting, and the immunizations were performed with either crushed gel slices (ARS-2) or DMSO-dissolved nitrocellulose strips (ARS-3) containing the 90 K LH/CG receptor polypeptide. In the former case, the antigen represents electrophoretically homogeneous, SDS-denatured receptor and in the latter case it is at least in part renatured as the Western blots binds $^{125}$I-hCG (2). Hence, antisera with different properties were to be expected.

The SDS-denatured and renatured LH/CG receptors do not essentially differ in antigenicity, as the antisera produced against them exhibit a similarly high antibody titre. They produced a distinct staining of a single 90 K polypeptide on Western blots of the affinity-purified receptor at dilutions up to 1:3000. Both antisera also showed a high specificity, since they yielded specific staining on only a single 90 K polypeptide on Western blots of the Triton X-100 extract of the pseudopregnant rat ovarian membranes. Irrespective of whether the antisera was raised against the SDS-denatured or renatured receptor, it seemed to recognize the native, SDS-denatured and SDS-denatured and reduced forms, the reaction being most intense with the native receptor in the case of both antisera, however. Thus, both antisera contain antibodies recognizing both conformational and peptide sequence epitopes.

The antisera described here differ most distinctly in two properties. Firstly, only ARS-3 contains detectable amounts of antibodies directed at or near the ligand-binding site of the receptor, as the binding of $^{125}$I-hCG to ovarian membranes pre-treated with different antisera dilutions was clearly inhibited in a dilution-dependent manner. The above difference together with the fact that they both readily recognize the native receptor, renders them particularly valuable, because they may be utilized to elucidate the role of the receptor binding region in evoking the hormonal signal. ARS-3 clearly resembled in its properties the earlier two receptor antisera produced by our laboratory against the native, affinity-purified receptor preparation, as they also contained antibodies to the ligand-binding site (4,5). ARS-3 must possess a higher specificity, however, as it was produced against the protein that had been further purified by SDS-PAGE from the affinity-purified receptor preparation. The property of ARS-2 of not inhibiting ligand binding to the membrane-bound receptor is consistent with that of the polyclonal antisera recently produced by Rosemblit et al. (3) against a similarly treated receptor. These findings support our previous view (5) that the antibodies to the hormone-binding region of the receptor are conformational ones and appear in abundance when the native receptor is used for immunization. Secondly, only ARS-3 contains sufficient amounts of antibodies capable of recognizing the LH/CG receptor in immunohistochemical tissue sections fixed with aldehyde or Carnoy fluid. The distribution of the LH/CG receptors on the luteal cell surfaces revealed by ARS-3 was identical to that seen in our earlier work (4,5) and in that of others (12-
ARS-3 recognized occupied receptors both on dot blots and in immunohistochemistry, as did also our earlier receptor antiserum (5). This property of ARS-3 extends its usefulness as it makes it applicable to studies of the fate of occupied receptors during hCG-induced down-regulation by both im-

Fig. 6.
Visualization of the LH/CG receptor prior to (Panels a-d; dilutions 1:20, 1:50, 1:100, 1:200, respectively) and 2 h after an iv injection of 500 IU (Panel e; dilution 1:50) in pseudopregnant rat ovaries using ARS-3 and the PAP complex technique. Panel f (dilution 1:20) shows the unstained luteal cell (arrow) within the connective tissue capsule of the corpus luteum. Bar 20 μm. Magnification 800 x.
munohistochemical and blotting techniques, for instance.

To conclude, the polyclonal antisera to the native
and SDS-denatured LH/CG receptors described
here provide us with a useful tool for studying fur¬
ther the structure and function of the receptor and

Fig. 7.
Visualization of the LH/CG receptor in the same samples as in Fig. 6 using ARS-2 and the PAP complex technique. Panels a-d (dilutions 1:20, 1:50, 1:100 and 1:200, respectively) represent samples bearing free receptors and panel e (dilution 1:50) occupied receptors. Panel f (dilution 1:100) shows the pre-immune serum control. Bar 20 μm. Mag¬
nification 800 x.
particularly for studying receptor location and regulation immunohistochemically. The evidence that the Western blotted renatured antigen produces antibodies useful for immunohistochemical purposes may apply to other antigens as well.

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