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Characterization by Photoaffinity Labeling of a Steroid Binding Protein in Rat Liver Plasma Membrane

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Summary. The mechanism of steroid uptake by the cell remains controversial. [^{3}H]R5020 was utilized to characterize by photoaffinity labeling the steroid binding site in plasma membrane. This binding was saturable, reversible and had one type of binding site (K_d = 33 ± 4 nM, B_{max} = 32 ± 2 pmol/mg). [^{3}H]R5020 could be prevented from binding by a variety of steroids (cortisol, progesterone, deoxycorticosterone, and levonorgestrel); estradiol did not have affinity for this binding site. The kinetics of R5020 photoactivation was time dependent and saturable. SDS-PAGE showed a specific band which corresponded to a 53-kDa peptide. The sucrose density gradient analysis has revealed the existence of a protein with a sedimentation coefficient of 3.6 ± 0.2 S. This polypeptide shows different characteristics than cytosolic steroid receptor or serum steroid binding proteins. This binding protein could correspond to the steroid binding site previously found in the plasma membrane.

Key Words glucocorticoids · R5020 · cortisol · steroids · specific binding sites · photoaffinity labeling · plasma membrane · rat liver

Introduction

It is usually assumed that steroid hormones penetrate into the cell by passive diffusion; later they bind to cytoplasmic receptors, forming a hormone-receptor complex that translocates to the nucleus and alters the transcription of specific gene sequences (O'Malley et al., 1972).

Nevertheless, it has been reported that steroids penetrate into the target cells by a specific transport mechanism rather than by free diffusion (Fant, Harbison & Harrison, 1979; Allera, Rao & Breuer, 1980; Jonkers et al., 1980). Moreover, some authors have characterized binding sites in plasma membrane for estrogens (Pietras & Szego, 1980; Rambo & Szego, 1983; Berthois et al., 1986; Bression et al., 1986), progestins (Haukkamaa, 1984; Blondeau & Baulieu, 1985; Sadler, Bower & Maller, 1985; Yamada et al., 1990) and glucocorticoids (Suyemitsu & Terayama, 1975; Koch et al., 1977, 1978; Ambellan, Swanson & Davidson, 1981; Towel & Sze, 1983; Savart & Cabillic, 1985; Allera & Rao, 1986; Gametchu, 1987; Trueba et al., 1987, 1989*a*,*b*, 1990, 1991; Quelle et al., 1988; Chirino et al., 1989; Guendouz et al., 1989).

Steroid hormones bind to specific binding proteins with high affinity but in a reversible reaction. For this reason technics that use denaturing conditions, like SDS-PAGE, cannot be employed to characterize the steroid-protein complex in heterogeneous preparations. Photoaffinity labeling could represent one approach to this problem.

Photoaffinity labels are ligands showing an inherent affinity for a binding site which contain a photosensitive functional group. When photoactivated with UV light, this group is capable of forming a covalent bond at or near the binding site (Fedan, Hogaboom & O'Donell, 1984). Ketosteroids have been utilized successfully as photoaffinity reagents to steroids receptors. The synthetic steroid R5020 or promegestone (17α,21-dimethyl-19-nor-pregne-4,9-diene-3,20-dione) was previously employed to photolabel progesterone (Dure, Schrader & O'Malley, 1980) and glucocorticoid (Nordeen et al., 1981) cytosolic receptors and an oocyte plasma membrane steroid receptor (Sadler & Maller, 1982; Blondeau & Baulieu, 1984). This steroid was chosen because of its maximum absorption at 320 nm, thereby allowing its activation by UV light at wavelengths above 300 nm, where protein damage would be minimized.

Recently, we have characterized a specific binding site for cortisol (Trueba et al., 1989*b*), progesterone (Trueba et al., 1990) and corticosterone (Trueba et al., 1989*a*) in mouse liver, for corticosterone in rat kidney (Ibarrola et al., 1991),

and for corticosterone also in hepatocytes and rat liver plasma membrane (Trueba et al., 1991). The aim of this paper is to identify this corticoid binding protein using [^{3}H]R5020 to covalently label the steroid binding site in rat liver plasma membrane.

Materials and Methods

Chemicals

[^{3}H]R5020 (86 Ci/mmol) was purchased from New England Nuclear. [^{3}H]Cortisol (87 Ci/mmol) was obtained from Amersham International (England). R5020, RU38486, RU28362 and R1881 were a generous gift from Roussel-Uclaf (France). Other steroid hormones were from Sigma Chemical (St. Louis, MO). PPO, POPOP, scintillation grade toluene and Emusolv™ were supplied by Scharlau (Barcelona, Spain). All the other reagents were from Merck (Darmstadt, Germany).

Isolation of Plasma Membrane

Liver plasma membrane was obtained as described with slight modifications (Maeda, Balakrishnan & Mehdi, 1983). Livers from Sprague-Dawley male rats (weighing about 300 g) were perfused with NaCl 0.9%, removed and homogenized in 3 vol of 0.25 M sucrose, 20 mM Tris-HCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, pH 7.4. The homogenate was centrifuged for 5 min at 1500 × g, and the pellet was resuspended in the original volume and centrifuged in a 41% (wt/vol) sucrose gradient for 65 min at 95,000 × g. The interfacial plasma membrane fraction was washed for 25 min at 95,000 × g with TE buffer (20 mM Tris-HCl, 1.5 mM EDTA, pH 7.9) plus 140 mM NaCl to remove possible adsorbed proteins. During the isolation procedure the temperature was kept at 0–4°C.

Purified plasma membranes were frozen in liquid nitrogen and stored in homogenization buffer at −70°C. The purity of this fraction had been previously determined with marker proteins (Trueba et al., 1991). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Binding Assay of [^{3}H]R5020

Incubation mixtures for binding studies contained 0.1 ml of purified plasma membrane fraction (0.2 mg protein/ml final concentration), 0.1 ml of [^{3}H]R5020, and 0.8 ml of TE buffer. The presence of specific R5020 binding sites was determined by the difference between the radioactivity bound to the membranes incubated only with [^{3}H]R5020 (total binding) and that bound in the presence of an excess of unlabeled R5020 (nonspecific binding).

Incubation assays were performed in an ice bath. At the end of the incubation period, triplicate 800-μl aliquots were filtered through a GF/C glass fiber filter (Whatman, England) placed in a 12-place filter manifold (Millipore). Filters were immediately washed with 12 ml of iced incubation buffer. The filters were placed in vials and counted in 5 ml of a Emusolv™-toluene based scintillation cocktail in a Packard Tricarb 2000 CA model which had an efficiency of 65%.

Saturation data were analyzed by means of the Kinetic, EBDA, Ligand, Lowry software provided by Biosoft (Cambridge, UK) using an IBM-PC computer. The SEM of several samples did not exceed 10% in any case.

Photoaffinity Labeling

The preparation of plasma membrane and steroid was incubated in Pyrex tubes in the dark and allowed to reach binding equilibrium in TE buffer in ice bath. The samples were then photolyzed in a cold room (temperature was kept below 7°C), placing the sample tubes 6 cm below an UV lamp (OSRAM HQV 125 W, λ_{max} = 350 nm). Incident light was filtered through a 1-cm thick layer of saturated $CuSO_4$ solution for a more effective elimination of wavelengths below 300 nm (Birnbaumer, Schrader & O'Malley, 1983). After the beginning of the irradiation, aliquots were taken at different times for the determination of the amount of radioactive ligand covalently bound to protein. This was accomplished by precipitation with 10% (wt/vol) trichloroacetic acid for 15 min at 0°C, collecting the precipitates on GF/C glass fiber filters. The filters were washed twice with 4 ml cold 5% (wt/vol) trichloroacetic acid and twice with 3 ml acetone/0.1% HCl (Westphal, Fleischmann & Beato, 1981) and counted for radioactivity.

SDS-Polyacrylamide Gel Electrophoresis

After 45 min of irradiation, membrane samples were centrifuged (14,000 × g, 10 min), resuspended in sample buffer (20 mM Tris-HCl, 3% (wt/vol) SDS, 5% (vol/vol) glycerol, 5 mM dithiothreitol), boiled 6 min and electrophoresed overnight in slab gels (80 × 140 × 2.7 mm) according to the method of Laemmli (1970). The acrylamide composition in the stacking and the running gels was 3% (wt/vol) and 10% (wt/vol), respectively.

Gels were frozen and sliced (2-mm thickness). Slices were soaked overnight in 0.5 ml of 30% (vol/vol) H_2O_2 at 40°C followed by the addition of 5 ml of scintillation cocktail.

Molecular weight determination was performed using the following standard proteins: β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

Sucrose Gradient Ultracentrifugation

Photoaffinity-labeled samples were solubilized with 0.5% (wt/vol) CHAPS for 30 min and centrifuged (14,000 × g, 30 min). Linear (5–20%) sucrose gradient containing 0.5% CHAPS in TE buffer was prepared using the layering diffusion method (Stone, 1974). Supernatant (0.2 ml) was layered on the top of the gradient, and the tubes were centrifuged at 150,000 × g and 4°C for 16 hr. The standard proteins, γ-globulin (9.6 S), bovine serum albumin (4.6 S), and ovoalbumin (3.5 S), were run separately. Fractions of four drops were collected by piercing the bottom of the tubes. The positions of protein standards were determined by $A_{280\ nm}$ measurements. Fractions of samples were dissolved in 5 ml of scintillation cocktail and counted for radioactivity.

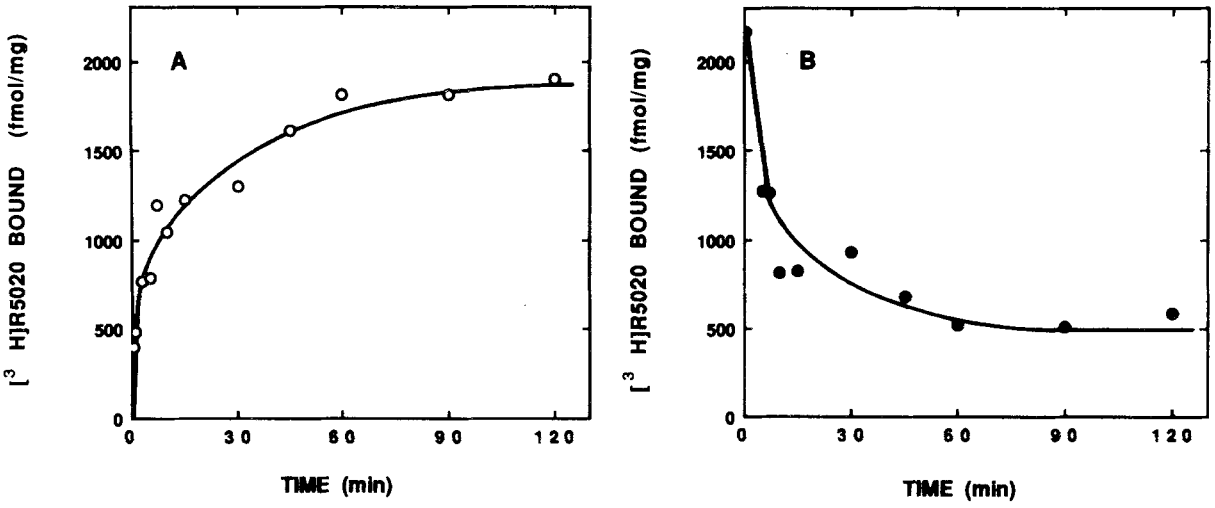

Fig. 1. Association (*A*) and dissociation (*B*) kinetics of 4 nM [^{3}H]R5020 binding to rat liver plasma membrane as a function of the incubation time. In *B*, after 1 hr of preincubation, 25 μl of 4 $\times$ 10^{-4} M cold R5020 was added. Binding was assayed as described in the text.

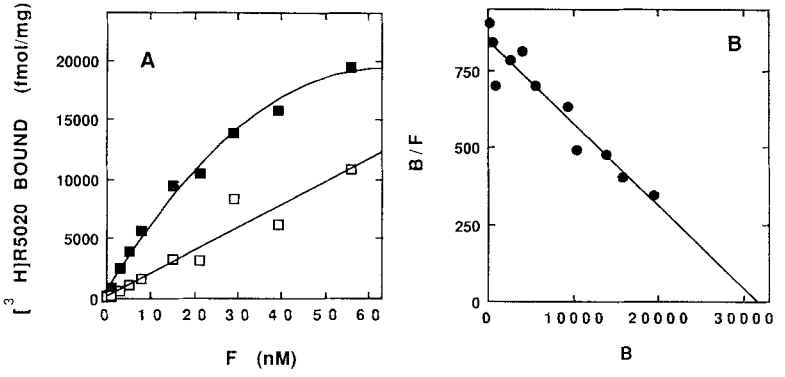

Fig. 2. (*A*) Equilibrium binding of [^{3}H]R5020 to rat liver plasma membrane. Two sets of incubation were carried out simultaneously in the absence and in the presence of unlabeled R5020 10 μM. (■) Specific binding and (□) nonspecific binding. (*B*). Scatchard plot of the specific binding data. *F*: R5020 free (nM); *B*: R5020 bound (fmol/mg of protein).

Results

Displacement of [^{3}H]Cortisol by Photolabile Steroids

In order to characterize cortisol binding to rat liver plasma membranes we first checked the ability of a number of synthetic steroids to compete for the [^{3}H]cortisol binding site. The displacement of 4 nM [^{3}H]cortisol bound to rat liver plasma membrane was carried out in the presence of increasing concentrations of the following unlabeled synthetic steroids: R5020, R1881, RU28362 and RU38486. R5020 was the most effective competitor with an IC_{50} = 288 ± 35 nM (concentration of unlabeled steroid which caused a 50% inhibition of specific binding). R1881 and RU28362, previously employed also as photolabels (Gyorki et al., 1986; Hermann, Schramm & Ghraf, 1987), were less effective in displacing such binding with a 10-fold higher IC_{50}, and the powerful antiglucocorticoid RU38486 showed no affinity by the corticoid binding site.

Characterization of [^{3}H]R5020 Binding to Liver Plasma Membranes

Association and dissociation kinetics (Fig. 1) have been performed with 4 nM [^{3}H]R5020. A saturable binding profile was obtained with 60 min of incubation at 2°C and pH 7.9. The rate data were calculated in the proper form as a linearized function (Weiland & Molinoff, 1981). The values obtained from the first order reaction were k_{+1} = 9.1 $\times$ 10^{-4} nM^{-1} min^{-1} and k_{-1} = 0.02 min^{-1}, and the calculated dissociation constant was K_d = 22 ± 3 nM. The binding was

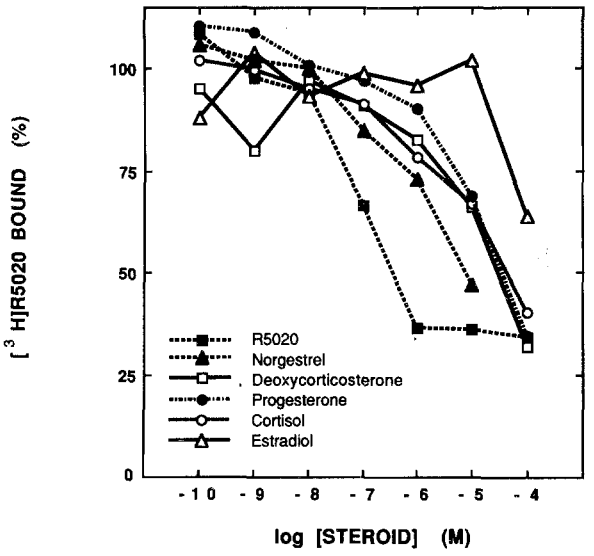

Fig. 3. Displacement of [^{3}H]R5020 bound to rat liver plasma membrane by increasing concentrations of the following steroids: (■) R5020, (□) deoxycorticosterone, (○) cortisol, (●) progesterone, (▲) levonorgestrel and (△) 17β-estradiol. The concentration of [^{3}H]R5020 was 4 nM, and the time of incubation was 1 hr.

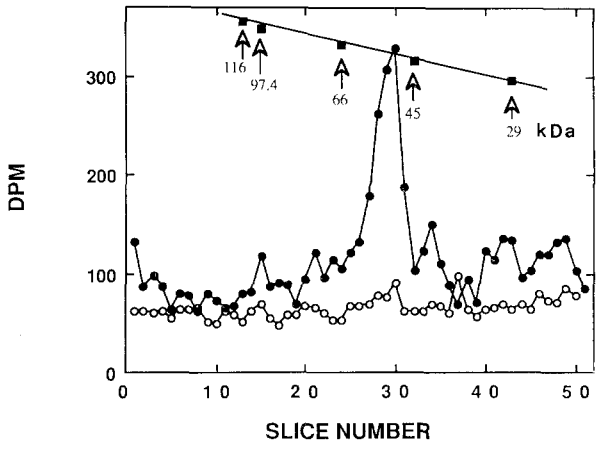

Fig. 4. SDS-PAGE profiles of [^{3}H]R5020 covalently bound to rat liver plasma membrane. Plasma membrane 0.1 mg/ml was incubated with 1 μM [^{3}H]R5020 in the absence (●) and in the presence (○) of unlabeled R5020 100 μM for 60 min in the dark, irradiated for 45 min, centrifuged (14,000 × g, 10 min), resuspended in 100 μl of sample buffer and electrophoresed overnight. The gels were sliced into 2-mm fractions for radioactivity determinations.

also saturable with protein concentrations higher than 0.4 mg/ml (*data not shown*).

The specific binding of [^{3}H]R5020 to plasma membrane (Fig. 2*A*) was maximum to 60 nM of free ligand. Nonspecific binding was determined in the presence of 10 μM cold R5020.

Scatchard (1949) analysis of the binding of [^{3}H]R5020 (Fig. 2*B*) yields a straight line, indicating a single type of binding site. The kinetic parameters determined from this data were: equilibrium dissociation constant K_d = 33 ± 4 nM and maximum capacity of binding B_{max} = 32 ± 2 pmol/mg of protein.

The results obtained from competition studies (Fig. 3) showed that cold R5020 was the most effective molecule to displace the binding with an IC_{50} = 51 ± 4 nM. Other steroids tested (cortisol, progesterone, levonorgestrel and deoxycorticosterone) showed an IC_{50} 100-fold higher, and 17β-estradiol did not displace R5020 from its binding site. From the IC_{50} value using the Chen-Prusoff equation (Weiland & Molinoff, 1981), the K_i = 45 ± 5 nM for unlabeled R5020 can be calculated; this value is very closed to the K_d obtained from the equilibrium studies mentioned above.

Photoaffinity Labeling of Plasma Membranes

The absorbance spectra of R5020 after different times of UV irradiation show a time-dependent decrease in the absorption peak (maximum at 315 nm), which is almost eliminated in 10 min (*data not shown*).

Irradiation at different times of the plasma membrane (preincubated in darkness for 60 min with 1 μM [^{3}H]R5020, 1 mCi/mmol) results in an increase in the radioactivity covalent attached into trichloroacetic acid-precipitable material up to 45 min.

SDS-Polyacrylamide Gel Electrophoresis Analysis

The membranes were photolyzed in the presence of 1 μM [^{3}H]R5020 and then analyzed by SDS-PAGE in slab gels (Fig. 4). A peak of covalently attached radioactivity was found corresponding to an apparent molecular mass of 53 ± 3 kDa which is completely suppressed by a 100-fold molar excess of unlabeled R5020.

Sucrose Density Gradient Analysis

The sedimentation profile of R5020 membrane binding protein is shown in Fig. 5. Specific [^{3}H]R5020 bound was found to be associated with a component in the plasma membrane, sedimenting at 3.6 ± 0.2 Svedberg in relation to ovoalbumin, bovine serum albumin and γ-globulin as markers.

Discussion

The reported studies demonstrate that rat liver plasma membrane contain R5020 binding sites with the characteristics of membrane receptors. These

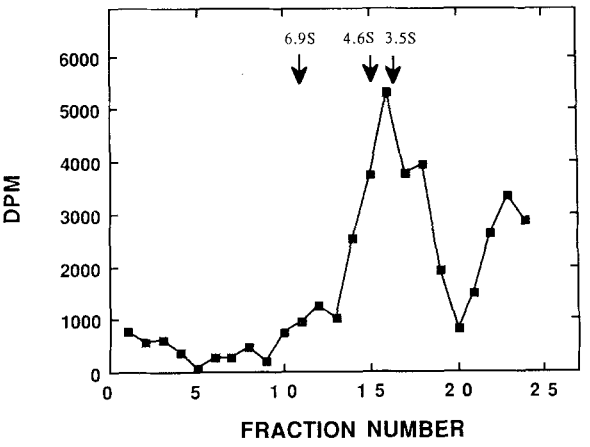

Fig. 5. Sucrose density gradient analysis of solubilized [^{3}H]R5020 covalently bound to rat liver plasma membrane. Plasma membrane 0.1 mg/ml was incubated with 1 μM [^{3}H]R5020 in the absence and in the presence of unlabeled R5020 100 μM for 60 min in the dark, irradiated for 45 min, solubilized with 0.5% CHAPS for 30 min, centrifuged (14,000 × *g* 30 min) and layered on 5–20% (wt/vol) sucrose gradient. Fractions (four drops) were collected for radioactivity determinations. The figure plots specific binding. The arrows show standard proteins: γ-globulin, bovine serum albumin and ovoalbumin.

sites are saturable and specific and have high affinity. Therefore, the use of a photoaffinity label has allowed the identification of this steroid binding protein in the plasma membrane.

Previous works have demonstrated the presence of specific binding sites in membranes for estrogens (Pietras & Szego, 1980; Rambo & Szego, 1983; Berthois et al., 1986; Bression et al., 1986), progestins (Haukkamaa, 1984; Blondeau & Baulieu, 1984, 1985; Sadler & Maller, 1982; Sadler et al., 1985; Yamada et al., 1990) and glucocorticoids (Suyemitsu & Terayama, 1975; Koch et al., 1977, 1978; Pietras & Szego, 1980; Ambellan et al., 1981; Rambo & Szego, 1983; Towel & Sze, 1983; Savart & Cabillic, 1985; Allera & Rao, 1986; Gametchu, 1987; Quelle et al., 1988; Chirino et al., 1989; Guendouz et al., 1989). In our laboratory we have characterized specific steroid binding sites in liver (Trueba et al., 1987, 1989*a,b*, 1990, 1991) and kidney (Ibarrola et al., 1991) plasma membrane. Allera and Rao (1986) have described a glucocorticoid *carrier* in rat liver plasma membrane. Moreover, Sadler and Maller (1982) and Sadler et al. (1985) have shown the existence of a progesterone receptor in *Xenopus* oocyte plasma membrane which causes the release of a second messenger such as cAMP or Ca^{2+}.

In the current work we have utilized [^{3}H]R5020 as photoaffinity label because of its ability to displace [^{3}H]cortisol from its binding site in rat liver plasma membrane; other photoaffinity steroids such as R1881 and RU28362 were less effective, and RU38486 showed no effect on the cortisol binding.

Firstly, the results indicated that R5020 plasma membrane binding was saturable and reversible. From the Scatchard analysis, an equilibrium dissociation constant of 33 ± 4 nM could be obtained. This value is about sixfold higher than the K_d previously found in the case of cortisol and corticosterone binding (Trueba et al., 1989*b*, 1991), but twofold higher for progesterone (Trueba et al., 1990), in plasma membrane. A value of the same order has been reported for the binding of R5020 to cytosolic glucocorticoid receptor (Raynaud, 1977; Nordeen et al., 1981) although values about 0.5–5 nM have been described for the binding of natural steroids.

In order to determine the binding specificity of [^{3}H]R5020 to plasma membrane we tested different types of steroids. The results showed that only unlabeled R5020 was able to displace that binding with high affinity (IC_{50} = 51 ± 4 nM), and natural steroids such as glucocorticoids, mineralocorticoids and progestins had lower affinity. Finally, the estrogen 17β-estradiol did not compete with R5020 for this binding site. The high level of unspecific binding (about 25%) could be due to the greatly R5020 hydrophobic structure.

The experiments carried out using the steroid photoactivation technic showed that R5020 was able to form covalent bonds with its specific binding sites in rat liver plasma membrane; this labeling being saturable with the time of photolysis. After irradiation, only one radioactive peak was detected in denaturing electrophoresis which was not observed in the presence of 100-fold excess of unlabeled steroid.

The SDS-PAGE showed binding to a peptide of about 53 kDa. This value does not correspond to any known cytosolic or nuclear steroid receptor, which has higher molecular sizes. LaCasse, Howel and Lefebvre (1990) have previously demonstrated the labeling of a microsomal binding site with the electrophyllic reagent dexamethasone 21-mesylate. The binding site we found does not correspond to this one since the authors identified the microsomal membrane binding site as a peptide of 45 kDa.

On the other hand, the existence of receptors for corticosteroid binding globulin (CBG) and sex hormone binding globulin on cell membranes has been reported (Hryb et al., 1986; Strel'chyonok & Avvakumov, 1990). However, rat CBG has a close molecular mass and sedimentation coefficient, but this serum protein shows low or no affinity to R5020 (Horwitz & McGuire, 1975; Chan & Slaunwhite, 1977). Moreover, the liver was extensively washed with saline solution prior to membrane extraction in order to avoid nonmembrane adsorbed proteins. CBG also has high affinity to steroids of 21 carbon

atoms (such as cortisol and progesterone), and this R5020 binding site does not. Therefore, levonorgestrel, a synthetic steroid unable to bind to CBG (Srivastava et al., 1983), displaced [^{3}H]R5020 from its binding site (Fig. 4). For all these reasons the binding site appeared different from CBG.

In conclusion, we have found that the plasma membrane steroid binding site of male rat liver appears to be a new type of steroid binder, as arises from the ligand specificity and the limited number of sites of this binding site, as it has been described in the present and previous papers (Trueba et al., 1987, 1989*a*,*b*, 1990, 1991). Moreover, our results demonstrated the presence of a 53-kDa peptide in liver plasma membrane which specifically binds [^{3}H]R5020. The sucrose density gradient analysis has revealed that the plasma membrane protein has a sedimentation coefficient of 3.6 ± 0.2 *S*. This protein could correspond to the binding site previously found in plasma membrane because of the affinity of R5020 to displace [^{3}H]cortisol and could explain some physiological and biochemical effects due to nongenomic action of steroid hormones (Gómez-Muñoz et al., 1989). In subsequent studies in our laboratory, we will attempt to purify this binding site.

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