J. Membrane Biol. 120, 115–124 (1991)
0022263191000382

The Journal of
Membrane Biology

Specific Binding Sites for Corticosterone in Isolated Cells and Plasma Membrane from Rat Liver

Miguel Trueba, Iñaki Ibarrola, Kepa Ogiza, Aida Marino, and José María Macarulla
Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad del País Vasco/Euskal Herriko Unibertsitatea, 48080 Bilbao, Spain

Summary. The specific binding of [^{3}H]corticosterone to hepatocytes is a nonsaturable, reversible and temperature-dependent process. The binding to liver purified plasma membrane fraction is also specific, reversible and temperature dependent but it is saturable. Two types of independent and equivalent binding sites have been determined from hepatocytes. One of them has high affinity and low binding capacity (K_D = 8.8 nM and B_{max} = 1477 fmol/mg protein) and the other one has low affinity and high binding capacity (K_D = 91 nM and B_{max} = 9015 fmol/mg). In plasma membrane only one type of binding site has been characterized (K_D = 11.2 nM and B_{max} = 1982 fmol/mg). As it can be deduced from displacement data obtained in hepatocytes and plasma membrane the high affinity binding sites are different from the glucocorticoid, progesterone nuclear receptors and the Na^+,K^+-ATPase digitalis receptor. Probably it is of the same nature that the one determinate for [^{3}H]cortisol and [^{3}H]corticosterone in mouse liver plasma membrane. Beta- and alpha-adrenergic antagonists as propranolol and phentolamine did not affect [^{3}H]corticosterone binding to hepatocytes and plasma membranes; therefore, these binding sites are independent of adrenergic receptors. The binding sites in hepatocytes and plasma membranes are not exclusive for corticosterone but other steroids are also bound with very different affinities.

Key Words corticosterone · hepatocytes · liver plasma membranes · specific binding sites · glucocorticoids · steroids · receptors

Introduction

Classically, the mechanism of action of steroid hormones includes the following steps: it is generally accepted that the steroid enters the target cell by simple diffusion and combines with a high affinity cytoplasmic receptor. The steroid-protein complex then undergoes activation and is transferred to the nucleus where it binds to selective sites in the chromatin (Edelman, 1975). The interaction between the steroid-receptor complex and the genome leads to a modulation of RNA and specific protein synthesis responsible for the physiological response to the hormone. On the other hand, it is now possible to define the characteristics of any steroid action which represents a nongenomic effect. They are: instantaneous or with a very short lag period and insensitive to RNA and protein synthesis inhibitors. These are direct steroid actions, not mediated through nuclear receptor occupancy (Baulieu et al., 1978). Some of these steroid effects are: anaesthetics (Holzbauer, 1976) and changes on cell excitability (Batra, 1980; Van Wilgenburg, 1982), on cyclic nucleotide levels (Sadler & Maller, 1982; Durant, Duval & Homo, 1983), on extraneuronal uptake (Rascher et al., 1980), on ion (Wehling, Kasmayr & Theisen, 1989), glucose (Livingston & Lockwood, 1975) and nucleoside (Gagne, Homo & Duval, 1980) transport, on lysosomes (Szego & Pietras, 1984) and as regulators of enzyme activities (Ackerman et al., 1981). In our laboratory we have found a short-time glycogenolytic effect in liver after steroid injection that is protein synthesis independent, and it provokes a decrease of cAMP levels with several steroid hormones (Diez et al., 1984; Sancho et al., 1986, 1988; Vallejo et al., 1986; Sanchez-Bueno et al., 1987).

Several nonreceptor-mediated actions can be direct effects of a steroid-plasma membrane interaction. In most of the experiments carried out to study glucocorticoid binding to isolated plasma membranes there is a common feature which supports their existence, distinct from the classical nuclear receptors (Suyemitsu & Terayama, 1975; Koch, 1978; Fant, Yeakley & Harrison, 1983; Omrani et al., 1983; Towle & Sze, 1983; Savart & Cabillic, 1985; Allera & Rao, 1986; Quelle et al., 1988). We have characterized the specific binding sites for cortisol in chicken (Trueba et al., 1987) and mouse (Trueba et al., 1989*b*) liver plasma membranes and corticosterone in mouse liver plasma membranes (Trueba et al., 1989*a*).

Other authors have provided biochemical evidences for the presence of corticosteroid binding globulin (CBG) or a CBG-like binder in various glucocorticoids and progesterone target organs. A CBG-like molecule has also been identified in liver plasma membrane fraction (Suyemitsu & Terayama, 1975). By immunocytochemical visualization some authors have shown the intracellular CBG localization in liver (Perrot-Applanat, David-Ferreira & David-Ferreira, 1981; Kuhn et al., 1986) and pituitary cells (Perrot-Applanat, Racadot & Milgrom, 1984). Recently the existence of specific binding of corticosteroid-binding globulin to cell membranes has been demonstrated (Hryb et al., 1986; Singer, Khan & Rosner, 1988).

We want to help bring to light the complex problem of the extragenomic effect of steroids. The aim of this study was to determine the kinetic parameters of the binding of corticosterone to rat hepatocytes and purified liver plasma membrane fraction. Also we studied the specificity of the binding sites using other natural and synthetic steroids.

Materials and Methods

Chemicals

[1,2,6,7-^{3}H]Corticosterone (75 Ci/mmol) was purchased from Amersham International (Amersham, UK). Collagenase from *Clostridium histolyticum*, 0.15 U/ml, and trypsin soybean inhibitor were supplied by Boehringer (Mannheim, Germany). Albumin fraction V, all steroid molecules, propranolol and HEPES were from Sigma Chemical (St. Louis, MO). RU28362 (11β,17β,-dihydroxy-6-methyl-17α-(1-propynyl)androsta-1,4,6-triene-3-one) and RU38486 (17β-hydroxy-11β,4-dimethylaminophenyl-17α-propynyl-estra-4,9-diene-3-one) were kindly donated by Roussel-Uclaf (Romainville, France). Phentolamine was free provided by Ciba-Geigy Laboratories (Barcelona, Spain). PPO, POPOP and scintillation grade toluene were supplied by Scharlau (Barcelona, Spain). All other reagents were from Merck (Darmstadt, Germany).

Preparation and Incubation of Liver Cells

Isolated hepatocytes were prepared by in vivo perfusion of liver from 200–250 g male Sprague-Dawley rats as described by Shears and Kirk (1984) with slight modifications. The flow rate during the perfusion was maintained at 25 ml/min, and the medium did not contain TES (N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid). The isolated cells were washed three times at room temperature by suspension in the Krebs-Ringer buffer followed by centrifugation at 50 x g for 2 min in a bench centrifuge. After washing, the cells were preincubated for 20 min at 37°C with 2-ml portions of Krebs-Ringer buffer + 10 mM glucose + 2% (wt/vol) bovine serum albumin + 1.25 mM $CaCl_2$. For the assay the buffer was changed by centrifugation and resuspension of the cells in the presence of incubation HEPES buffer (115 mM NaCl + 5.4 mM KCl + 1.8 mM $CaCl_2$ + 0.8 mM $MgCl_2$ + 5 mM glucose + 25 mM HEPES, pH 7.4) to a final concentration of 1.5 $\times$ 10^5 cells/ml. The viability of the cells was checked by the trypan blue test and measurement of the cellular lactate dehydrogenase activity.

Isolation of Plasma Membranes

Livers were perfused and removed from male Sprague Dawley rats weighing about 250 g, homogenized in threefold (wt/vol) excess of 20 mM Tris buffer, pH 7.4, containing 0.25 M sucrose, 1 mM dithiothreitol and 0.01 mM phenyl-methyl-sulfonyl-fluoride. The tissue was homogenized using a Ultra-Turrax T-25 homogenizer by means of three bursts of 15 sec each at 9,500 rpm. The homogenate was separated into cell fractions as described by Maeda, Balakrisnan & Mehdi (1983). After a first centrifugation at 1,500 $\times$ g for 5 min, the pellet was resuspended, homogenized and filtered. The filtrate was layered over 10 ml of a 41% solution of sucrose in the homogenization buffer and centrifuged at 95,000 $\times$ g for 1 hr in a Kontron TST 28.38 swinging bucket rotor. The white interfacial band of membranes was collected, the sucrose was diluted by adding a fourfold excess of the incubation buffer, and the membranes were pelleted by centrifugation at 95,000 $\times$ g for 25 min and suspended in the incubation Tris buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.0 mM glucose and 25 mM Tris/HCl, pH 7.4) for the binding studies.

Purified plasma membranes were stored at −70°C in an ultralow temperature freezer (Selecta Conbatemp model, Barcelona, Spain). Proteins were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Membrane Marker Enzymes

The activity of several specific enzymes has been determined for plasma membrane: 5′-nucleotidase (Aronson & Touster, 1974), ATPase (Mg^{2+}-ATPase + Na^+,K^+-ATPase (Quigley & Gotterer, 1969), and phosphodiesterase I (Aronson & Touster, 1974) as purification criteria of rat liver plasma membrane. Also, the glucose-6-phosphatase (Aronson & Touster, 1974) was assayed as endoplasmic reticulum marker.

Binding Assay of [^{3}H]Corticosterone

Incubation mixtures for binding studies contained 0.1 ml of hepatocyte suspension in HEPES buffer (1.5 $\times$ 10^5 cells/ml final concentration) or 0.1 ml of purified plasma membrane fraction in Tris buffer (0.2 mg protein/ml final concentration), 0.1 ml of [^{3}H]corticosterone at different concentrations, and 0.8 ml of different incubation buffers. The presence of specific corticosterone binding sites was determined by the difference between the radioactivity bound to the hepatocytes or membranes incubated only with [^{3}H]corticosterone (total binding) and the radioactivity bound in the presence of an excess of unlabeled deoxycorticosterone or corticosterone (nonspecific binding), respectively. The incubations were carried out in ice bath for 2 hr with hepatocytes and plasma membranes. The viability of cells was always higher than 80%.

Incubation with plasma membrane was performed in an ice bath. At the end of the incubation period, triplicate 800-μl aliquots were filtered through GF/C fiber filter (Whatman, England) placed in a 12-place filter manifold (Millipore). Filters were immediately

Table 1. Marker enzyme analysis of rat liver plasma membranes[a]

	Homogenate	Plasma membranes
5'-Nucleotidase[b]	2.3 ± 0.5	21.1 ± 3.4
Total ATPase[b]	3.4 ± 0.3	19.1 ± 2.8
Phosphodiesterase I[c]	1.0 ± 0.2	8.5 ± 0.6
Glucose-6-phosphatase[b]	2.8 ± 0.8	6.6 ± 1.4

[a] Rat liver tissue was homogenized as described in Materials and Methods. Plasma membranes were prepared by the Maeda et al. (1983) technique. Marker enzymes were analyzed in the crude homogenate and in the membrane preparations. Values are presented as mean ± SE of triplicate determinations.
[b] μmol of inorganic phosphate releasing/mg protein per hour.
[c] μmol of *p*-nitrophenol release/mg protein per hour.

washed with 10 ml of iced incubation buffer without glucose. The dried filters were placed in vials and counted in 5 ml of scintillation cocktail (4 g PPO, 0.05 g POPOP, 1 liter toluene) in a Packard Tricarb 2000 CA model with an efficiency of 65%.

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

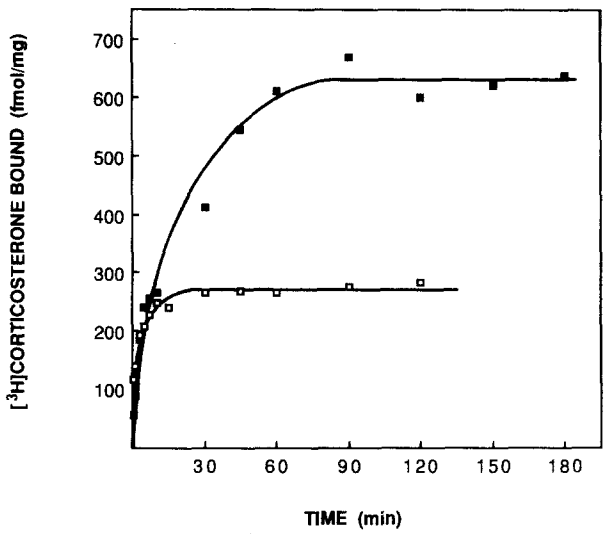

Fig. 1. Time course of the association of [^{3}H]corticosterone using rat hepatocytes (■) and liver plasma membranes (□) at 2°C. Hepatocytes (1.5 × 10^5 cells/ml) and membranes (0.2 mg of protein/ml) were incubated with 4 nM [^{3}H]corticosterone in HEPES and Tris buffers, pH 7.4, respectively. The points are the mean ± SEM of quadruplicate determinations

Results

Plasma Membrane Fraction Purity

The purity of rat liver plasma membrane fragments has been checked by means of marker enzyme analysis. The results are summarized in Table 1. The specific activities of marker enzymes for rat liver plasma membrane were much enhanced in this fraction. On the other hand, the glucose-6-phosphatase activity determined in plasma membrane was much lower than that found in microsomes (61.2 μmol of inorganic phosphate release/mg protein per hour).

Association and Dissociation Kinetics

These experiments have been performed with 4 nM [^{3}H]corticosterone, according to previous studies (Trueba et al., 1989*a*,*b*) and 1.5 × 10^5 cells/ml of hepatocytes or 0.2 mg protein/ml of membranes as final concentrations.

First, we have determined that [^{3}H]corticosterone binding in function of protein concentration (from 0.05 to 1.00 mg/ml) is a lineal process until 0.5 mg/ml for hepatocyte and plasma membrane systems. Thus, we have chosen the protein concentration mentioned above. HEPES buffer has been used for the [^{3}H]corticosterone-hepatocyte binding study because it assures a larger survival of the cells (>85%) at the end of the incubation. For membranes the highest binding was obtained with Tris/HCl + cations buffer (Trueba et al., 1987); therefore, this buffer was chosen for the following experiments.

The binding of [^{3}H]corticosterone to plasma membrane fractions occurred rapidly at 2°C and pH 7.4 (Fig. 1). The uptake to hepatocytes was slower, 2.4-fold greater than the membrane, and reached the saturation at 60 min of incubation and remained constant to 180 min. Membrane binding was complete in 10 min and remained unaltered for 120 min. The dissociation kinetics showed a biphasic aspect with a speed and a slow component, reaching the maximum displacement at 2 hr in both systems (Fig. 2). From association and dissociation plots, by means of a logarithmic transformation (Weiland & Molinoff, 1981) the following parameters can be calculated: k_{+1} = 0.62 × 10^6 M^{-1} × min^{-1}, k_{-1} = 6.07 × 10^{-3} min^{-1} and k_{+1} = 0.7 × 10^6 M^{-1} × min^{-1}, k_{-1} = 6.38 × 10^{-3} min^{-1} for hepatocyte and membrane systems, respectively. The K_D values obtained from these parameters are: 2.0 and 9.0 nM for hepatocytes and membrane, respectively. These results mean that high affinity binding sites exist in both systems.

Binding as a Function of [^{3}H]Corticosterone Concentration

The kinetic parameters of corticosterone binding to both plasma membrane and hepatocytes have been determined using the Scatchard analysis (Scatchard,

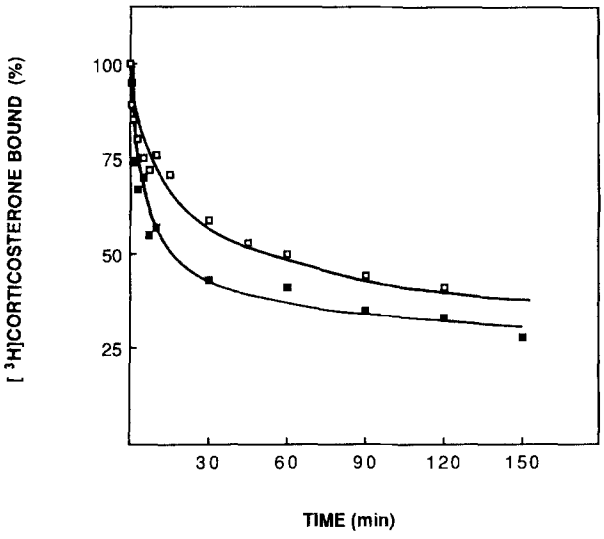

Fig. 2. Time course of the dissociation of [³H]corticosterone from hepatocytes in the presence of 10 μM of deoxycorticosterone (■) and from liver plasma membranes in the presence 10 μM of corticosterone (□). Hepatocytes (1.5×10^5 cells/ml) and membranes (0.2 mg/ml) were incubated with 4 nM [³H]corticosterone at 2°C in HEPES and Tris buffers, pH 7.4, respectively. The points are the mean ± SEM of triplicate determinations

1949). The incubated samples contained [³H]corticosterone at different final concentrations, from 0.4 to 25 nM. To determine unspecific binding, 10 μM of deoxycorticosterone or corticosterone were used as displacers for hepatocyte and membrane, respectively.

In experiments with hepatocytes it was necessary to differentiate between the uptake of corticosterone into the cell and the steroid bound to membrane and other subcellular structures. Thus, two different series have been made simultaneously. In one the samples were frozen in liquid nitrogen after 2 hr of incubation. The cells were broken, as it was corroborated by optical microscopy, and the [³H]corticosterone trapped into cytosol was liberated by ultrafiltration. In the other one the samples were filtered without freezing. The binding values obtained from the unfrozen series were 15% higher than those from frozen series. This percentage could be considered as the free corticosterone trapped into the cytosol. In Fig. 3*A* it is observed that the specific binding of the frozen and broken cells series does not reach the saturation. From the Scatchard plot (Fig. 3*B*) two different sites can be determined. The kinetic parameters were calculated by the least-squares method. The high affinity site has a K_D = 8.8 nM and B_{max} = 1477 fmol/mg protein and the low affinity one has a K_D = 91 nM and B_{max} = 9015 fmol/mg protein. Binding for plasma membrane is a saturant process (Fig. 4*A*) and only one type of reversible binding site for corticosterone can be determinate from the Scatchard plot (Fig. 4*B*). A maximum binding of 1982 fmol/mg protein with a dissociation constant, K_D, of 11.2 nM was reached. From the binding data the calculated Hill coefficient was 0.985.

Stereospecificity of Corticosterone Binding

The specificity of the [³H]corticosterone binding sites for hepatocytes and plasma membranes was determined using different steroids that competed for the same binding site. Glucocorticoids and other steroids have been assayed in concentrations that ranged from 10^{-10} to 10^{-4} M (Figs. 5–7).

To be able to compare the different affinities, we have calculated the IC_{50} value, which indicates the concentration of steroid competitor that cause 50% of displacement of [³H]corticosterone taking this one alone at 100% (Table 2). In this table it is observed that from all the steroids assayed, using either hepatocytes or membranes, only cortisol, deoxycorticosterone, progesterone, testosterone, aldosterone, prednisolone, norgestrel and 20β-hydroxyprogesterone displace with higher or lower affinity the [³H]corticosterone from its binding site. Other steroids, as estradiol, dexamethasone, triamcinolone acetonide, RU38486 and ouabain, have no affinity for the binding site (Figs. 5–7).

Influence of Adrenergic Antagonist on [³H]Corticosterone Binding

As possible displacers of [³H]corticosterone from its site, alpha- and beta-adrenergic antagonists were investigated. Final concentrations of propranolol and phentolamine between 0.1 nM to 10 μM were assayed for hepatocytes and plasma membranes (Fig. 8). As it is observed in the figure neither propranolol nor phentolamine displaced significantly the [³H]corticosterone bound to hepatocytes or purified plasma membrane fraction.

Discussion

Our results demonstrated the presence of specific binding sites for corticoids in rat hepatocytes and liver plasma membranes. This binding is reversible and temperature dependent for both systems and it is saturable only with plasma membranes.

The presence of enzymes that metabolize corti-

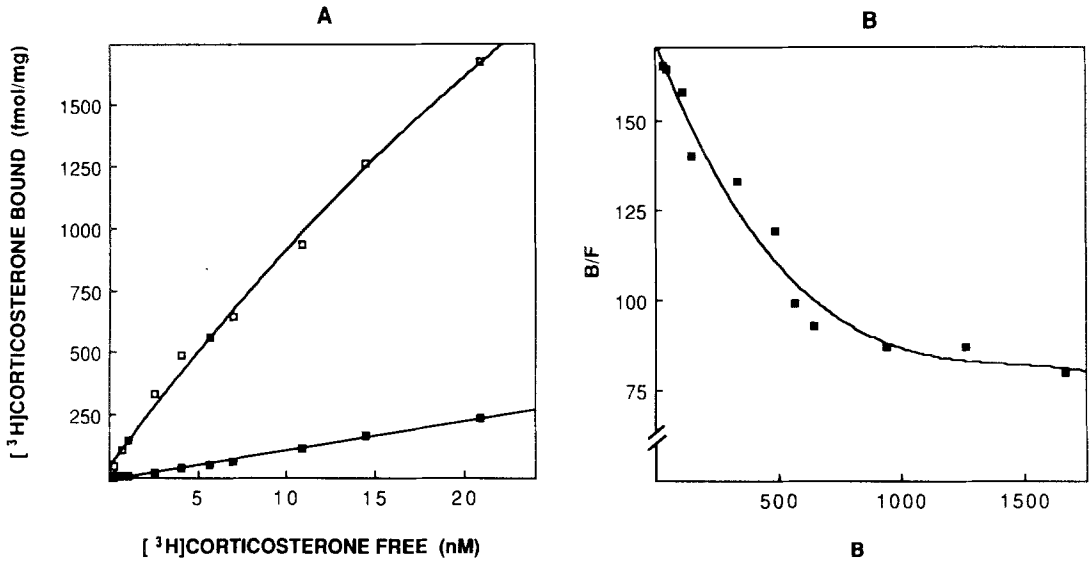

Fig. 3. (*A*) Binding of [^{3}H]corticosterone as a function of radioactive steroid concentration (0.4–25 nM) to rat hepatocytes at 2°C in HEPES buffer, pH 7.4. Two sets of incubation were carried out simultaneously with the same cells (1.5×10^5 cells/ml) in the presence and absence of deoxycorticosterone 10 μM for 2 hr. After incubation all the samples were frozen in liquid nitrogen and thawed afterwards. Specific binding (□) and nonspecific binding (■). Points indicate the means ± SEM of quadruplicate determinations. (*B*) Scatchard analysis of the specific binding data. Kinetic parameters were calculated by the least-squares method. The correlation values obtained were 0.98 and 0.94. *B*, [^{3}H]corticosterone bound (fmol/mg protein); *F*, [^{3}H]corticosterone free (nM)

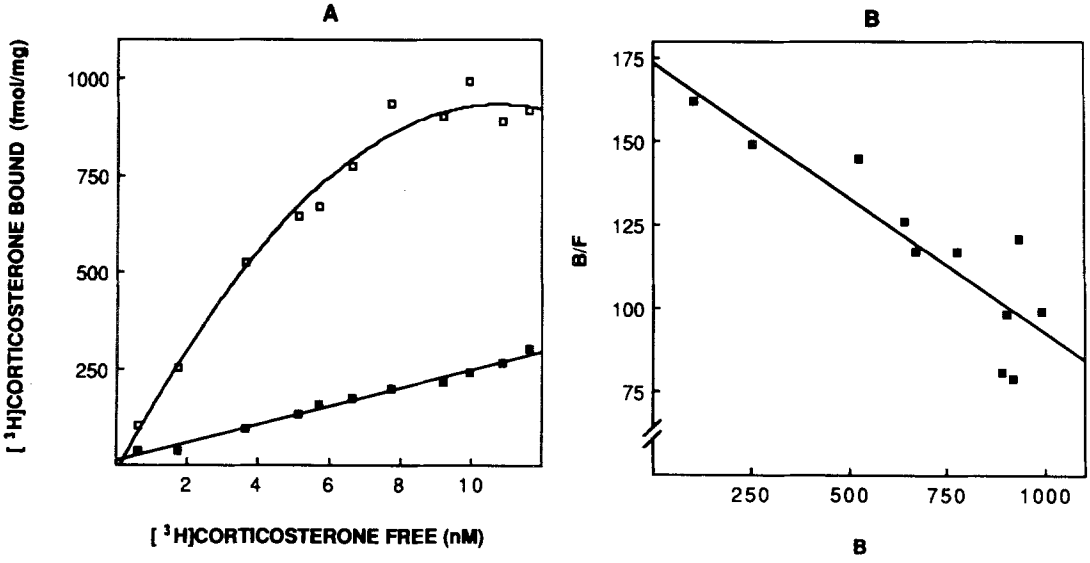

Fig. 4. (*A*) Binding of [^{3}H]corticosterone to rat liver plasma membranes in function of radioactive steroid concentration (0.4–25 nM) at 2°C in Tris buffer, pH 7.4. Two sets of incubation were carried out simultaneously with the same plasma membranes (0.2 mg/ml) in the presence and absence of corticosterone 10 μM for 2 hr. Specific binding (□) and nonspecific binding (■). Points indicate the mean ± SEM of quadruplicate determinations. (*B*) Scatchard plot of the specific binding data of *A* obtained by least-squares method (r = 0.985). *B*, [^{3}H]corticosterone bound (fmol/mg protein); *F*, [^{3}H]corticosterone free (nM)

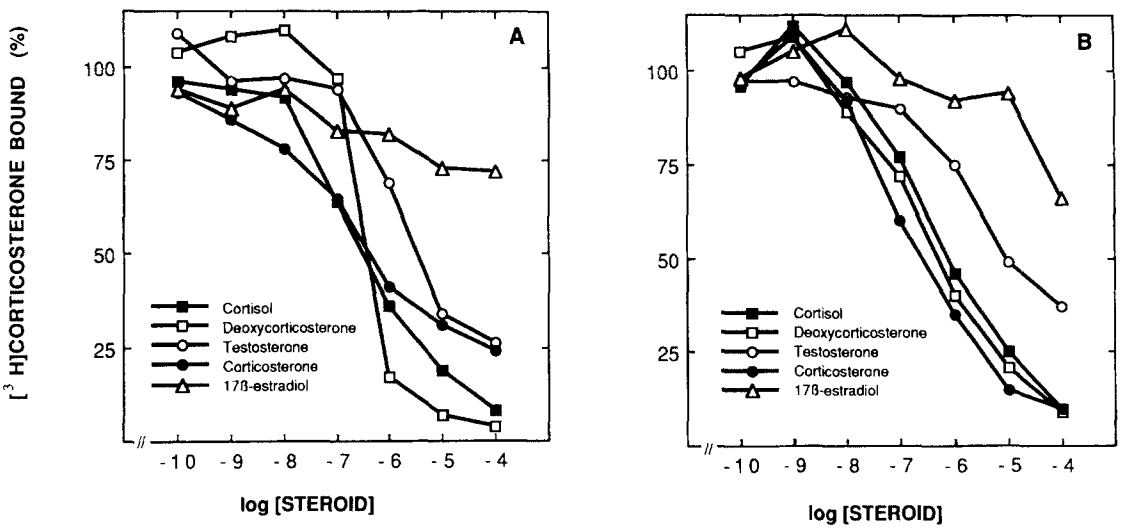

Fig. 5. Influence of various steroids on the binding of [^{3}H]corticosterone to rat hepatocytes (*A*) and liver plasma membranes (*B*). Hepatocytes (1.5×10^5 cells/ml) and membranes (0.2 mg protein/ml) were incubated for 2 hr at 2°C with 4 nM [^{3}H]corticosterone in HEPES and Tris buffers, pH 7.4, respectively. The incubations were carried out in the absence (100% of binding) and presence of different concentrations (0.1 nM–0.1 mM) of steroids. The points are the mean ± SEM of quadruplicate determinations

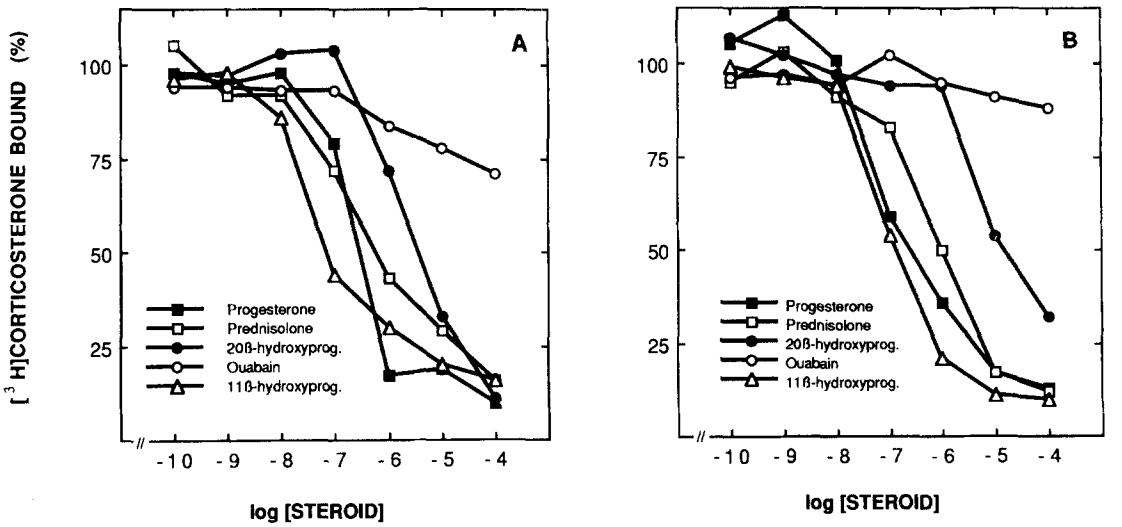

Fig. 6. Influence of various steroids on the binding of [^{3}H]corticosterone to rat hepatocytes (*A*) and liver plasma membranes (*B*). Conditions of incubation were similar to those described in Fig. 5

costeroids cannot explain this binding because these enzymes can only be found in microsomes and sarcoplasmic reticulum fractions; the data obtained from binding studies and marker enzyme analysis confirm our results. The physiological role of steroid occurs in a limited range of concentrations and the uptake can only occur with high affinity receptors (Duval, Durant & Homo-Delarche, 1983). It has been also described that the steroid penetrate into target cells by specific carrier mechanisms (Rao, 1981; Szego & Pietras, 1981; Allera & Rao, 1986). On the other hand, binding sites or receptors for glucocorticoids have been reported for liver plasma membranes (Suyemitsu & Terayama, 1975; Allera, Rao & Breuer, 1980; Trueba et al., 1987; Quelle et al., 1988; Guendouz et al., 1988; Trueba et al., 1989*b*), pituitary gland (Koch et al., 1978; Koch, Sakly & Lutz-Butcher, 1981), skeletal muscle (Savart & Cabillic, 1985) and *Xenopus* oocytes (Sadler & Maller, 1982; Sadler, Bower & Maller, 1985).

The [^{3}H]corticosterone-hepatocyte binding shows a pattern for two independent types of sites.

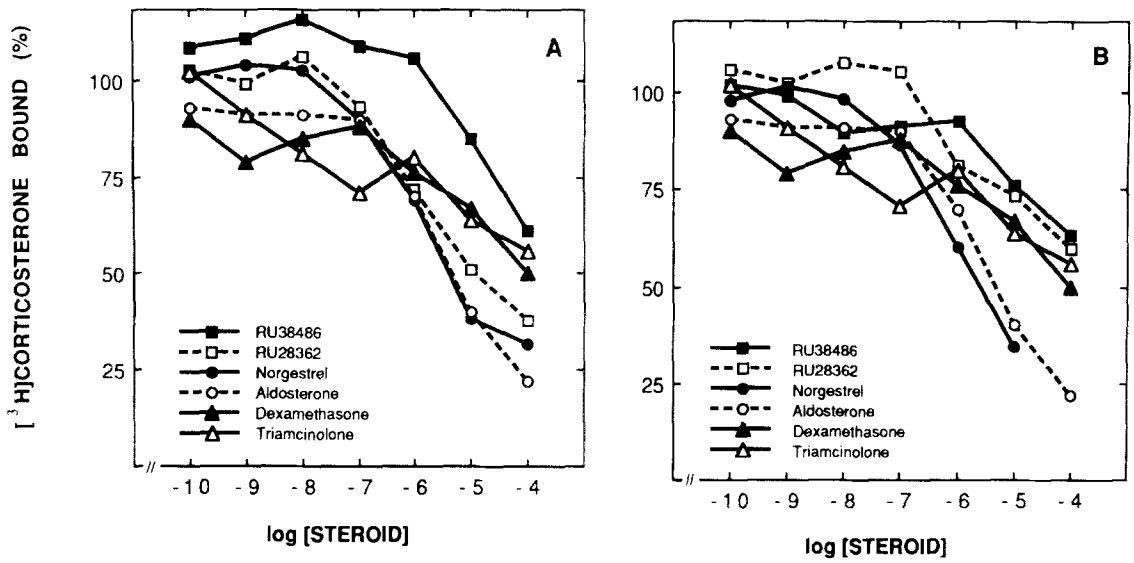

Fig. 7. Influence of various steroids on the binding of [^{3}H]corticosterone to rat hepatocytes (*A*) and liver plasma membranes (*B*). Conditions of incubation were similar to those described in Fig. 5

Table 2. Competition for [^{3}H]corticosterone binding sites in plasma membranes and hepatocytes of various unlabeled steroids

Unlabeled steroid	IC_{50} (nM)	
	Membranes	Hepatocytes
11β-Hydroxyprogesterone	128	209
Progesterone	243	355
Corticosterone	256	407
Deoxycorticosterone	496	339
Cortisol	676	245
Prednisolone	985	549
Norgestrel	3162	4677
Aldosterone	6046	4890
Testosterone	9087	3890
20β-Hydroxyprogesterone	15150	4570
RU28362	not displaced	4677

Results are presented as competitor concentration that displaces the 50% of the [^{3}H]corticosterone binding which was taken as 100% (IC_{50}). Plasma membranes from liver were incubated with 4 nM [^{3}H]corticosterone alone or in the presence of different concentrations of unlabeled steroids. The results are the mean of quadruplicate determinations.

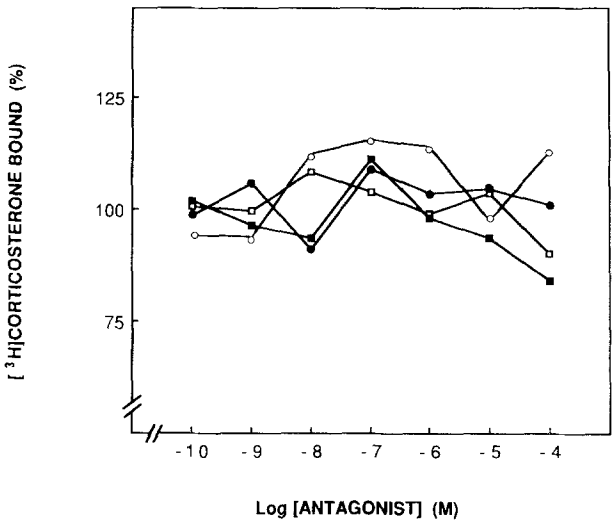

Fig. 8. [^{3}H]corticosterone binding to rat hepatocytes (open symbols) and liver plasma membranes (filled symbols) in presence of phentolamine (○, ●) and propranolol (□, ■). Conditions of incubation were similar to those described in Fig. 5, but in the presence of different concentrations (0.1 nM–0.1 mM) of adrenergic antagonists. The points are the mean ± SEM of triplicate determinations

The first site has high affinity and low capacity and the second one has low affinity and high capacity of binding. In contrast, the model for [^{3}H]corticosterone-plasma membrane interaction corresponds to only one independent and equivalent site. We think that this site in the membrane and the one of high affinity in hepatocytes are the same. This hypothesis can be confirmed because the high affinity dissociation constants and the maximum capacity of binding obtained are similar in both systems. The presence of the two kinds of binding sites in hepatocytes could be explained by the fact that [^{3}H]corticosterone can also be bound to other subcellular membrane structures.

From competition experiments with other steroids, it is necessary to mention that estradiol, dexa-

methasone, triamcinolone acetonide, ouabain and RU38486 do not compete for these binding sites in both systems. RU28362 show a different effect in membrane (do not displace) and hepatocytes (displace with low affinity). RU38486 and RU28362 are specific antagonists for cytosolic glucocorticoid receptor (Moguilewsky & Philibert, 1984; Groyer et al., 1985; Hermann, Schramm & Ghraf, 1987); therefore, we can infer that the binding site is different from the nuclear estrogen receptor, the nuclear glucocorticoid receptor and the Na^+,K^+-ATPase digitalis receptor (Labella et al., 1985).

From the displacement studies of [^{3}H]corticosterone bound to plasma membrane, we have determined the affinity order for the competitor steroids based on the IC_{50} values (Table 2). This order decreases as follows: 11β-hydroxyprogesterone > progesterone, corticosterone > deoxycorticosterone, cortisol > prednisolone > norgestrel > aldosterone, testosterone > 20β-hydroxyprogesterone. Thus, our binding site binds progestin and corticoids preferentially, on the contrary, the nuclear progesterone receptor has no affinity by glucocorticoids and has high affinity to RU38486 (Groyer et al., 1985). Therefore, we can say that this corticoid binding site is different from the nuclear progesterone receptor. In the same way, it is different to CBG because of norgestrel does not bind this serum protein (Qi-Gui & Hümpel, 1990). The relatively high affinity of progesterone and progesterone derivatives towards the plasma membrane binding site is related to its structural similarity with natural glucocorticoids (common structure: 3-oxo-4-ene).

Our data are according with those found by Suyemitsu & Terayama (1975), Koch (1978), Koch et al. (1978), Savart & Cabillic (1985), Trueba et al. (1989*a*,*b*), and prove that this specific binding site is not exclusive for corticosterone and shows preference by progestins and glucocorticoids.

The dissociation constant for high affinity corticosterone binding to membranes (11.2 nM) and hepatocytes (8.8 nM) are similar to that calculated from the association and dissociation kinetics (9.0 and 9.8 nM, respectively) which demonstrate the validity of the methodology employed. This K_D value is of the same order of magnitude that the one obtained by other authors with steroids and membranes (Suyemitsu & Terayama, 1975; Koch et al., 1978; Savart & Cabillic, 1985; Trueba et al., 1987, 1989*b*). Based on K_D and B_{max} values and steroid affinities we can say that this high affinity site determined for [^{3}H]corticosterone is possibly the same that was characterized previously with cortisol (Trueba et al., 1989*b*) and corticosterone in mouse liver (Trueba et al., 1989*a*).

On the other hand, the existence of a plasma membrane steroid hormone receptor has been reported (Sadler & Maller, 1982; Blondeau & Baulieu, 1984). The binding of the steroid to that receptor or binding site, in our case, could cause the release of a second messenger such as Ca^{2+} into the intracellular medium, since we have already described in our system that the early glycogenolytic effect is independent of the cAMP levels (Diez et al., 1984; Sancho et al., 1986, 1988; Vallejo et al., 1986; Sanchez-Bueno et al., 1987).

Furthermore, it has been demonstrated that estradiol activates methylating enzymes involved in the conversion of phosphatidylethanolamine to phosphatidylcholine in membranes (Drouva et al. 1986), and we have found an increased phospholipid methylation and glycogen phosphorylase activation after a single dose of dexamethasone in mouse liver (Marino et al., 1988).

The specific binding site determined in plasma membrane is different from the recently characterized binding of corticosteroid binding globulin to rat cell membranes (Singer et al., 1988). This binding was a little specific at 4°C, and maximal binding was obtained at 37°C. The kinetic parameters were K_D = 0.84 μM and a binding capacity of 39 pmol/mg membrane protein. Thus, the K_D and B_{max} are 100- and 2-fold higher than that obtained for the corticoid binding sites in this work.

Also, Su, London and Jaffe (1988) have identified specific σ binding sites in mammalian brain and lymphoid tissue. The findings suggest that interactions of progesterone and other steroids with σ receptors may constitute an important link between the endocrine, immune and central nervous systems, as for instance, in the anti-inflammatory action.

Recent studies suggest the presence of specific glucocorticoid binding sites on rat liver microsomal membranes. Chromatography on DEAE Trisacryl and heparin Ultrogel confirmed that the solubilized protein is different from CBG and the nuclear glucocorticoid receptor (Guendouz et al., 1989). Their binding site, according to K_D and competition experiments with steroids, coincide with ours obtained from purified plasma membrane fraction.

Our results show a plasma membrane corticoid binding site that may play a role in glucocorticoid hormone action. For instance, a defect or complete loss of this steroid binding site may be the cause of steroid resistance and/or loss of response to steroid hormones already known, in the presence of functionally intact nuclear receptors.

This work was supported by grants PB85-0461 from the "Comisión Asesora de Investigación Científica y Técnica", PGV-8612 from the "Departamento de Educación, Universidades e Investigación del Gobierno Vasco" and from "Iberduero, S.A."

References

Ackerman, G.E., McDonald, P.C., Gudelsky, G., Mendelson, C.R., Simpson, E.R. 1981. Potentiation of epinephrine-induced lipolysis by catechol estrogens and their methoxy derivates *Endocrinology* **109:**2084–2088

Allera, A., Rao, G.S. 1986. Characteristics and specificity of glucocorticoid "carrier" of rat liver plasma membrane. *In*: Steroid Hormone Resistance. Mechanisms and Clinical Aspects. G.P. Chrousos, D.L. Loriaux, and M.B. Lipsett, editors. pp. 53–65. Plenum, New York

Allera, A., Rao, G.S., Breuer H. 1980. Specific interaction of corticosteroids with component of the cell membrane which are involved in the translocation of the hormone into the intravesicular space of purified rat liver plasma membrane vesicles. *J. Steroid Biochem.* **12:**259–266

Aronson, N.N., Touster, O. 1974. Isolation of rat liver plasma membrane fragments in isotonic sucrose. *Methods Enzymol.* **31:**90–102

Batra, S. 1980. Strogen and smooth-muscle function. *Trends Pharmacol. Sci.* **1:**388–391

Baulieu, E.E., Godeau, F., Schorderet, M., Schorderet-Slathrine, S. 1978. Steroid-induced meiotic division in *Xenopus* oocytes surface and calcium. *Nature (London)* **275:**593–598

Blondeau, J.P., Baulieu, E.E. 1984. Progesterone-receptor characterized by photoaffinity-labeling in the plasma-membrane of *Xenopus laevis* oocytes. *Biochem. J.***219:**785–792

Díez, A., Sancho, M., Egaña, M., Trueba, M., Marino, A., Macarulla J.M. 1984. An interaction of testosterone with cell membranes. *Horm. Metab. Res.* **16:**475–477

Drouva, S.V., Laplante, E., Lablanc, P., Bechet, J.J., Clauser, H., Kordon, C. 1986. Estradiol activates methylating enzyme(s) involved in the conversion of phosphatidylethanolamine to phosphatidylcholine in rat pituitary membranes. *Endocrinology* **119:**2611–2622

Durant, S., Duval, D., Homo, F. 1983. Potentiation by steroids of the β-adrenergic agent-induced stimulation of cyclic AMP in isolated mouse thymocytes. *Biochim. Biophys. Acta* **762:**315–324

Duval, D., Durant, S., Homo-Delarche, F. 1983. Non genomic effects of steroids. Interactions of steroids molecules with membrane structure and function. *Biochim. Biophys. Acta* **737:**409–442

Edelman, I.S. 1975. Mechanism of action of steroid-hormones. *J. Steroid Biochem.* **6:**147–159

Fant, M.E., Yeakley, J., Harrison, R.W. 1983. Evidence for carrier-mediated transport of glucocorticoids by human placental membrane-vesicles. *Biochim. Biophys. Acta* **731:**415–420

Gagne, D., Homo, F., Duval, D. 1980. Steroid-induced inhibition of nucleoside uptake in isolated mouse thymocytes. *Biochim. Biophys. Acta* **603:**27–35

Groyer, A., Le Bouc, Y., Joab, I., Radanyi, C., Renoir, J.M., Robel, P., Baulieu, E.E. 1985. Chick oviduct glucocorticosteroid receptor. Specific binding of the synthetic steroid RU 486 and immunological studies with antibodies to chick oviduct progesterone receptor. *Eur. J. Biochem.* **149:**445–451

Guendouz, F., Blanchardie, P., Denis, M., Orsonneau, J.L., Lustenberger, P. 1989. Occurrence of glucocorticoid binding sites in solubilized microsomes from rat liver. *J. Steroid Biochem.* **34:**325–330

Hermann, T., Schramm, K., Ghraf, R. 1987. Photoaffinity labeling with [^{3}H]RU28362: A powerful tool for the study of rat brain glucocorticoid receptors. *J. Steroid Biochem.* **26:**417–423

Holzbauer, M. 1976. Physiological aspects of steroids with anesthetic properties. *Med. Biol.* **54:**227–242

Hryb, D.J., Khan, M.S., Romas, N.A., Rosner, W. 1986. Specific binding of human corticosteroid-binding globulin to cell membranes. *Proc. Natl. Acad. Sci. USA* **83:**3253–3256

Koch, B. 1978. Inverse effects of corticosterone and thyroxine on glucocorticoid binding site in the anterior pituitary gland. *Endocrinol. Acta* **88:**29–37

Koch, B., Lutz-Butcher, B., Briaud, B., Mialhe, C. 1978. Specific interaction of corticosteroids with binding sites in plasma membranes of rat anterior pituitary gland. *J. Endocrinol.* **79:**215–222

Koch, B., Sakly, M., Lutz-Butcher, B. 1981. Modulation by transcortin-like binding-sites of uptake and distribution of glucocorticoid by dispersed pituitary cells. *Clin. Endocrinol.* **22:**169–178

Kuhn, R., Green, A., Raymoure, W., Siiteri, P. 1986. Immunocytochemical localization of corticosteroid-binding globulin in rat tissues. *J. Endocrinol.* **108:**31–36

Labella, F.S., Bihler, I., Templeton, J., Kim, R.S., Hnatowich, M., Rohrer, D. 1985. Progesterone derivatives that bind to the digitalis receptor: Effects on Na^+,K^+-ATPase and isolated tissues. *Fed. Proc.* **44:**2806–2811

Livingston, J.N., Lockwood, D.H. 1975. Effect of glucocorticoids on glucose-transport system of isolated fat-cells. *J. Biol. Chem.* **250:**8353–8360

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275

Maeda, T., Balakrishnan, K., Mehdi, S.Q. 1983. A simple and rapid method for the preparation of plasma membranes. *Biochim. Biophys. Acta* **731:**115–120

Marino, A., Bartolomé, B., Trueba, M., Sancho, M.J., Macarulla, J.M. 1988. Increased phospholipid methylation and glycogen phosphorylase activation after a single dose of dexamethasone in mouse liver. *Exp. Clin. Endocrinol.* **92:**314–322

Moguilewsky, M., Philibert, D. 1984. RU38486: Potent antiglucocorticoid activity correlated with strong binding to the cytosolic glucocorticoid receptor followed by an impaired activation. *J. Steroid Biochem.* **20:**271–276

Omrani, G.R., Furukawa, H., Sherwood, J.A., Loeb, J.N. 1983. [^{3}H] labeled dexamethasone binding by rat liver microsomes. Effects of age, sex and adrenal status. *Endocrinology* **112:**178–186

Perrot-Applanat, M., David-Ferreira, J.F., David-Ferreira, K.L. 1981. Immunocytochemical localization of corticosteroid binding globulin (CBG) in guinea pig hepatocytes. *Endocrinology* **109:**1625–1633

Perrot-Applanat, M., Racadot, O., Milgrom, E. 1984. Specific localization of plasma corticosteroid-binding globulin immunoreactivity in pituitary corticotrophs. *Endocrinology* **115:**559–569

Qi-Gui, L., Hümpel, M. 1990. Serum protein binding characteristics of cyproterone acetate, gestodene, levonorgestrel and norethisterone in rat, rabbit, dog, monkey and man. *J. Steroid Biochem.* **35:**319–326

Quelle, F.W., Smith, R.V., Hrycyna, C.A., Kaliban, T.D., Crooks, J.A., O'Brien, J.M. 1988. [^{3}H]Dexamethasone binding to plasma membrane-enriched fractions from liver of nonadrenalectomized rats. *Endocrinology* **123:**1642–1651

Quigley, J.P., Gotterer, G.S. 1969. Distribution of (Na^+,K^+)

stimulated ATPase activity in rat intestinal mucosa. *Biochim. Biophys. Acta* **173**:456–469

Rao, G.S. 1981. Mode of entry of steroid and thyroid hormones into cells. *Mol. Cell. Endocrinol.* **21**:97–108

Rascher, W., Dietz, R., Schomig, A., Burkart, G., Gross, F. 1980. Reversal of corticosterone-induced super-sensitivity of vascular smooth-muscle to noradrenaline by arachidonic-acid and prostacyclin. *Eur. J. Pharmacol.* **68**:267–273

Sadler, S.E., Bower, M.A., Maller, J.L. 1985. Studies of a plasma membrane steroid receptor in *Xenopus* oocytes using the synthetic progestin RU 486. *J. Steroid Biochem.* **22**:419–426.

Sadler, S.E., Maller, J.L. 1982. Identification of a steroid receptor on the surface of *Xenopus* oocytes by photoaffinity labeling. *J. Biol. Chem.* **257**:355–361

Sanchez-Bueno, A., Sancho, M.J., Trueba, M., Macarulla, J.M. 1987. An early effect of estradiol at hepatic level, previous to its protein synthesis activation. *Int. J. Biochem.* **19**:93–96

Sancho, M.J., Egaña, M., Trueba, M., Marino, A., Macarulla, J.M. 1986. Two independent effects of cortisol in chicken liver. *Exp. Clin. Endocrinol.* **88**:249–255

Sancho, M.J., Gómez-Muñoz, A., Sánchez-Bueno, A., Trueba, M., Marino, A. 1988. Glycogen phosphorylase activation by progesterone in liver. *Exp. Clin. Endocrinol.* **92**:154–160

Savart, M., Cabillic, Y. 1985. Specific binding of dexamethasone to plasma membranes from skeletal muscle. *Biochim. Biophys. Acta* **813**:87–95

Scatchard, G. 1949. The attraction of proteins for small molecules and ions. *Ann. NY Acad. Sci.* **51**:660–672

Shears, S.B., Kirk, C.J. 1984. Characterization of a rapid cellular-fractionation technique for hepatocytes. *Biochem. J.* **219**:375–382

Singer, C.J., Khan, M.S., Rosner, W. 1988. Characteristics of the binding of corticosteroid-binding globulin to rat cell membranes. *Endocrinology* **122**:89–96

Su, T.P., London, E.D., Jaffe, J.H. 1988. Steroid binding at σ receptors suggest a link between endocrine, nervous and immune systems. *Science* **240**:219–221

Suyemitsu, T., Terayama, H. 1975. Specific binding sites for natural glucocorticoids in plasma membranes of rat liver. *Endocrinology* **96**:1499–1508

Szego, C.M., Pietras, R.J. 1981. Membrane recognition and effector sites in steroid hormone action. *In*: Biochemical Actions of Hormones. G. Litwack, editor. Vol. 8, pp. 307–463. Academic, New York

Szego, C.M., Pietras, R.J. 1984. Lysosomal functions in cellular activation: Propagation of the action of hormones and other effectors. *Int. Rev. Cytol.* **88**:1–302

Towie, A.C., Sze, P.Y. 1983. Steroid binding to synaptic plasma membrane differential binding of glucocorticoids and gonadal steroids. *J. Steroid Biochem.* **18**:135–143

Trueba, M., Guantes, J.M., Vallejo, A.I., Sancho, M.J., Marino, A., Macarulla, J.M. 1987. Characterization of cortisol binding sites in chicken liver plasma membrane. *Int. J. Biochem.* **19**:957–962

Trueba, M., Ibarrola, I., Vallejo, A.I., Sancho, M.J., Marino, A., Macarulla, J.M. 1989*a*. Characterization of specific binding sites for corticosterone in mouse liver plasma membrane. *Membr. Biochem.* **8**:229–239

Trueba, M., Vallejo, A.I., Rodriguez, I., Ibarrola, I., Sancho, M.J., Marino, A., Macarulla, J.M. 1989*b*. Evidence for the presence of specific binding sites for corticoids in mouse liver plasma membranes. *J. Membrane Biol.* **108**:115–124

Vallejo, A., Trueba, M., Sancho, M.J., Egaña, M., Marino, A., Macarulla, J.M. 1986. Glycogenolytic effect of cortisol previous to its interaction with cell nucleus in mouse liver. *Exp. Clin. Endocrinol.* **87**:201–207

Van Wilgenburg, H., Djie Njio, K., Belling, G.A.C., Van Den Hoven, S. 1982. Effects of corticosteroids on the myoneural junction-A morphometric and electro-physiological study. *Eur. J. Pharmacol.* **84**:129–137

Wehling, M., Käsmayr, J., Thiesen, K. 1989. Fast effects of aldosterone on electrolytes in human lymphocytes are mediated by the sodium-proton-exchanger of the cell membrane. *Biochem. Biophys. Res. Commun.* **164**:961–967

Weiland, G.A., Molinoff, P.B. 1981. Quantitative analysis of drugs-receptor interactions. I. Determination of kinetic and equilibrium properties. *Life Sci.* **29**:313–330

Received 30 April 1990; revised 13 August 1990