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Characterization of specific corticosterone binding sites in adrenal cortex plasma membrane and their localization by autoradiographic studies

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Abstract. Specific corticosterone binding to calf adrenal cortex plasma membrane was measured using the biologically active radioligand [^{3}H]corticosterone. Corticosterone binding was found to be time-dependent, saturable and reversible, and was reduced by more than 70% when membranes were pretreated with proteases. The population of corticosterone binding sites in calf adrenal cortex plasma membrane was homogeneous and displayed the following characteristics: equilibrium dissociation constant $K_d = 77 \pm 8$ nM and maximum specific binding capacity $B_{max} = 70{,}378 \pm 6{,}385$ fmol/mg protein. The relative affinities of several structural analogues of steroids were deduced from competition assays. From these experiments we can conclude that the plasma membrane binding site characterized is selective for corticosterone and progesterone derivatives, and different from nuclear glucocorticoid, mineralocorticoid, estrogen and progestin receptors. Likewise, this corticosterone binding site is independent of mineralocorticoid and Na^+, K^+-ATPase digitalis receptors. From autoradiographic studies we suggest these corticosterone binding sites are located in the whole adrenal cortex.

Key words. Corticosterone; steroid hormones; calf adrenal cortex; plasma membrane; specific binding sites; glucocorticoid membrane receptor; autoradiographic studies.

Steroid hormone action in most cellular systems studied so far involves hormone entry into the cell and binding to nuclear receptors with subsequent modulation of transcription. This classical mechanism of steroid hormone action has been extensively investigated [1, 2]. However, several reports of steroid hormone action in different cell types, such as oocytes [3], hepatocytes [4], neurons [5] and spermatozoa [6] suggest the involvement of a mechanism different from that based on genomic receptors. Some of the earliest evidence that not all steroid hormones act via intracellular receptors is that their effects occur with latencies of minutes, seconds or even milliseconds, too fast to be explained by gene transcription and protein synthesis. In most of these cases, the hormone effect occurs at the membrane level. These rapid responses to steroid hormones led to the universality of the genomic model being questioned and to the proposal that steroid hormones may also act through alternative, nongenomic mechanisms [7]. There is now additional documentation of rapid steroid responses, as well as growing evidence that gonadal and adrenal steroid hormones bind to receptor proteins in the plasma membrane and modulate ligand-gated ion channels as well as G-protein coupled responses [8]. Moreover, it has been described that cortisol and other steroid hormones modify calcium metabolism and alter the cyclic nucleotide levels, inducing the stimulation of calcium binding to liver plasma membrane [9–11]. It

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has been reported that testosterone, estradiol, progesterone, dexamethasone and cortisol provoke a rapid activation of glycogen phosphorylase in liver and cultured rat hepatocytes [12, 13]. This glycogenolytic effect is earlier than and independent of the stimulation of protein synthesis and it is not accompanied by a rise in cAMP levels. We have also previously characterized steroid hormone binding sites in chicken, mouse and rat kidney and liver [14–20].

The aim of the present study was to characterize corticosterone binding sites in cortex plasma membrane purified from calf adrenal gland, the main tissue for corticoid synthesis, determining the saturation, reversibility, specificity and kinetic parameters, and to establish the tissue localization of this binding protein in the gland by autoradiographic studies.

Materials and methods

Chemicals and reagents. [1,2,6,7-^{3}H]corticosterone (80 Ci/mmol) was purchased from Amersham International (England). Nonradioactive steroid hormones were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RU-steroids were a gift from Roussel-Uclaf (Paris, France). PPO, bis-MSB, scintillation grade toluene and Emusolv® were supplied by Scharlau (Barcelona, Spain). All other reagents were from Merck (Darmstadt, Germany). GF/C glass fibre filters used were purchased from Whatman (Whatman International Ltd. Maidstone, UK).

Isolation of plasma membranes. Adrenal glands from male calves slaughtered at a local abattoir were placed in ice and transported immediately to the laboratory in ice-cold Hank's balanced salt solution. Glands were cleaned of adhering fat with scissors and cortex was separated from medulla by dissection under a magnifying glass. Adrenal cortex plasma membranes were isolated as previously described by Maeda et al. [21]. Briefly, adrenal cortex was minced and homogenized in 3 volumes 20 mM Tris, pH 7.4, containing 0.25 M sucrose, 0.5 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulphonyl fluoride (homogenization buffer) using a Polytron homogenizer (IKA Ultra-Turrax T25) 3 times at 9500 rpm for 15 sec. The homogenate was centrifuged for 5 min at 1500 $\times$ g and the pellet was resuspended in the original volume and centrifuged in a 41% (w/v) sucrose gradient for 65 min at 95,000 $\times$ g. The interfacial plasma membrane fraction was washed for 30 min at 95,000 $\times$ g with Tris buffer (140 mM NaCl, 20 mM Tris, 1.5 mM EDTA, pH 7.4). During the isolation procedure the temperature was kept at 0 to 4 °C. Aliquots of purified plasma membranes were frozen in liquid nitrogen and stored in Tris buffer at −70 °C until use.

[^{3}H]corticosterone binding assays. Incubation mixtures for binding studies contained 50 µl of purified plasma membrane fraction (0.2 mg protein/ml final concentration), 50 µl of [^{3}H]corticosterone, and 400 µl of incubation buffer (140 mM NaCl, 20 mM Tris, 5 mM glucose, 1.5 mM EDTA, pH 7.4). The presence of specific corticosterone binding sites was determined by the difference between the radioactivity bound to the membranes incubated only with [^{3}H]corticosterone (total binding) and the radioactivity bound in the presence of an excess of unlabelled corticosterone (nonspecific binding). Incubation assays were performed in an ice bath. At the end of the incubation period, triplicate 400 µl aliquots were filtered under vacuum through GF/C glass microfibre filters (Whatman Ltd., England) placed in a twelve place filter manifold (Millipore Corporation, USA), and immediately washed 3 times with 4 ml of ice-cold Tris buffer to remove unbound steroid. The dried filters were placed in vials and equilibrated with 5 ml of an Emusolv®-Toluene based scintillation cocktail and the radioactivity was measured in a Packard Tricarb 2700 TR model with a tritium efficiency of 65%.

Treatment of membranes with proteases. Plasma membranes of adrenal cortex (0.2 mg/ml protein final concentration) were preincubated with trypsin from bovine pancreas (30 µg protein/ml) and proteinase K from *Tritirachium album* (20 µg protein/ml) for 15 min at 4 °C in 20 mM Tris, 140 mM NaCl, 1.5 mM EDTA, 5 mM glucose, pH 7.4. Control samples were always included and incubated in the absence of proteases. Subsequently, the membranes were assayed under standard conditions.

Autoradiography. Slices (30 µm) obtained with a Bright 5030 microtome from calf adrenal gland at −20 °C were prepared and fixed on gelatin (1%)-chrome alumn (0.1%). The sections were subsequently kept frozen at −70 °C until use. Sections were preincubated for 15 min in ice-cold buffer (20 mM Tris-HCl, pH 7.4). The binding procedure was performed in a humidified chamber for 15 min at 4 °C under agitation. Each slice was covered with 400 µl of 20 mM Tris-HCl, 1.5 mM EDTA, 140 mM NaCl and 5 mM glucose solution, pH 7.4 and 100 µl of 30 nM [^{3}H]corticosterone. Nonspecific binding was assessed by incubating parallel sections in the presence of 10 µM nonradioactive corticosterone. After incubation the slices were rapidly washed twice for 5 min each time in ice-cold incubation buffer without glucose, and rinsed briefly in ice-cold distilled water. After drying under a cold air stream, the [^{3}H]corticosterone-exposed cryosections were attached to Hyperfilm-MP (Amersham) and placed in a cassette. The samples were stored at −70 °C for 3 months, and the emulsion-coated cover slips were then developed and fixed.

Protein determination. Protein was measured by the method of Bradford [22] with the Bio-Rad protein assay kit and BSA as standard reference protein.

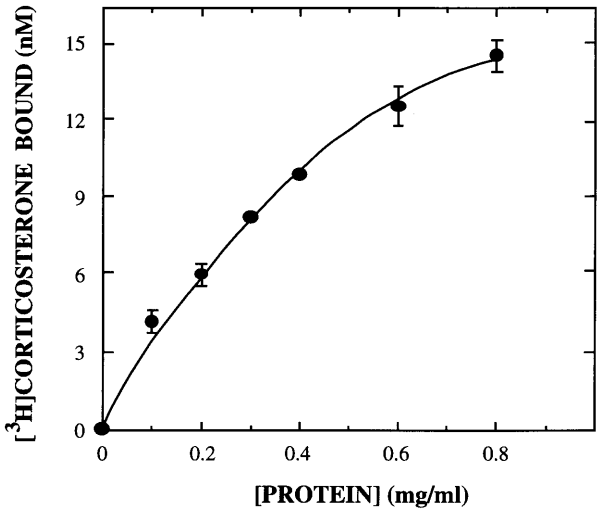

Figure 1. Binding of [^{3}H]corticosterone (40 nM) as a function of the protein concentration from calf adrenal cortex plasma membranes. The incubation time was 25 min. The data are the mean ± SEM of 2 experiments performed in triplicate.

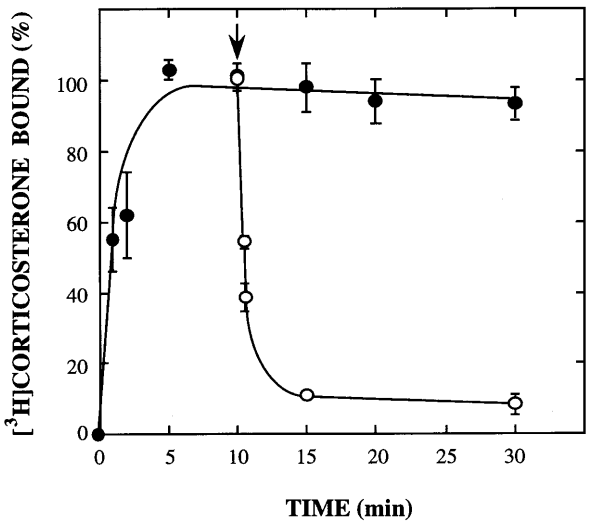

Figure 3. Kinetics of binding of [^{3}H]corticosterone to plasma membranes from calf adrenal cortex. Membranes (0.2 mg of protein/ml) were incubated with [^{3}H]corticosterone (30 nM) in incubation buffer pH 7.4 at 4 °C (association, ●). At 10 min an excess (10 μM) of unlabelled corticosterone was added and the kinetics of displacement of the radioligand were followed (dissociation, ○). The data are the mean ± SEM of 3 experiments performed in triplicate.

Results

Plasma membrane fraction purity. The purity of calf adrenal cortex plasma membrane fractions has been assessed by determining the activity of the following membrane-specific enzyme markers: 5′-nucleotidase (for the plasma membrane), glucose 6-phosphatase (for endoplasmic reticulum) and succinate dehydrogenase (for the mitochondria). 5′-nucleotidase activity was enhanced 15-fold in the adrenal cortex membrane (24.2 μmol/h per mg protein) with respect to the whole homogenate. Glucose 6-phosphatase activity was 3-fold higher in the whole homogenate (1.2 μmol/h per mg protein) but much lower than that found in microsomes. On the other hand, succinate dehydrogenase activity was not detected in the purified plasma membranes while in the whole homogenate the activity was 0.3 μmol/h per mg protein.

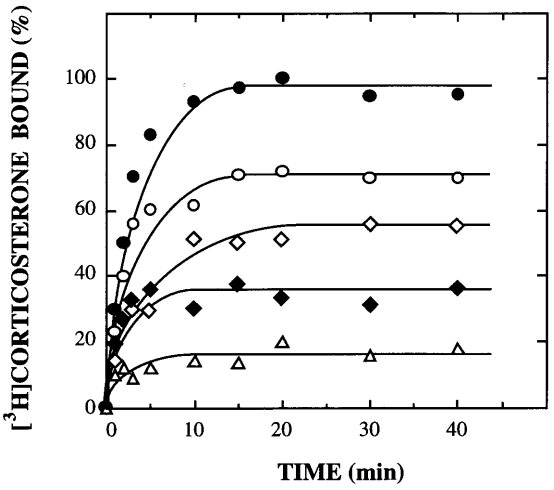

Figure 2. Temperature dependence of [^{3}H]corticosterone binding to adrenal cortex plasma membranes. Binding was assayed at different temperatures: 4 °C (●), 25 °C (○), 37 °C (◇), 50 °C (◆) and 70 °C (△). The data are the mean ± SEM of 3 experiments performed in triplicate.

Binding of [^{3}H]corticosterone to calf adrenal cortex plasma membranes. In order to optimize the assay conditions we determined the ideal concentration of protein. The results showed a linear correlation between the corticosterone binding and the protein added up to 0.4 mg of protein/ml (fig. 1). It was also observed that the binding of the radioligand was strongly dependent on pH. The optimum pH for the binding was 7.4, whereas at higher and lower pH the binding descended dramatically (data not shown). According to these results the conditions selected for further incubations were 0.2 mg/ml for the final protein concentration and a pH of 7.4. The binding was shown to be temperature dependent with an optimum at 4 °C. When temperature increased the binding of [^{3}H]corticosterone decreased (fig. 2). If we take the binding at 4 °C and 20 min of incubation as 100%, the binding of [^{3}H]corticosterone at 25, 37, 50 and 70 °C was 70%, 57%, 34% and 15% respectively. The binding was also affected by the pres-

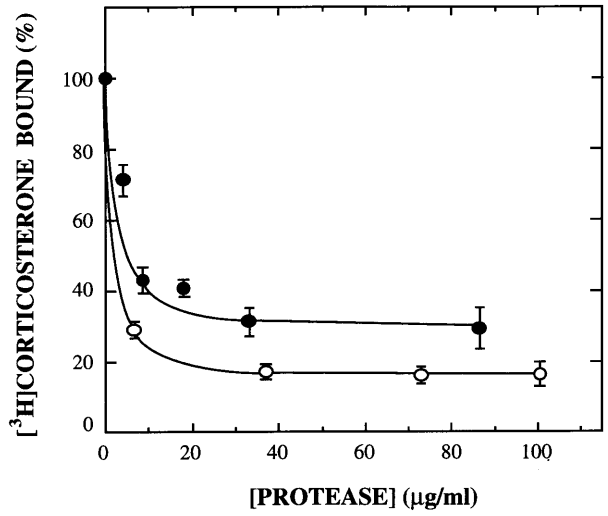

Figure 4. Influence of pretreatment with proteases (trypsin and proteinase K) on subsequent binding of [^{3}H]corticosterone to plasma membranes from calf adrenal cortex. Membranes were preincubated separately with both enzymes, trypsin (●) and proteinase K (○), for 15 min as described in Materials and methods. The data are the mean ± SEM of 3 experiments performed in duplicate.

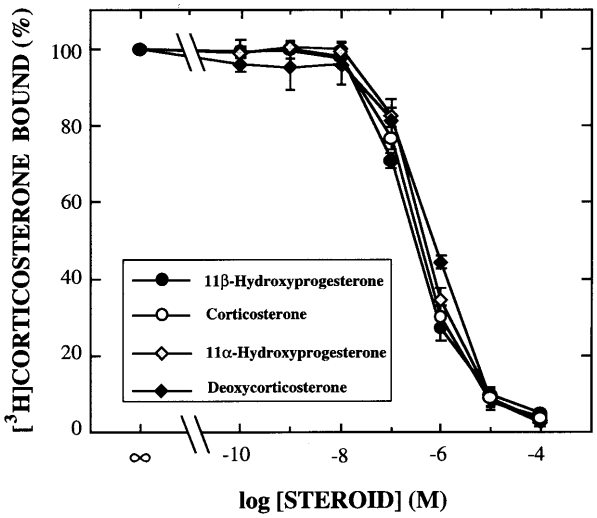

Figure 6. Influence of various steroids on the binding of [^{3}H]corticosterone to adrenal cortex plasma membranes. Binding was determined at 30 nM [^{3}H]corticosterone as described in Materials and methods. The incubations were carried out in the absence (100% binding) and presence of different concentrations (0.1 nM–0.1 mM) of unlabelled steroids. Values are the mean ± SEM of 4 determinations performed in triplicate.

ence of the sulphydryl agent benzylmaleimide. This reagent blocked the binding of [^{3}H]corticosterone by more than 90%, suggesting the presence of disulphide bridges in the steroid binding domain of this binding protein (data not shown).

Association and dissociation kinetics (fig. 3) have been performed under standard conditions (30 nM [^{3}H]corticosterone final concentration, 30 min incubation, 4 °C and pH 7.4). A saturable binding was obtained rapidly within 5 min of incubation. Maximal dissociation of the site-ligand complex was observed 5 min after addition of 10 μM of unlabelled corticosterone, reaching 88% displacement from total binding. For further experiments 15 min was chosen as the incubation time for the assays.

Treatment of membranes with proteases. When plasma membranes were preincubated with trypsin (30 μg/ml) and proteinase K (20 μg/ml), 70% and 83% respectively of the subsequent binding of [^{3}H]corticosterone was removed (fig. 4). Thus, these data show that trypsin and

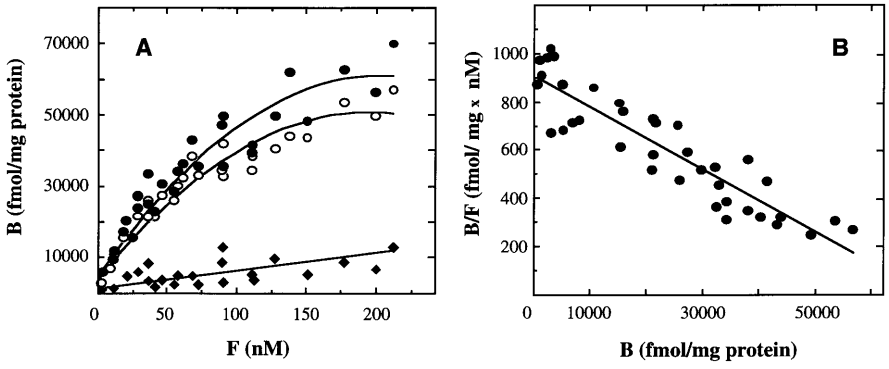

Figure 5. Radioligand binding studies using [^{3}H]corticosterone to adrenal cortex plasma membranes. (*A*) Equilibrium saturation binding. Two sets of incubations with increasing concentrations of [^{3}H]corticosterone were carried out simultaneously with the same plasma membranes (0.2 mg/ml) in the presence and absence of 10 μM corticosterone for 15 min. Specific binding (○) equals total binding (●) minus nonspecific binding (◆). (*B*) Scatchard plot of the specific binding data of (*A*) obtained by least-square method ($r = 0.910$). B: [^{3}H]corticosterone bound, in fmol/mg protein; F: free [^{3}H]corticosterone concentration, in nM.

Table 1. Competition for [^{3}H]corticosterone binding sites in plasma membranes from calf adrenal cortex of various unlabelled steroids.

Unlabelled steroid	IC_{50} (nM)
Corticosterone	387
11β-Hydroxyprogesterone	335
11α-Hydroxyprogesterone	456
Deoxycorticosterone	748
Progesterone	1585
Cortisone	2799
17β-Estradiol	3511
Prednisolone	9550
R5020	17783
17α-Hydroxyprogesterone	21877
Cortisol	22387
Testosterone	25119
Dexamethasone	25586
Norgestrel	28184
Megestrol acetate	31623
RU 38486	35481
5α-Dihydrocortisol	36308
Triamcinolone	50119
Aldosterone	79432
5-Pregnenolone	n.d.
Ouabain	n.d.

Results are presented as competitor concentration that displaces 50% of the [^{3}H]corticosterone binding, taken as 100% (IC_{50}). Plasma membranes from calf adrenal cortex were incubated with 30 nM [^{3}H]corticosterone alone or in the presence of different concentrations of unlabelled steroids. The results are the mean of triplicate determinations (n.d.: no displacement).

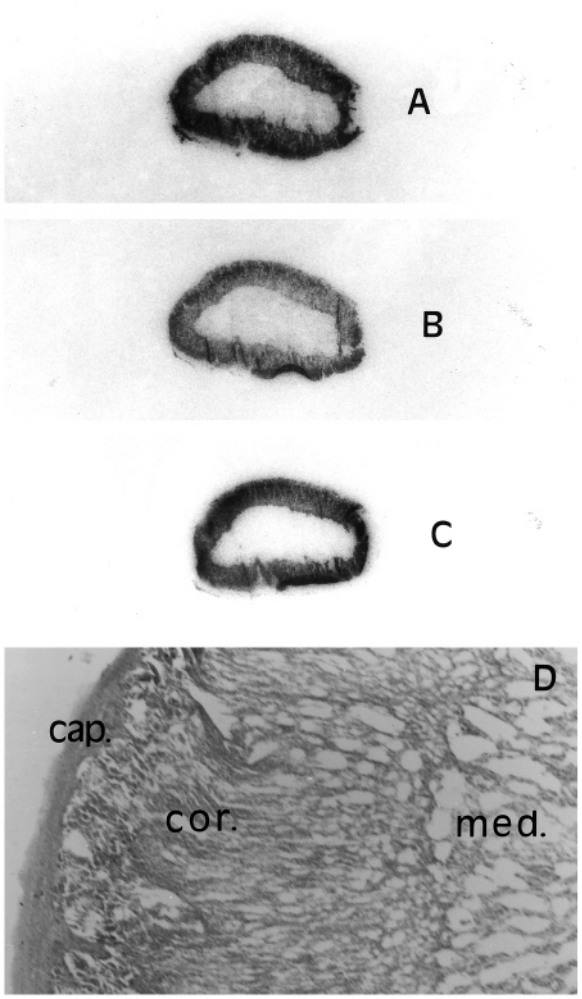

Figure 7. Autoradiography of transverse sections from calf adrenal gland. 30 µm slices from adrenal gland were preincubated for 15 min with 30 nM [^{3}H]corticosterone, as described detailed in Materials and methods, with vehicle (total binding) (*A*); in the presence of 10 µM unlabelled corticosterone (nonspecific binding) (*B*); in the presence of 10 µM 5-pregnenolone (*C*); panel *D* is a photomicrograph of a fixed 30 µm section (×100) stained with hematoxylin and eosin, and shows the morphological appearance of calf adrenal gland. cap., capsule; cor., cortex; med., medulla.

proteinase K are potent inhibitors of [^{3}H]corticosterone binding to adrenal cortex plasma membranes.

Binding of [^{3}H]corticosterone in the equilibrium. The affinity constant was determined from saturation experiments, in which 0.2 mg/ml protein was incubated with increasing concentrations of [^{3}H]corticosterone, from 1 to 200 nM in the absence and presence of 10 µM of unlabelled corticosterone. A Michaelis-Menten plot of corticosterone binding to adrenal cortex plasma membrane is shown in figure 5A. Scatchard analysis of the data showed high affinity (fig. 5B). The specific binding data were analyzed using an iterative nonlinear curve-fitting program, best-fit to one-site model ($p < 0.05$), indicating the presence of a single membrane binding site. These sites are equivalent and independent, because the Hill's slope value was 0.98. The results revealed the following kinetic parameters: equilibrium dissociation constant $K_d = 77 \pm 8$ nM and maximum capacity of binding $B_{max} = 70{,}378 \pm 6{,}385$ fmol/mg protein.

Specificity of [^{3}H]corticosterone binding sites. A range of unlabelled steroids was tested for the ability to compete with [^{3}H]corticosterone for the binding sites. The binding site showed high specificity for corticosterone and progesterone derivatives over the other steroids tested (fig. 6). These data are in accordance with findings of others works [23, 24]. In table 1 the IC_{50} values calculated are shown. Metyrapone and aminoglutethimide (corticoid biosynthesis inhibitors that act on cytochrome P-450) were also investigated to determine their capacity for displacing [^{3}H]corticosterone from its binding site. Both inhibitors gave negative results (data not shown).

Tissue localization of [^{3}H]corticosterone binding sites. Autoradiography showed the highest accumulation of silver grains corresponds to [^{3}H]corticosterone binding sites in the calf adrenal cortex (fig. 7A). In the adrenal cortex, where the density of silver grains is about 95.7% higher than in the medulla, the radioligand is accumu-

lated primarily in the cortex (zona glomerulosa plus zona fasciculata-reticularis). In the presence of 10 μM unlabelled corticosterone this dense zone partially disappears (fig. 7B) and in the presence of 10 μM 5-pregnenolone, an antagonist with very low affinity for these binding sites, the highest accumulation in cortex reverts to its original state (fig. 7C). The microscopic-morphological appearance of calf adrenal gland stained with hematoxylin and eosin is shown (fig. 7D). Here, the adrenal cortex can easily be differentiated from the medulla. From the silver grain density difference between figures 7A and 7B and the pattern of figure 7D we can deduce that corticosterone binding sites or receptors are present in the whole calf adrenal cortex.

Discussion

Our results demonstrate the presence of specific class of binding sites for corticosterone in the plasma membrane of calf adrenal cortex. This binding is specific, saturable and reversible at 4 °C and pH 7.4, and shows high affinity (average K_d 77 nM) and a capacity of binding of 14 nM (average concentration). These parameters fall within the typical range of hormone receptors. This binding is more stable at low temperatures, in line with the results obtained by other studies [18, 25]. In the presence of trypsin or proteinase K the corticosterone binding was drastically reduced, indicating the protein nature of this binding site.

We have previously shown the existence in different cell types of specific binding sites for cortisol [14, 16], corticosterone [15, 18, 19], R5020 [20] and progesterone [17]. The K_d values for the corticosterone binding sites found in this work vary from five to 20-fold higher than those reported by previous studies for glucocorticoids assayed in other tissues and animals, and the binding site concentration was 35-fold and 114-fold higher than those found in liver [18] and kidney [19].

From the displacement studies of [^{3}H]corticosterone bound to adrenal cortex plasma membrane, we have determined the affinity order for the competitor steroids based on the IC_{50} values (table 1). This order decreases as follows: corticosterone, 11β-hydroxyprogesterone, 11α-hydroxyprogesterone > deoxycorticosterone > progesterone > cortisone > 17β-estradiol > prednisolone > R5020 > 17α-hydroxyprogesterone, cortisol, testosterone, dexamethasone, norgestrel > megestrol acetate, RU38486, 5α-dihydrocortisol > triamcinolone acetonide > aldosterone > 5-pregnenolone. Thus, this binding site binds corticosterone and progestin derivatives preferentially due to their common structure 3-oxo-4-ene. Androgens such as testosterone, estrogens such as 17β-estradiol, and mineralocorticoids such as aldosterone, were relatively poor or ineffective competitors for the corticosterone binding site.

We can deduce that the specific binding observed is not to the glucocorticoid cytosolic receptor, based on the affinities of dexamethasone and triamcinolone acetonide for that receptor [1]. The binding is not to the progesterone cytosolic receptor either, since this has no affinity for glucocorticoids and has high affinity to RU38486 [26]. The very low affinity of aldosterone and ouabain for the plasma membrane binding site allows it to be distinguished from both the mineralocorticoid and Na^+-K^+-ATPase digitalis receptors.

Cozza et al. [27] showed that cortisol and corticosterone bind specifically to the inner membrane of zona glomerulosa mitochondria. Cortisol binding to cytochrome P-450 was inhibited by pretreatment with sodium dithionite, and displaced by corticosterone, deoxycorticosterone and metyrapone (an inhibitor of the 11β-hydroxylation reaction). These authors suggest that cortisol and corticosterone bind cytochrome P-450. Conversely, in our case, metyrapone and aminoglutethimide (both inhibitors of the conversion of cholesterol to corticosteroids, through interaction with cytochrome P-450) do not displace [^{3}H]corticosterone from its specific binding site. Thus, these results prove that corticosterone in our system does not bind in adrenal cortex mitochondria.

From autoradiographic studies we suggest this corticosterone binding protein is present all over the adrenal cortex. Nevertheless, we consider that the nonspecific binding shown in the autoradiography can be due in part to the P-450 cytochrome component of the mitochondrial inner membrane.

It is possible that the physiological actions of corticosterone would take place at a limited range of concentrations, and the uptake of this hormone would occur only via high affinity receptors. Thus, Allera and Wildt [28] proposed a carrier for corticosterone in rat liver plasma membrane. On the other hand, the existence of a plasma membrane receptor for steroid hormones has been extensively reported [29–31]. Megestrol, a 17-hydroxyprogesterone derivative, is specific for the membrane progesterone receptor, and was found to be an antagonist of the ability of the hormone to elevate intracellular free calcium concentration in sperm cells through a surface progesterone receptor [32]. In our case, it had no effect. It has been suggested that the membrane-bound progesterone receptor may itself be a Ca^{2+} channel or a binding site of the Ca^{2+} channel [33]. Such a situation was found on neurons, where the progesterone receptor was shown to be the Cl^- ion channel itself and to contribute to its activation in response to some stimuli [30].

The results obtained from affinity and displacement experiments are different from those found for corticosteroid-binding globulin (CBG); moreover, we consider that this binding protein is different from CBG for several reasons. The adrenal gland first, and the purified

membrane after, were washed with saline solution in order to avoid adsorbed proteins. Moreover, the steroid binding to CBG is optimum at the relatively high pH of 9 to 10 and in our system the optimum pH is 7.4. CBG has affinity for steroids of 21 carbon atoms (such as cortisol, corticosterone, progesterone), but the adrenal binding site shows no affinity for cortisol and dexamethasone.

Recently, a cortisol binding protein from rat liver plasma membranes has been solubilized and purified [34]. The activity of the corticosteroid receptor characterized on amphibian neuronal membranes appears to be modulated by guanyl nucleotides [30]. Wehling et al. [35] have demonstrated rapid effects of aldosterone on intracellular pH in vascular smooth cells, which include a final alkalization not seen after exposure to angiotensin II or PDGF.

Accumulated data from in vitro experiments have suggested that 18-hydroxysteroids may be stored within the intact rat adrenal zona glomerulosa [36]. The results suggest that 18-hydroxydeoxycorticosterone is the major steroid sequestered within the rat adrenal zona glomerulosa and that this sequestration is attributable to the association of 18-OH-DOC with a high-density lipid-protein component of the plasma membrane. These data are at variance with the usual view of steroid secretion by simple diffusion.

On the other hand, P-glycoprotein, the product of the multidrug resistance (MDR1) gene, is an ATP-driven transmembrane pump that increases the resistance of cells by actively exporting toxic chemicals. Interestingly, recent experiments suggest that steroid hormones may be physiological substrates for P-glycoprotein. In fact, various steroids have recently been shown to affect photoaffinity labelling of P-glycoprotein by axidopine and to inhibit the export of an anticancer drug and/or to reverse multidrug resistance. Progesterone (10–100 μM), the most potent steroid tested, increased accumulation of vinblastine in COS-7 kidney cells [37]. The mechanism of these transporters is not well understood, but their localization in the adrenal cortex suggests that P-glycoprotein might be involved in steroid transport, and these hormones may be natural substrates [38]. Porcine LLC-PK1 cells that expressed human P-glycoprotein showed a multidrug-resistant phenotype, transporting cortisol, aldosterone and dexamethasone but not progesterone [39]. In addition, there exists a family of transporter genes with high structural homology to P-glycoprotein, the so-called ABC (ATP-binding casette) family. In yeast plasma membrane the ABC transporter LEM1 alters the biological potency of glucocorticoids from that predicted by their binding affinities for the glucocorticoid receptor, and selectively pumps dexamethasone and triamcinolone, but not deoxycorticosterone [40]. In mammalian L929 cells but not in HeLa cells, FK506-binding protein potentiated dexamethasone accumulation, without altering the hormone-binding properties of the glucocorticoid receptor [41]. Thus, steroid transporters may modulate, in a cell-specific manner in an initial step of the signalling cascade, the availability of hormone by the nuclear receptor. According to these results, it is unlikely that the corticosterone binding protein would be a transmembrane transporter, but the possibility of binding to these transporters cannot be excluded.

In conclusion, we provide evidence for the existence and localization of a specific binding protein for corticosterone in purified plasma membrane from calf adrenal cortex. Further studies should be done to elucidate its physiological significance and the signal transduction mechanism involved. One possibility is that steroid substrates with different metabolic fates may be stored in discrete pools, allowing a more precise control over the secretion of hormones with varying biological activities, in accordance with physiological demands.

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