Journal of Bioenergetics and Biomembranes, Vol. 23, No. 6, 1991

Steroid Hormone Specifically Binds to Rat Kidney Plasma Membrane

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Received June 28, 1990

Abstract

A high-affinity and low-capacity corticosterone specific binding was detected in the purified plasma membrane preparation from rat kidney using an *in vitro* steroid hormone binding assay. The specific-bound hormone was efficiently distinguished from the irreversible-bound hormone with 10 μM corticosterone. Under standardized conditions of pH 7.4 at 2°C and 30 min incubation time, the binding was saturable and showed $K_d = 13 \pm 3$ nM and $B_{max} = 616 \pm 34$ fmol/mg of protein. Competitive binding studies with analogue steroids indicated that corticosterone binding to kidney plasma membrane is hormone-specific. Results indicated that the possible nongenomic effects of steroids could be mediated by their interaction with plasma membrane.

Key Words: Steroid hormone; kidney plasma membrane; corticosterone binding site; steroid receptors.

Introduction

It has generally been reported that steroids enter cells by passive diffusion through the plasma membrane because of their small molecular size and lipophilic nature (Peck *et al.*, 1973; Lovell-Smith and García-Webb, 1986). Allera *et al.* (1980) and Allera and Rao (1986) recently demonstrated that the uptake of corticosterone by the plasma membrane vesicle was a saturable and reversible process. Many studies have suggested that plasma membranes are involved in nongenomic effects of steroid hormones (Suyemitsu and Terayama, 1975; Pietras and Szego, 1979; Fant *et al.*, 1979; Duval *et al.*, 1983; Sadler *et al.*, 1985; Weis and Gurpide, 1988; Muldoon *et al.*, 1988; Cozza *et al.*, 1990). Several of these investigators pointed to the possibility that steroid

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0145-479X/91/1200-0919$06.50/0

receptors were localized in the membrane (Sadler and Maller, 1982; Blondeau and Baulieu, 1985). Recently, we have reported evidence for the presence of specific binding sites for corticoids, with high affinity and low capacity of binding, in chicken and mouse liver plasma membranes (Trueba *et al.*, 1987, 1989a, b, 1991), and progestins (Trueba *et al.* 1990). This interaction could be the origin of the different effects that steroid hormones have at short time (Egaña *et al.*, 1981; Vallejo *et al.*, 1986; Gomez-Muñoz *et al.*, 1989). We show here that these binding sites are not only into rat liver plasma membranes but also in kidney, and examine the binding kinetic parameters and the specificity of different steroid hormone analogues.

Materials and Methods

[1,2,6,7-^{3}H]Corticosterone (75 Ci/mmol) was purchased from Amersham International (Amersham, U.K.). The radiochemical purity determined by thin-layer chromatography on silica gel in chloroform : acetone (20 : 80) was 98%, and in chloroform : ethanol (70 : 30) was 99%. All steroid hormones and analogues, propranolol, dithiothreitol, and phenylmethylsulfenyl fluoride (PMSF) were purchased from Sigma Chemical (St. Louis, Missouri). Phentolamine was kindly donated by Ciba Geigy Laboratories (Barcelona, Spain). PPO, POPOP, and scintillation-grade toluene were supplied by Scharlau (Barcelona, Spain). All the other components were obtained from Merck (Darmstadt, Germany).

Livers were perfused and removed from male rat weighing about 250 g under ether anesthesia, homogenized in 3-fold (w/v) excess of 20 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 0.5 mM EDTA, 1 mM dithiothreitol, and 5 μM PMSF as described by Maeda *et al.* (1983). The tissue was homogenized using a Ultra-Turrax T-25 homogenizer (Stanfen, Germany) by means of three bursts of 15 s each at 9,500 rpm. The homogenate was filtered through two fine and then four coarse layers of cheese cloth. After a first centrifugation at 1,000 $\times$ *g* for 5 min the supernatant was discarded. Pellet was resuspended and homogenized again. A 25-ml portion of the homogenate was layered over 10 ml of a solution of 41% sucrose in the homogenization buffer and centrifuged at 95,000 $\times$ *g* for 1 h in a Kontron TST 28.38 swinging bucket rotor in a Beckman L8-50M/E model ultracentrifuge. The white interfacial band of membranes was collected, and the sucrose was diluted by adding a 3-fold excess of the incubation 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). Membranes were washed twice and pelleted by centrifugation at 95,000 $\times$ *g* for 20 min and resuspended in the sawe buffer to use them in binding studies. Purified plasma membranes were

frozen in liquid nitrogen and stored at −70°C in a ultralow-temperature freezer Selecta Conbatemp 80 model (Barcelona, Spain).

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Incubation mixtures for binding studies contained 0.1 ml of purified plasma membrane fraction (with final concentration of 0.2 mg protein/ml), 0.1 ml of 4 nM [^{3}H]corticosterone, and 0.8 ml of incubation buffer. The presence of specific corticosterone binding sites was determined by the difference between the radioactivity bound to the membranes incubated 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, three 800-μl aliquots were filtered through 25 mm GF/C glass fiber filter (Whatman, England) placed in a twelve place filter manifold from Millipore (Bedford, Massachusetts). Filters were immediately washed with 10 ml of iced-cold incubation buffer. The dried filters were placed in vials with 5 ml scintillation cocktail (4 g PPO, 0.05 g POPOP, and 1 liter toluene) and counted 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, U.K.) in its version for an IBM PC computer. The S.E.E. of the samples did not exceed 10% in any case.

Results

The plasma membrane fraction integrity has been checked by marker enzyme analysis. The specific activities of marker enzymes for plasma membranes were much enhanced in the purified plasma membranes as compared with the homogenate. 5′-Nucleotidase (Aronson and Touster, 1974): 16.0 ± 0.8 and 2.3 ± 0.5 μmol Pi/mg × h, respectively; ATPase (Quigley and Gotterer, 1969): 51.9 ± 5.7 and 3.4 ± 1.2 μmol Pi/mg × h and phosphodiesterase, respectively (Aronson and Touster, 1974): 14.1 ± 0.7 and 0.8 ± 0.2 μmol Pi/mg × h, respectively.

Negative membranes for corticosterone have not been found. Crude membranes from brain and lung showed a very long binding. Therefore, we have used these membranes in the following experiments. First, the binding with crude membranes from brain and lung was 42 and 50 fmol/mg protein respectively. Second, the binding with purified membranes from brain and lung after incubation with liver cytosol was 34 and 61 fmol/mg of protein, respectively. These results agree with the fact that the specific binding of

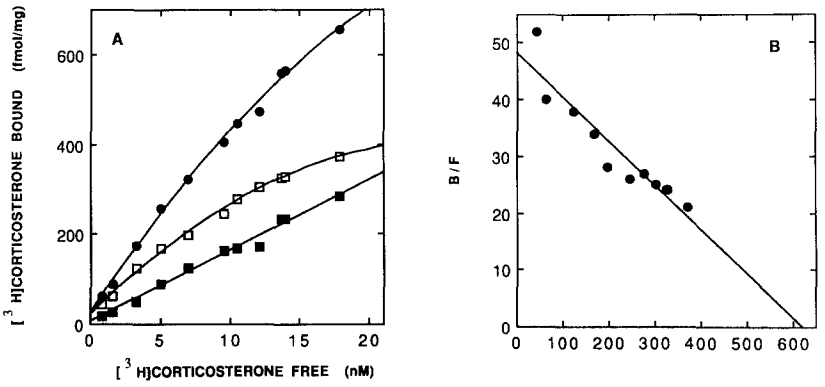

Fig. 1. (A) Binding of [^{3}H]corticosterone to rat kidney plasma membranes as a function of the radioactive steroid concentration (0.6–25 nM) at 2°C in incubation buffer, pH 7.4. Two sets of incubation were carried out simultaneously with the same plasma membranes (0.2 mg/ml) in the presence and absence of 10 μM corticosterone for 60 min. Specific binding (□), nonspecific binding (■), and total binding (●). Points indicate the mean ± SEM of quadruplicate determinations. (B) Scatchard plot of the specific binding data. Kinetic parameters were calculated by the least-squares method. *B*, [^{3}H]corticosterone bound (fmol/mg protein); *F*, [^{3}H]corticosterone free (nM).

[^{3}H]corticosterone is located mainly in the kidney and liver (Trueba *et al.*, 1991) plasma membrane and is not due to a cytosolic contamination.

Corticosterone binding to kidney plasma membrane was maximal at 30 min of incubation, and this remains up to 120 min. The specific binding was measured at 2°C and pH 7.4 (Trueba *et al.*, 1987, 1989a).

Binding was shown to be a linear function of the membrane protein concentration up to 0.4 mg protein/ml of membrane suspension (data not shown).

Affinity constant was determined from saturation experiments, in which 0.2 mg/ml protein was incubated with increasing concentrations of [^{3}H]corticosterone, from 0.6 to 25 nM (Fig. 1) in the absence and presence of 10 μM of unlabeled corticosterone. Scatchard analysis of the data showed high-affinity and low-capacity binding sites for kidney plasma membranes (Fig. 1B). The kinetic parameters were calculated using the program LIGAND, on the basis of the best-fit analysis (lowest mean-square values with $p < 0.05$). The best fit was obtained for the specific binding analyzed as one site model. The following binding characteristics were obtained: dissociation constant $K_d = 13 \pm 3$ nM and maximum capacity of binding $B_{max} = 616 \pm 34$ fmol/mg protein. From Hill's plot we have found a slope of 0.99 (data not shown), so there was no cooperativity in the binding.

The specificity study (Fig. 2) showed that the binding is specific for corticoids and progesterone compounds. 17β-Estradiol, triamcinolone

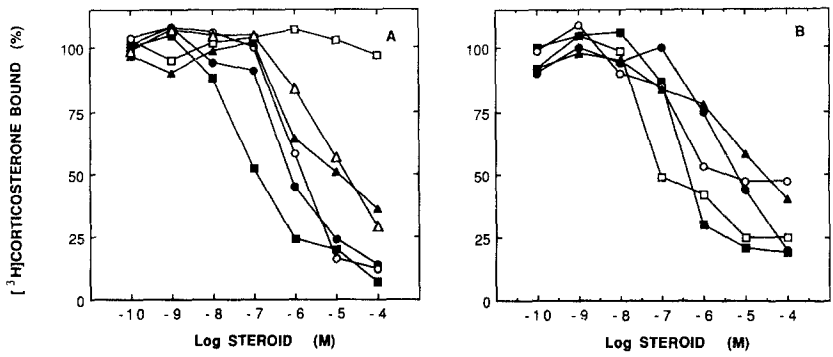

Fig. 2. (A) Influence of various steroids on the binding of [^{3}H]corticosterone to rat kidney plasma membranes. Corticosterone (■), cortisol (●), deoxycorticosterone (○), aldosterone (△), testosterone (▲), and 17β-estradiol (□). (B) Progesterone (■), 11β-hydroxyprogesterone (□), 20β-hydroxyprogesterone (▲), 20α-hydroxyprogesterone (○), and 11α-hydroxyprogesterone (●). Membranes (0.2 mg protein/ml) were incubated for 2 h at 2°C with 4 nM [^{3}H]corticosterone in incubation buffer, pH 7.4. The incubations were carried out in the absence (100% of binding) and presence of different concentrations (0.1 nM to 0.1 mM) of steroids. The points are the mean ± SEM of quadruplicate determinations.

acetonide, dexamethasone, and ouabain had no affinity for the binding sites labeled with [^{3}H]corticosterone. The values of the affinities of steroid competitors with respect to IC_{50} are presented in Table I. We can calculate the constant K_i or K_d, using the Cheng–Prusoff (1973) equation, because the

Table I. Affinity of Several Steroid Analogues for the Binding of [^{3}H]Corticosterone to Rat Kidney Plasma Membrane[a]

Competitor steroid	IC_{50} (nM)
Corticosterone	30 ± 4
11β-Hydroyprogesterone	47 ± 5
20α-Hydroxyprogesterone	209 ± 18
Progesterone	251 ± 20
Cortisol	474 ± 32
Prednisolone	562 ± 53
Testosterone	631 ± 61
Deoxycorticosterone	1010 ± 85
20β-Hydroxyprogesterone	2399 ± 217
11α-Hydroxyprogesterone	3162 ± 270
Digitoxigenine	4467 ± 409
Aldosterone	6310 ± 613
Cortisolone	7079 ± 629

[a] Incubation of 4 nM [^{3}H]corticosterone and plasma membranes was performed in ice bath in the presence of various concentrations of unlabeled steroid during 60 min of incubation time. IC_{50} is the concentration of unlabeled steroid which inhibits specific [^{3}H]corticosterone binding by 50% at equilibrium. Values for inhibition of binding at each concentration are means of three determinations.

bound ligand is a small percentage of the total ligand and K_d is much greater than the concentration of binding sites. The K_d value obtained for corticosterone was 23 ± 4 nM. This value is near the value determined in equilibrium studies.

Discussion

The data of this paper indicate that [^{3}H]corticosterone binds to one type of binding site with high affinity in the kidney plasma membranes. The binding is specific for corticoids and progesterone analogues. In reference to membrane extraction methods their identity was shown by membrane-specific enzyme markers, whose activities were between 7- and 15-fold higher in membrane than in homogenates.

The blood concentration of steroid hormones is very low (0.1–100 nM). Moreover, most is bound to carrier plasma proteins which do not have free access to target cells. So, it is possible that the physiological action of these hormones would take place at limited range concentrations, and the uptake of these hormones would occur only via high-affinity receptors (Duval *et al.*, 1983). In previous work, Harrison and Yeakley (1979) found that glucocorticoids enter into At-20 cells by a membrane-mediated process; Jonkers *et al.* (1980) determined the existence of a saturable and energy-dependent process for the corticosterone uptake by rat hepatocytes; and Allera and Rao (1986) proposed a carrier with high affinity and low capacity of binding to corticosterone in rat liver plasma membrane.

Results showed that the binding is specific, saturable, and reversible at 2°C and pH 7.4. This binding is more stable at low temperatures, according to the results obtained by other coworkers (Suyemitsu and Terayama, 1975; Pietras and Szego, 1980; Bression *et al.*, 1986). From equilibrium studies we can deduce that only one type of binding site exists; independent and equivalent with high affinity.

The binding-site concentration was 123 pM, three times lower than those found in liver (Trueba *et al.*, 1991). So, we can say the liver is a target organ that binds glucocorticoids more extensively than kidney.

Other K_d values, previously obtained for corticosterone binding, were 3.2 nM for pituitary gland plasma membrane (Koch *et al.*, 1978), 3.95 nM for rat liver plasma membrane (Allera and Rao, 1986), and 4.06 nM for mouse liver plasma membrane (Trueba *et al.*, 1989b). The affinity order for the natural and synthetic steroid assayed was: corticosterone, 11β-hydroxyprogesterone > 20α-hydroxyprogesterone, progesterone > cortisol, prednisolone, testosterone > deoxycorticosterone, 20β-hydroxyprogesterone,

11α-hydroxyprogesterone > digitoxigenine, aldosterone, cortisolone. Synthetic steroids as dexamethasone and triamcinolone acetonide do not have any affinity for the binding site, contrary to their high affinity by the nuclear receptors. Because of this, our binding site is different from that receptor.

On the other hand, this specific binding site has high affinity for C_{21}, very low affinity for C_{19} (as testosterone), and no one for C_{18} steroids (as 17β-estradiol). About the hydroxyl group position the binding is higher in C_{11} than in C_{20} steroids. These results agree with those found previously by Allera and Rao (1986) and Trueba *et al.* (1989b). Ouabain and digitoxigenine are NA^+, K^+-ATPase inhibitors that bind to digitalis receptor. In our case these compounds do not have high affinity for the corticosterone binding site. The affinity constant determined was four times higher than the corticosterone binding site from rat liver.

The results obtained from affinity and displacement experiments are similar to those found for corticosteroid binding globulin (CBG), but it is discarded in our case because the R5020 has high affinity by the corticosterone binding site (data not shown) and none by CBG (Barile *et al.*, 1979).

Thus, we propose that a corticoid plasma membrane binding site or receptor exists also in kidney which could be implicated in the hormone internalization process and in the nongenomic steroid response that occurs prior to the nucleus–hormone interaction.

Acknowledgments

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

Allera, A., and Rao, G. S. (1986). In Steroid Hormone Resistance. Mechanisms and Clinical Aspects, (Chrousos, G. P., Loriaux, D. L., and Lipsett, M. B., eds.), Plenum Press, New York, pp. 53–65.

Allera, A., Rao, G. S., and Breuer, H. (1980). *J. Steroid Biochem.* **12,** 259–266.

Aronson, N. N., and Touster, O. (1974). *Methods Enzymol.* **31,** 90–102.

Barile, G., Sica, G., Corradini, M., and Iacobelli, S. (1979). *Eur. J. Obstet. Gynec. Reprod. Biol.* **9,** 237–242.

Blondeau, J. P., and Baulieu, E. E. (1985). *J. Biol. Chem.* **219,** 3617–3625.

Bression, D., Michard, M., LeDafniet, M., Pagesy, P., and Peillon, F. (1986). *Endocrinology* **119,** 1048–1051.

Cheng, Y. C., and Prusoff, W. H. (1973). *Biochem. Pharmacol.* **22,** 3099–3108.

Cozza, E. N., Fecking, M. F., and Gomez-Sanchez, C. E. (1990). *J. Steroid Biochem.* **35,** 511–514.

Duval, D., Durant, S., and Homo-Delarche, F. (1983). *Biochim. Biophys. Acta* **737,** 409–442.

Egaña, M., Sancho, M. J., and Macarulla, J. M. (1981). *Horm. Metab. Res.* **13,** 609–611.
Fant, M. E., Harbison, R. D., and Harrison, R. W. (1979). *J. Biol. Chem.* **254,** 6218–6221.
Gomez-Muñoz, A., Hales, P., Brindley, D. N., and Sancho, M. J. (1989). *Biochem. J.* **262,** 417–423.
Guendouz, F., Blanchardie, P., Denis, M., Orsonneau, J. L., and Lustenberger, P. (1989). *J. Steroid Biochem.* **34,** 325–330.
Harrison, R. W., and Yeakley, J. (1979). *Mol. Cell. Endocrinol.* **15,** 13–18.
Jonkers, G. H., Timmermans, H. A., deJong, F. H., Lambert, S. W. J., and Birkenhager, J. C. (1980). *FEBS Lett.* **111,** 403–406.
Koch, B., Lutz-Bucher, B., Briaud, B., and Mialhe, C. (1978). *J. Endocrinol.* **79,** 215–222.
Lovell-Smith, C. J., and Garcia-Webb, P. (1986). *Biochem. Biophys. Res. Commun.* **135,** 160–165.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193,** 265–275.
Maeda, T., Balakrishnan, K., and Mehdi, S. Q. (1983). *Biochim. Biophys. Acta* **731,** 115–120.
Muldoon, T. G., Watson, G. H., Graigevans, A., Jr., and Steinsapir, J. (1988). *J. Steroid Biochem.* **30,** 23–31.
Peck, E. J., Burgner, J., and Clark, J. (1973). *Biochemistry* **12,** 4596–4602.
Pietras, R. J., and Szego, C. M. (1979). *J. Steroid Biochem.* **11,** 1471–1483.
Pietras, R. J., and Szego, C. M. (1980). *Biochem. J.* **191,** 743–760.
Quigley, J. P., and Gotterer, G. S. (1969). *Biochim. Biophys. Acta* **173,** 456–469.
Sadler, S. E., and Maller, J. L. (1982). *J. Biol. Chem.* **257,** 355–361.
Sadler, S. E., Bower, M. A., and Maller, J. L. (1985). *J. Steroid Biochem.* **22,** 419–426.
Suyemitsu, T., and Terayame, H. (1975). *Endocrinology* **96,** 1499–1508.
Trueba, M., Guantes, J. M., Vallejo, A. I., Sancho, M. J., Marino, A., and Macarulla, J. M. (1987). *Int. J. Biochem.* **19,** 957–962.
Trueba, M., Vallejo, A. I., Rodriguez, I., Ibarrola, I., Sancho, M. J., Marino, A., and Macarulla, J. M. (1989a). *J. Membr. Biol.* **108,** 115–124.
Trueba, M., Ibarrola, I., Vallejo, A. I., Sancho, M. J., Marino, A., and Macarulla, J. M. (1989b). *Membr. Biochem.* **8,** 229–239.
Trueba, M., Rodriguez, P., Vallejo, A. I., Marino, A., Sancho, M. J., and Macarulla, J. M. (1990). *Exp. Clin. Endocrinol.* **95,** 169–180.
Trueba, M., Ibarrola, I., Ogiza, K., Marino, A., and Macarulla, J. M. (1991). *J. Membr. Biol.* **120,** 115–124.
Vallejo, A. I., Trueba, M., Sancho, M. J., Egaña, M., Marino, A., and Macarulla, J. M. (1986). *Exp. Clin. Endocrinol.* **87,** 201–207.
Weis, D. J., and Gurpide, E. J. (1988). *J. Steroid Biochem.* **31,** 671–676.