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Purification of a cortisol binding protein from hepatic plasma membrane

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Abstract

A cortisol binding protein from rat liver plasma membranes has been solubilized in active form by using the zwitterionic detergent CHAPS. Two types of binding sites have been characterised in both native and solubilized membranes. The first is of high affinity and low binding capacity (12 nM; 946 fmol/mg) and the other one is of low affinity and high capacity of binding (344 nM; 12 677 fmol/mg) for solubilized membranes. The purified material retained a binding activity comparable to that displayed by the original membrane. The specific binding activity was enriched about 12 700-fold, with an 8% yield. Analysis of the purified preparation on sodium dodecyl sulphate-polyacrylamide gel electrophoresis showed two protein subunits with molecular mass of 52 000 and 57 000 Da. The new cortisol-specific binding membrane protein could be related to the nongenomic effects previously described for this hormone.

Keywords: Cortisol; Liver plasma membrane; Cortisol binding protein; Solubilization; Purification

1. Introduction

It is usually assumed that steroid hormones enter the cell by passive diffusion, and bind cytoplasmic receptors, forming a hormone-receptor complex that translocates to the nucleus and modifies the transcription of specific gene sequences [1]. Nevertheless, it has been reported that steroids penetrate into the target cells by a specific transport mechanism rather than by free diffusion [2,3].

Moreover, some authors have characterised binding sites in plasma membrane for oestrogens, progestins and glucocorticoids [4,5]. Thus, we have characterised specific binding sites for corticosterone in mouse [6], rat liver [7] and kidney [8] and cortisol in mouse liver [9] plasma membrane. Ketosteroids have been utilised successfully as photoaffinity reagents for steroid receptors. The synthetic steroid R5020 or promegestone was employed to photolabel a plasma membrane specific protein [10]. SDS-PAGE showed significant R5020 binding to a 53 kDa molecule, that did not occur in the presence of unlabeled R5020 100 μM [10]. Recent reports indicate that activation of membrane oestrogen receptors causes a rapid release of intracellular calcium [11]. Moreover, glucocorticoids inhibit the formation of inositol phosphates in macrophages [12]. In vascular smooth muscle and endothelial cells an increase in IP3 was demonstrated 15–30 s after aldosterone administration. Pertussis toxin completely inhibited this response, whereas cholera toxin was without effect. These findings suggest the involvement of G proteins in signal transduction by mineralocorticoids [13]. In the present paper, the isolation and further purification of a cortisol binding protein from liver plasma membrane, that had not been reported so far, are described.

2. Materials and methods

2.1. Chemicals

[^{3}H]Cortisol (87 Ci/mmol) was purchased from New England Nuclear. Other steroid hormones and WGA-agarose were from Sigma Chemical Co. (St. Louis, MO, USA). Hydroxyapatite resin Biogel HTP was from Biorad. GF/C and GF/B filters were obtained from Whatman.

Abbreviations: CBG, corticosteroid binding globulin; CHAPS, 3-[(3-cholamidopropyl) dimethyl-ammonia]-1-propanesulfonate; CMC, critique micellar concentration; mCBP, membrane cortisol binding protein; NAG, N-acetylglucosamine; PMSF, phenylmethanesulfonyl fluoride; R5020, 17α,21-dimethyl-19-nor-pregne-4,9-diene-3,20-dione; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; WGA, wheat germ agglutinin.

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PPO, bis-MSB, scintillation grade toluene and Emusolv™ were supplied by Scharlau (Barcelona, Spain). All the other reagents were from Merck (Darmstadt, Germany).

2.2. Isolation of liver plasma membrane

Liver plasma membrane was obtained as described with slight modifications [14]. Livers from Sprague–Dawley male rats (weighting about 300 g) were perfused with NaCl 0.9%, removed and homogenised in 3 vol 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 $1500 \times g$ 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 25 min at $95\,000 \times g$ with TE buffer (20 mM Tris-HCl, 1.5 mM EDTA, pH 7.9) plus 140 mM NaCl to remove possible adsorbed proteins. The temperature was kept at 0–4°C during the isolation procedure. Purified plasma membranes were frozen in liquid nitrogen and stored in homogenisation buffer at −70°C. The purity of this fraction has been previously determined with marker proteins [7].

2.3. Cortisol binding assays

The standard mixture contained 20 mM Tris-HCl, 0.5 mM EDTA (pH 7.9), a sample, 10 nM [^{3}H]cortisol in a 1 ml total volume. Incubation was performed at 4°C for 45 min and terminated by the addition of 4 ml of cold 20 mM Tris-HCl, 0.5 mM EDTA (pH 7.9). The mixture was then filtered in vacuum through a GF/B filter, pre-soaked with 0.3% polyethylenimine (PEI) for 4 h [15]. The filter was washed two times with 4 ml of the same buffer. When membrane samples were assayed, it was carried out in the same way but filtered directly on GF/C filters without PEI treatment [7].

2.4. Solubilization

Non-ionic (Brij 35, octylglucoside, Triton X-100, Tween 20) and zwitterionic (CHAPS) detergents were examined for their capacity to solubilize the [^{3}H]cortisol binding activity from plasma membrane. All detergents tested solubilize 40–60% of the binding activity. CHAPS was selected because of its high CMC and little interference with the assays. Moreover, CHAPS has been used to isolate a number of receptor proteins in their active form. Liver plasma membrane fraction (about 4 mg protein/ml), containing 0.1 mM PMSF was stirred with 6 mM CHAPS for 15 min at 4°C. The mixture was centrifuged at $100\,000 \times g$ for 60 min. The supernatant was recovered as the solubilized extract. Thus CHAPS solubilize 60% of the membrane steroid binding protein and the active form is maintained for over 10 days at 4°C with a 20% loss (data not shown).

2.5. Hydroxyapatite chromatography

40 mM of Na_2HPO_4 was added to the solubilized extract and applied to a 25-ml hydroxyapatite column which had been equilibrated and eluted in 40 mM Na_2HPO_4, 4 mM CHAPS (pH 6.9) buffer. Eluted fraction was collected by gravity and assayed as described. The procedure was carried out in cold room.

2.6. WGA-agarose chromatography

Eluted fractions from hydroxyapatite were applied on a 4-ml column of WGA bound to agarose at 0.4 ml/min, washed with 20 ml of a buffer 40 mM Na_2HPO_4, 4 mM CHAPS (pH 6.9). Active fractions were eluted with 10 ml of 50 mM N-acetylglucosamine in the same buffer and assayed as described. The procedure was carried out in cold room.

2.7. High resolution size-exclusion chromatography

Active fractions from WGA-agarose chromatography were pooled and concentrated with a ultrafiltration device (Centriprep 10 Amicon) from 10 ml to 600 μl. 200–400 μl of samples were applied on a ProteinPack SW300 glass column (Waters) (300 × 8 mm) in a Smart micro purification system (Pharmacia) at room temperature. Chromatography was performed at a flow rate of 0.3 ml/min with buffer 20 mM Tris-HCl, 1.5 mM EDTA, 100 mM NaCl, 10% glycerol, 6 mM CHAPS (pH 7). 250-μl fractions were collected and aliquots assayed as described in Section 2.3.

2.8. High resolution ionic exchange chromatography

It was carried out in a Smart system at room temperature. A 100-μl mono-Q column (1.65 × 50 mm) was equilibrated with buffer 20 mM piperazine, 10% glycerol, 8 mM CHAPS (pH 5). Pooled active fractions (1 ml) from the previous step were applied at 50 μl/min. The column was washed with 1 ml of buffer and the protein eluted with a NaCl gradient with three isocratic steps at 100, 160 and 200 mM. Fractions of 100 μl were collected and aliquots assayed as described in Section 2.3.

2.9. SDS-polyacrylamide gel electrophoresis

Protein samples were precipitated by the addition of 10% trichloroacetic acid, centrifuged at $14\,000 \times g$ 15 min and washed twice with acetone as described [16]. The precipitates were dissolved in 40 μl of Laemmli buffer and subjected to electrophoresis in a 12.5% gel. Molecular

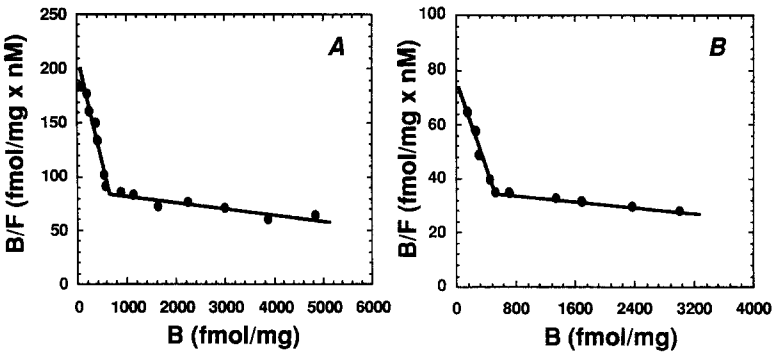

Fig. 1. Part A: Scatchard-analysis of the specific binding of [^{3}H]cortisol to rat liver plasma membranes as a function of radioactive steroid concentration (0.5–80 nM). Two sets of incubation were carried out simultaneously with the same plasma membranes fraction (0.2 mg/ml of protein) in the presence and absence of 10 μM cortisol for 60 min at 2°C in Tris buffer (pH 7.9). Part B: Scatchard plot of the specific binding data obtained in the same conditions from hepatic plasma membranes solubilized with 6 mM CHAPS. Points indicate the mean ± S.E.M. of quadruplicate determinations. B, [^{3}H]cortisol bound (fmol/mg protein); F, [^{3}H]cortisol free (nM).

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). Proteins were silver stained according to the Bio-Rad kit method.

2.10. Protein assays

Proteins were determined according to Bradford [17] with the Bio-Rad protein assay kit, using BSA as reference standard. In the last purification step, the protein concentration was calculated from absorbance at 280 nm, calibrating with a known amount of protein.

3. Results

3.1. Cortisol binding assays

Two types of binding sites have been characterised in rat plasma membrane. The first is of high affinity and low binding capacity (5 ± 1 nM and 1088 ± 76 fmol/mg protein) and the other one is of low affinity and high binding capacity (155 ± 12 nM and 13976 ± 1258 fmol/mg protein) (Fig. 1A). Scatchard-analysis of binding site data in the membrane solubilized by 6 mM CHAPS also showed the existence of two different and independent binding proteins. The kinetic parameters determined were: equilibrium dissociation constant 12 ± 3 and 344 ± 31 nM, and maximal binding capacity 946 ± 97 and 12677 ± 1160 fmol/mg protein for the high and low affinity binding sites respectively (Fig. 1B). The Hill coefficient calculated from the binding data were 1.058 (from native) and 0.949 (from solubilized membranes). We can infer that there are not cooperativity in both binding process. The binding characterised in native and solubilized membranes show a heterogeneous population of sites.

The kinetic parameters determined for high affinity binding sites in native and solubilized membrane are similar to that obtained by us and other authors with different corticoids and plasma membranes [6–9,18–20]. In order to characterise cortisol binding to rat liver plasma membrane, we checked the ability of different steroids to compete for the cortisol binding protein. The displacement of 4 nM [^{3}H]cortisol bound to rat liver plasma membrane high affinity binding site was carried out in the presence of increasing concentrations of unlabeled steroids. Their affinities vary in the following order: cortisol, prednisolone, 11β-hydroxyprogesterone, progesterone, deoxycorticosterone, corticosterone > 11α-hydroxyprogesterone > 20α-hydroxyprogesterone > 20β-hydroxyprogesterone > aldosterone > testosterone > dexamethasone > triamcinolone acetonide (data not

Table 1
Purification of the cortisol binding protein (mCBP) from rat liver plasma membrane

	Protein (μg/ml)	Volume (ml)	Total Prot. (μg)	Spec. Act. (fmol/μg)	Total Act. (fmol)	Recovery (%)	Purification (fold)
Membrane	4440	50	222000	0.6	123913	–	–
Supernatant	3427	45	154215	0.4	62586	100.0	1
Hydroxyapatite	110	50	5500	9.0	49585	79.7	22
WGA-agarose	20	10	200	90.7	18138	29.0	223
TSK300	7	1.5	10	1569.8	16483	26.3	3867
Mono-Q	2.5	0.4	1 [a]	5172.0	5172	8.3	12739

[a] Final protein concentration obtained by the integration of the A_{280} peak area.
For more methodological details see Section 2.

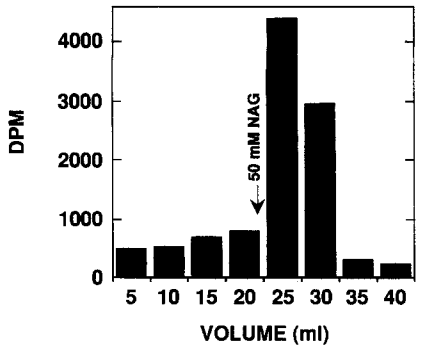

Fig. 2. WGA-agarose chromatography. mCBP-containing fractions eluted from the hydroxyapatite column were applied on a column of WGA-agarose at 0.4 ml/min. Active fractions were eluted with 10 ml of 50 mM NAG in the same buffer. Chromatography was performed as described in Section 2.6 and the aliquots binding activity were displayed.

shown). We can deduce that the high specific binding site observed is not the glucocorticoid cytosolic receptor, in view of the affinities that dexamethasone and triamcinolone acetonide have to that receptor [21]. The binding does not occur on the progesterone cytosolic receptor either, because the latter does not bind cortisol nor corticosterone. Also, the low affinity for aldosterone of the plasma membrane binding site allows to differentiate it from the glucocorticoid type-I receptor.

3.2. Purification of the membrane cortisol binding protein (mCBP)

First, the solubilized extract is subjected to hydroxyapatite chromatography (Table 1). The eluate usually contains 80–90% of the [^{3}H]cortisol binding activity and only about 5% of the initial protein. About 22-fold purification is achieved in this first step. This eluate is adsorbed onto WGA-agarose and the binding activity released by addition of 50 mM NAG (Fig. 2). This second step reaches

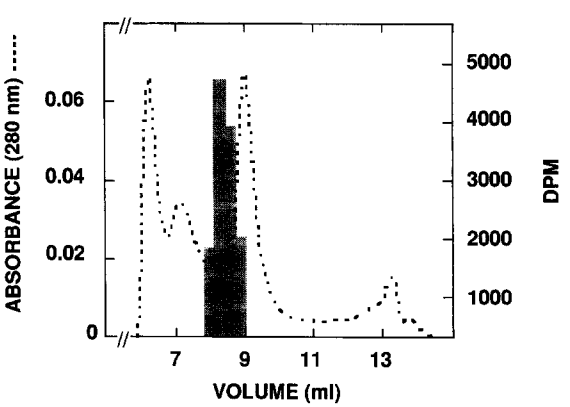

Fig. 3. Size-exclusion chromatography. Active fractions from WGA-agarose chromatography were pooled and concentrated with an ultrafiltration device [Centriprep 10 Amicon]. A 400-μl sample of the mCBP containing fractions was applied on a Protein Pack SW300 glass column (Waters) (300×8 mm) in a Smart System (Pharmacia). Chromatography was performed as described in Section 2.7. Absorbance 280 nm (broken line) and the aliquots binding activity (shaded area) were represented.

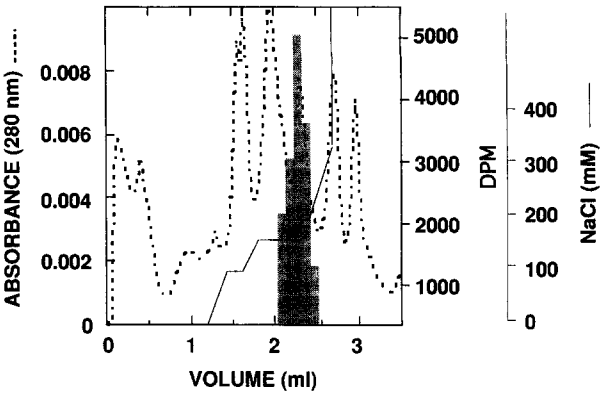

Fig. 4. Anion exchange chromatography. Pooled active fractions from the size-exclusion chromatography were injected on a Mono-Q™ column (1.6×50 mm) equilibrated with buffer 20 mM piperazine, 10% glycerol, 8 mM CHAPS (pH 5) at 50 μl/min in a Smart System (Pharmacia). Chromatography was performed as described in Section 2.8. Absorbance 280 nm (broken line) and the aliquots binding activity (shaded area) were represented.

223-fold purification. The NAG active fractions are concentrated and subjected to size-exclusion chromatography. The elution profile is shown in Fig. 3. Glycerol is added to the buffer in order to improve recovery [22]. The binding

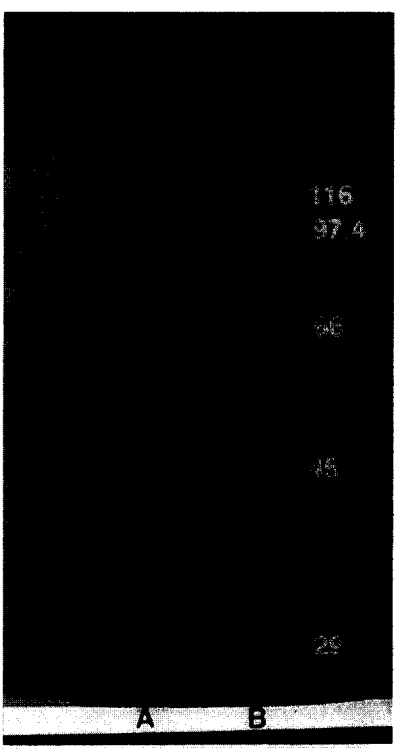

Fig. 5. SDS-polyacrylamide gel electrophoresis of purified mCBP. Protein samples were dissolved in 40 μl solubilization buffer (3% SDS, 10% glycerol, 20 mM Tris, 0.01% bromophenol blue) and subjected to electrophoresis in a 12.5% polyacrylamide gel. Lane A, active fractions from Mono-Q™ column. Lane B, molecular weight markers: 1. β-galactosidase (116 kDa), 2. phosphorylase b (97.4 kDa), 3. BSA (66 kDa), 4. ovalbumin (45 kDa), and 5. carbonic anhydrase (29 kDa). Electrophoresis was performed as described in Section 2.9.

activity is eluted in a symmetrical peak at around 8.5 ml retention volume. This corresponds to a protein of molecular mass of 120 kDa (according to molecular weight markers). The major impurity elutes at 9 ml. A 3867-fold purification is attained after this step. The last step of purification is carried out in an optimised system for protein micropurification (Smart System) with a mono-Q PC 1.6/50 mm column. The buffer is piperazine pH 5 and a higher CHAPS concentration (8 mM) is used to improve resolution. In addition, the gradient has three isocratic steps to better resolve the different peaks. The active fractions are obtained at 200 mM NaCl (Fig. 4) with a final purification of 12 700-fold over the solubilized extract (Table 1). The silver-stained SDS-PAGE gel (Fig. 5) shows two main bands of *m* 52 and 57 kDa. A representative purification of mCBP is shown in Table 1. About 12 700-fold purification is obtained from the solubilized extract with a recovery of 8%. According to Table 1, the specific activity reached (5172 fmol/μg protein) is about 50% of the theoretical maximum activity that could be obtained from the binding of one mol of [^{3}H]cortisol to one mol of 55 kDa protein. These results guarantee the preparation purity.

4. Discussion

The presence of specific steroid binding to plasma membranes has been reported by different authors [23–25]. Previous work from our laboratory shows the existence of specific steroid binding to plasma membranes [6–9]. In this study the cortisol binding to rat hepatic native and solubilized plasma membranes has been characterised and the kinetic properties are similar to those determined previously.

We also had characterised by [^{3}H]R5020 photoaffinity labelling the presence of a 53 kDa protein, that binds R5020 specifically [10]. In order to determine the analogy of the purified R5020 binding protein, photolabelling experiments were carried out with [^{3}H]R5020. We found that R5020 binds a different protein from cortisol, with similar molecular weight, in base to the different size exclusion chromatography elution profile and autoradiographic results.

Other authors have investigated the identity of membrane steroid binding protein in different systems. Gametchu et al. [26], labelling S-49 lymphoma cells with dexamethasone-21-mesylate, found multiple bands with molecular mass 170–500 kDa. A 50 kDa protein was labelled with [^{125}I]aldosterone in mononuclear leukocytes [27]. Ozegovic et al. [28] used SDS to solubilize an aldosterone binding protein in rat kidney plasma membrane. No further characterisation or purification has been reported and many important questions remain unanswered yet. This paper describes a rapid mCBP micropurification method using high resolution chromatographic methods. The first phase, hydroxyapatite chromatography, was a critical step decreasing the total protein amount with high recovery. The fact that this protein adsorbs to WGA-agarose and can be eluted with NAG suggested that mCBP could be a glycoprotein as most membrane receptors are. If a molecular weight of 55 000 for this protein band and one ligand site per molecule are assumed, the final specific activity was about 50% of the theoretical value. Since quantitative binding of the ligand was in all probability not obtained, one would expect that the purified material was essentially homogeneous and that the specific activity smaller than the theoretical one was due to denaturation of some of the hormone binding sites. The two different bands, to 52 and 57 kDa, could be due to the existence of a heterodimer with a native M_r of 110 000. This last value was the size-exclusion *m* obtained, but we can think of a possible hydrodynamic change produced by the binding of detergent molecules on a hydrophobic membrane protein. It is also known that sugars in glycoproteins greatly increase the Stokes radius. On the other hand, we can also deduce that these two bands could correspond with the two different types of binding proteins characterised, one of high and another one of low affinity for cortisol.

Some characteristics of this mCBP remind those of the serum steroid carrier, CBG or transcortin. In addition, some authors have described this membrane steroid binding protein as a transcortin-like protein. An alternative possibility was the plausible existence of two different types of CBG molecules: a soluble one in plasma and a second one anchored to the plasma membrane. These two kinds of proteins exist in the cases of IgM, insulin receptor, HLA antigens, interleukin-2 receptor and transferrin [29]. However, the negative results with anti-CBG antibodies, both in ELISA and Western blot techniques (data not shown), rather suggest that the membrane steroid binding protein and the corticosteroid binding globulin are unrelated proteins. Therefore, this new membrane cortisol binding protein could be related to the rapid effects of glucocorticoids on liver glycogen metabolism [30] and others nongenomic responses [31]. Recently, Sze and Iqbal [32] have reported the modulation by glucocorticoids of [^{125}I]calmodulin binding in synaptic plasma membranes. The present work about the purification of a cortisol binding protein from hepatic plasma membrane will allow in the future the micro sequencing and cloning of gene that codified this protein.

Acknowledgements

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