Identification of dexamethasone-binding sites on male-rat liver plasma membranes by affinity labelling

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Binding studies with [³H]dexamethasone identified two binding sites on plasma membranes prepared from the male rat liver, a low-capacity site with a K_D of 7.0 nM and a higher-capacity site with a K_D of 90.1 nM. Both sites exhibited glucocorticoid responsiveness and specificity for glucocorticoids and progestins. Triamcinolone acetonide, which competes well for the binding of dexamethasone to the cytosolic glucocorticoid receptor, did not compete well for the binding of [³H]dexamethasone to the plasma-membrane binding sites. The binding sites were sensitive to protease and neuraminidase treatment, and resistant to extraction with NaCl, but were extracted with the detergent Triton X-100. As these experiments indicated the presence of plasma-membrane protein components which bind glucocorticoids at physiological concentrations, affinity-labelling experiments with dexamethasone mesylate were conducted. Two peptides were specifically labelled, one at approx. M_r 66000 and one at M_r 45000. The M_r -66000 peptide was not sensitive to glucocorticoids, and was extracted by NaCl, and so did not correspond to either of the sites identified in the dexamethasone-binding studies. The M_r -45000 entity, on the other hand, resembled the dexamethasone-binding sites in its response to glucocorticoid manipulation of the animal and in its resistance to salt extraction. This peptide was not present in rat serum. Thus we have identified a plasma-membrane peptide which binds dexamethasone. Whether this peptide is involved in transport of the glucocorticoid across the plasma membrane remains to be determined.

INTRODUCTION

The passage of steroids across the plasma membrane has generally been assumed to occur by simple diffusion, a non-specific process, because steroids are small, lipophilic and non-ionic molecules, and as such should pass easily through membranes. However, several lines of evidence support the concept that a carrier-mediated uptake process for steroids is operational in plasma membranes of steroid-hormone target tissues. Pietras & Szego [1] showed that endometrial and liver cells bind to a matrix of oestrogen immobilized on an inert support, whereas intestinal cells do not. These results suggest that plasma membranes of oestrogen-target cells possess oestrogen-binding sites, which may be involved in transport of the steroid across the plasma membrane. Others have investigated steroid uptake in whole cells and have concluded that carrier-mediated uptake is responsible for entry into the cell [2–4]. Cytochemistry has identified steroid-binding sites on plasma membranes [5]. Evidence has been reported for carrier-mediated uptake of glucocorticoids by plasma-membrane vesicles, used to avoid complications with cytoplasmic components [6–8], and binding sites on plasma-membrane vesicles have been reported for oestrogen [9,10], progesterone [11] and aldosterone [12]. Although all of these studies are consistent with a protein-mediated cellular uptake system for steroids, several other reports have questioned the studies and their conclusions. The whole-cell experiments have been criticized on the grounds that somewhat tenuous approximations had to be made to account for

the contribution of the intracellular receptor to the binding [13–15]. The possibility of artifactual localization of binding sites owing to redistribution has been raised both in the cytochemical studies [16] and in the plasma-membrane-vesicle studies [17,18].

One way of providing conclusive evidence for an active transport system for steroids in plasma membranes is reconstitution of the steroid-transport system in liposomes. This necessitates identification and purification of the plasma-membrane steroid-binding sites. To date several plasma-membrane steroid-binding sites have been characterized, but a steroid-binding site has not been directly identified. We now report the identification of dexamethasone-binding sites on male-rat liver plasma membranes, determined with the affinity reagent dexamethasone mesylate.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats weighing 200–250 g were obtained from Charles River Canada (Montreal, Que., Canada) and maintained on a diet of Wayne Lab Blox (Allied Mills, Chicago, IL, U.S.A.) and tap water *ad libitum*. The rats were killed by decapitation, and the livers were quickly removed, stripped of connective tissue, weighed and placed on ice. Rats were adrenalectomized under halothane anaesthesia and subsequently received 0.9% NaCl in their drinking water. These rats were used 7 days after surgery.

Abbreviations used: DTT, dithiothreitol; CBG, corticosteroid-binding globulin.

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Materials

[³H]Dexamethasone (38–48.9 Ci/mmol), [³H]dexamethasone mesylate (38–48.9 Ci/mmol) and unlabelled crystalline dexamethasone mesylate were purchased from New England Nuclear (Lachine, Que., Canada). Unlabelled steroids were from Steraloids (Wilton, NH, U.S.A.), except for cholesterol, triamcinolone, triamcinolone acetonide and triamcinolone diacetate, which were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sucrose (Ultra-pure) was from Schwartz–Mann (Cambridge, MA, U.S.A.). Taps and Trizma base were from Sigma. All chemicals for polyacrylamide-gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Neuraminidase was checked for the presence of contaminating proteolytic activity by its application to a casein/agar plate followed by overnight incubation at 20 °C. The plate did not detect any contaminating proteases.

Plasma-membrane preparation

Plasma membranes were prepared by modifications of the procedure of Ray [19]. Male rat liver was minced and suspended (1:12, w/v) in 1 mM-NaHCO₃ containing 0.5 mM-CaCl₂ and 1 mM-dithiothreitol (DTT) (Buffer A) and homogenized in a Dounce homogenizer (Kontes Scientific Glassware/Instruments, San Leandro, CA, U.S.A.) with 25 strokes of the loose-fitting pestle. The homogenate was then diluted with Buffer A (1:100, w/v) and left on ice for 5 min before centrifugation for 30 min at 1000 *g*. The supernatant was discarded and the pellet was resuspended in Buffer A (1:50, w/v) with five strokes of the loose-fitting pestle in the Dounce homogenizer. After centrifugation for 15 min at 1000 *g*, the supernatant was discarded and the pellet resuspended (1:25, w/v) before re-centrifugation for 16 min at 1000 *g*. Pellets were suspended to a final concentration of 48% (w/w) sucrose in Buffer A. The suspension was divided (12 ml/tube) among six tubes (Beckman SW28 plastic tubes) and overlaid with 7 ml of 45% sucrose, 10 ml of 41% sucrose and 3 ml of 37% sucrose before centrifugation at 100 000 *g* for 2 h. The membrane band at the 37%–/41%-sucrose interface was collected, suspended in Buffer A, but without DTT, and centrifuged at 33 000 *g* for 30 min. The final pellet was suspended either in 10 mM-Tris/HCl, pH 7.5, or in 25 mM-Taps, pH 8.6.

Binding assay

Unless stated otherwise, plasma-membrane suspension containing 100 µg of protein was incubated at 0–4 °C for 16 h in the presence of 50 nM-[³H]dexamethasone with or without 1000-fold excess of unlabelled dexamethasone or other unlabelled steroids in a total volume of 250 µl of 25 mM-Taps, pH 8.6. The incubations were stopped by centrifugation in a Microfuge B for 1 min and aspiration of the supernatant after the addition of 1 ml excess of buffer. The pellet was resuspended in 1 ml of buffer, the suspension was centrifuged again and the supernatant was aspirated. The tip of the tube containing the pellet was cut off and dissolved in 500 µl of NCS tissue solubilizer (Amersham Canada, Oakville, Ont., Canada) at 40–50 °C. The radioactivity was determined by scintillation counting in 5 ml of Ready-Solv HP (Beckman Instruments, Palo Alto, CA, U.S.A.). When the effect of various enzymes was being tested, enzymes were added to the assay tubes in 25 µl of 25 mM-Taps, pH 8.6. To

the control, buffer alone was added. The tubes were then incubated for 20 min at 20 °C, followed by another 20 min on ice to cool the tubes before termination of the assay. Salt and detergent extractions were done in two ways. (1) After the incubation, the membranes were washed with 2 × 1 ml of 25 mM-Taps, pH 8.6, and then the extractant in Taps was added. The suspension was left at 20 °C for 30 min, with resuspension by vortex-mixing every 10 min, before termination of the assay. Control tubes were incubated in buffer alone. (2) Alternatively 100 µg of membrane was made up to 1.00 ml in the extractant in 25 mM-Taps, pH 8.6. This suspension was left on ice for 30 min, then centrifuged, and the pellet was washed twice in 25 mM-Taps, pH 8.6, before the usual binding assay.

Affinity labelling

Unless stated otherwise, plasma membranes (150 µg of protein/tube) were incubated with 100 nM-[³H]dexamethasone mesylate in 25 mM-Taps, pH 8.6 (final volume 250 µl), at 0–4 °C for 12 h. Non-specific binding was determined by addition of the affinity label to samples which had been incubated with a 1000-fold excess of unlabelled dexamethasone for 12 h. The incubation was terminated as described previously for the binding assay, and the pellets were solubilized in SDS-sample buffer. The samples were run on SDS/polyacrylamide-gel electrophoresis, in 7.5%-acrylamide running gels. The gels were cut into 2 mm slices, dissolved in H₂O₂ and, after addition of 10³ units of catalase and 25 mg of ascorbic acid, dissolved in 10 ml of Ready-Solv HP for counting. Alternately, the gels were autoradiographed after treatment with Enhance (Beckman Instruments).

Biochemical analyses

Protein content of subcellular fractions was determined by the method of Lowry *et al.* [20], with bovine serum albumin as standard. The purity of the [³H]-steroids and the identity of the steroid bound to liver plasma membranes after incubation of the plasma membranes with radioactive dexamethasone were analysed by t.l.c. The chromatography plates were coated with silica gel (GF) (REDI-PLATE, Fisher Scientific Co., Pittsburgh, PA, U.S.A.), and the solvent system was ethyl acetate/chloroform (1:1, v/v) as described in ref. [21].

RESULTS

Characteristics of plasma-membrane preparation

As we had shown that DTT was necessary for the optimal binding of dexamethasone to nuclear envelopes [22], the effect of DTT on the plasma-membrane preparation described by Ray [19] was tested. In one experiment, addition of 1.0 mM-DTT improved the yield from 1.52 to 2.43 mg of protein/g of liver. DTT (1.0 mM) was therefore included in all homogenization buffers. Addition of protease inhibitors [benzamidine (10 mM), pepstatin (1 mg/l), EDTA (2 mM) and EGTA (2 mM)] to the homogenization buffer dramatically decreased the yield of membranes, and therefore they were not used. We obtained plasma membrane with an average yield of 1.89 ± 0.70 mg of protein/g of liver (*n* = 15), whereas Ray [19] reported a yield of 1.30 mg of protein/g of liver.

The membrane preparation contained 20.51 units of the plasma-membrane marker 5'-nucleotidase/mg of protein, which represented 29.4% of the homogenate

Fig. 1. Time- and temperature-dependent association of [3 H]dexamethasone to male-rat liver plasma membranes

Plasma membranes (100 μ g of protein/assay tube) were incubated in triplicate with 100 nM-[3 H]dexamethasone in the absence or presence of 100 μ M-dexamethasone at 0–4 $^{\circ}$ C, 20 $^{\circ}$ C or 37 $^{\circ}$ C. Specific binding was calculated from the difference in binding of [3 H]dexamethasone in the presence (non-specific binding) and absence (total binding) of unlabelled dexamethasone. Total, non-specific and specific binding at 0–4 $^{\circ}$ C are shown, but only specific binding at 20 $^{\circ}$ C and 37 $^{\circ}$ C is shown.

activity and a 33-fold enrichment over that in the homogenate. Importantly, lactate dehydrogenase was present with an activity of 11.28 units (0.55 unit/mg of protein), which represented only 0.09% of the homogenate activity, and a specific activity of 0.09 relative to the homogenate, indicating very little contamination of the plasma-membrane preparation with cytosolic components.

Characterization of dexamethasone-binding sites

Fig. 1 shows that specific binding of [3 H]dexamethasone to male-rat liver plasma membranes is thermolabile. At 22 and 37 $^{\circ}$ C, optimum binding occurred at 1 h. At 37 $^{\circ}$ C, the binding was never as high as at 22 $^{\circ}$ C, indicating that degradation of the binding sites was occurring at that temperature. After 1 h, degradation occurred at both 22 and 37 $^{\circ}$ C, with less residual binding occurring at the higher temperature. At 0–4 $^{\circ}$ C, optimum binding occurred at 6–7 h. At this temperature there was no evidence of degradation with up to 24 h of incubation. The time course in the presence of low concentrations of dexamethasone (1 nM) also showed that equilibrium had been reached at 12 h and no degradation occurred to 18 h. Therefore, equilibrium binding conditions were defined as a 12–16 h incubation at 0–4 $^{\circ}$ C. Fig. 1 also shows that non-specific binding at equilibrium represented 17% of the total binding. We verified that binding assays at pH 8.6 gave identical results with those obtained at pH 7.4 (results not shown), because we intended to perform affinity labelling studies at pH 8.6. When DTT was omitted from both the homogenization buffers and the binding assays, not only was the yield of plasma membrane decreased, as discussed above, but the binding of [3 H]dexamethasone was also decreased by 21%. When

Fig. 2. Analysis of [3 H]dexamethasone binding to rat liver membranes

Rat liver plasma membranes (100 μ g of protein) were incubated with concentrations of [3 H]dexamethasone (0.5–200 nM) in the absence or presence of a 1000-fold excess of unlabelled dexamethasone for 16 h at 0–4 $^{\circ}$ C. (a) Saturation curve of binding to liver plasma membranes from intact rats. Specific binding ($\blacktriangle$) was determined by subtraction of non-specific binding ($\circ$) from total binding ($\bullet$). (b) Scatchard analysis of specific [3 H]dexamethasone binding to liver plasma membranes obtained from intact rats: $\bullet$, plot obtained from the experimental data. The broken line A is the plot for the high-affinity binding site corrected for the low-affinity site, and the broken line B is the plot from the low-affinity site corrected for the high-affinity site [29].

DTT was used in buffers during preparation of the membranes, the further addition of DTT to the binding assay mixture did not alter the number of binding sites. Therefore, DTT was used in preparatory buffers, but not in the binding assay mixtures. Binding was shown to be a linear function of the membrane concentration to 0.80 mg of protein/ml. By using the assay conditions described above, the effect of three additional washes on the specific binding was investigated. The specific binding did not decrease, indicating that the binding components are not loosely attached to the membrane. Analysis of bound steroid by t.l.c. revealed that, after incubation of the membrane fraction with radiolabelled dexamethasone for 16 h, 92% of the recovered label was dexamethasone.

A saturation analysis of [3 H]dexamethasone binding to rat liver plasma membranes obtained from intact animals was then undertaken. Fig. 2 is the analysis of binding to one plasma-membrane preparation. Fig. 2(a) shows that the binding is saturable. When the data for specific binding were subjected to Scatchard [23] analysis, a curvilinear plot indicative of two binding sites was obtained (Fig. 2b). In the normal rat liver plasma membrane there is a low-capacity high-affinity site with a K_D of 7.0 ± 5.2 nM and a maximum binding capacity of

Table 1. Specificity of [³H]dexamethasone binding to male-rat liver plasma membranes

Rat liver plasma membranes (100 µg of protein) were incubated with 50 nM-[³H]dexamethasone for 16 h at 0–4 °C in the absence or presence of 10 µM, 20 µM and 50 µM (200-, 400- and 1000-fold excess) of the unlabelled steroid. The data are expressed as percentage displacement of total binding. Total binding of [³H]dexamethasone in these studies was 1.03 pmol/mg of protein.

Unlabelled hormone	Displacement (%)		
	10 µM	20 µM	50 µM
Dexamethasone	64.8	76.7	77.9
Corticosterone	52.0	61.4	61.0
Deoxycorticosterone	48.5	63.9	68.8
Cortisol	49.1	62.4	62.6
Triamcinolone	54.6	54.1	71.1
Triamcinolone diacetate	51.2	63.1	64.0
Triamcinolone acetonide	42.9	40.0	47.3
Progesterone	66.6	71.2	66.2
R5020	62.7	60.2	66.5
Aldosterone	3.7	23.7	30.2
Cholesterol	14.6	17.6	13.0
Testosterone	44.1	43.4	53.4
5α-Dihydrotestosterone	35.9	48.2	47.3
5β-Dihydrotestosterone	21.9	37.1	42.9
17β-Oestradiol	61.6	67.6	68.0
17α-Oestradiol	45.9	42.3	42.7
Diethylstilboestrol	59.6	57.9	62.4

155 ± 22 fmol/mg of protein, and a high-capacity low-affinity site with a K_D of 90.1 ± 20.3 nM and a maximum binding capacity of 2572 ± 347 fmol/mg of protein ($n = 2$). We next examined the glucocorticoid dependency of these rat liver plasma-membrane binding sites. Scatchard analyses of [³H]dexamethasone binding to membranes from 7-day-adrenalectomized rats showed that these membranes have only the high-capacity low-affinity sites ($K_D = 121.4 ± 38.2$ nM) present in lower numbers (maximum capacity = 706 ± 58 fmol/mg of protein) compared with the normal rat liver plasma membranes ($n = 2$). Thus adrenalectomy results in loss of the high-affinity binding sites from the plasma membrane.

To assess the steroid specificity of the [³H]dexamethasone-binding sites, competition studies with unlabelled steroids were performed (Table 1). The natural glucocorticoids, corticosterone, deoxycorticosterone and cortisol, competed for the binding of [³H]dexamethasone with almost the same potency as unlabelled dexamethasone. The synthetic glucocorticoid analogues, triamcinolone and triamcinolone diacetate, were also relatively effective competitors. However, triamcinolone acetonide was a poorer competitor than either of these two analogues. Progesterone, promegestone (R5020) and 17β-oestradiol were all effective competitors of [³H]dexamethasone for the binding, whereas the androgens testosterone and 5α-dihydrotestosterone competed less effectively. Aldosterone and cholesterol competed poorly for binding to the plasma membrane sites. Oestradiol-17α and 5β-dihydrotestosterone did not compete as well as their natural isomers.

The effect of various enzymes on the binding of [³H]dexamethasone to rat liver plasma membranes was investigated. Chymotrypsin and protease VI caused a marked loss of binding [75.0 ± 8.5% and 59.1 ± 9.8% respectively; mean ± s.d. ($n = 2$)]. On the other hand, the sites were resistant to trypsin treatment. Thus the binding sites appear to be protein in nature, but either of unusual amino acid content, such that they do not contain the basic residues cleaved by trypsin or, more likely, the basic residues may be inaccessible to trypsin. Neuraminidase caused a 30% loss of binding, indicating that the binding sites are glycoproteins.

The salt- and detergent-extractability of the binding sites was also examined. The binding sites were not extracted by 0.1 M-, 0.6 M- or 1.0 M-NaCl. The non-ionic detergent Triton X-100 extracted 99.9% of the binding sites. These results were obtained whether extraction with the salt or detergent was carried out before or after incubation of [³H]dexamethasone with the plasma membranes. Taken together, these extraction studies indicate that the binding sites are integral membrane proteins.

Affinity labelling of rat liver plasma-membrane dexamethasone-binding sites

We next affinity-labelled the plasma membrane with the electrophilic reagent dexamethasone mesylate [24] to identify the binding sites. To ensure that binding of dexamethasone mesylate was to the same sites as those binding [³H]dexamethasone, we did a competition study with unlabelled dexamethasone mesylate against

Fig. 3. Affinity labelling of the rat liver plasma membrane with [³H]dexamethasone mesylate

Plasma membranes (150 µg of protein/tube) were incubated for 12 h at 0–4 °C in 25 mM-Taps, pH 8.6, with 100 nM-[³H]dexamethasone mesylate. Non-specific binding was determined by addition of [³H]dexamethasone to plasma membranes which had been incubated with 100 µM-dexamethasone for 12 h. The labelled plasma membranes were boiled in SDS-sample buffer for 5 min and run on SDS/polyacrylamide (7.5%) gel electrophoresis. The gel was cut into 2 mm slices and dissolved in 150 µl of 50% H₂O₂/0.8% NH₃ (19:1, v/v) overnight at 60–70 °C. After addition of 10³ units of catalase and 25 mg of ascorbic acid in a total volume of 250 µl, the sample was counted for radioactivity in 10 ml of Ready-Solv. Total binding (●) and specific binding (○) are shown. Positions of M_r markers are shown at the top.

Fig. 4. Autoradiograms of SDS/polyacrylamide-gel-electrophoresis profiles of liver plasma membranes and serum after affinity labelling with dexamethasone mesylate

(-), Incubated with [3 H]dexamethasone mesylate; (+), incubated with [3 H]dexamethasone mesylate and dexamethasone. (a) Autoradiogram of the plasma membranes; (b) autoradiogram of affinity-labelled serum obtained from intact rats. Serum was adjusted to 70 % -satd. $(\text{NH}_4)_2\text{SO}_4$ with a saturated solution of $(\text{NH}_4)_2\text{SO}_4$. The pellet was obtained by centrifugation at 10000 g for 20 min and resuspended in 25 mM-Taps, pH 8.6. Samples (150 μ g of protein/tube) were affinity-labelled with dexamethasone mesylate and run on SDS/polyacrylamide-gel electrophoresis as described in the Experimental section.

[3 H]dexamethasone. Dexamethasone mesylate competed for [3 H]dexamethasone binding to rat liver plasma membranes, but with lower affinity than dexamethasone [IC₅₀ (concn. of competing steroid giving 50 % inhibition of [3 H]dexamethasone binding) of 2.15 nM, compared with 0.60 nM for dexamethasone]. As dexamethasone alone or dexamethasone together with dexamethasone mesylate caused the same displacement of [3 H]dexamethasone, the mesylate function of the dexamethasone mesylate is not causing non-specific binding of [3 H]dexamethasone to the membranes.

Fig. 3 shows the radioactivity profile of an SDS/polyacrylamide gel obtained after incubation of plasma membranes with [3 H]dexamethasone mesylate for 12 h at pH 8.6. The incubation with the affinity label resulted in two major specifically labelled peaks, one at M_r approx. 45000 and one at M_r approx. 66000. There is also evidence for a third labelled peptide of just slightly higher M_r than 45000. However, this peak was not consistently observed. When present, it was included in calculations with the M_r -45000 entity.

The extent of [3 H]dexamethasone mesylate binding to

Table 2. Effect of several parameters on the binding of [3 H]dexamethasone mesylate to rat liver plasma membranes

Affinity-labelling studies were done as described in the Experimental section: (a) and (b), and (c) and (d), are from the same plasma-membrane preparation. In (f) the plasma-membrane suspension was made up to 1 ml in the extractant in 25 mM-Taps, pH 8.6. This suspension was left on ice for 30 min, then centrifuged, and the pellet was washed twice in 25 mM-Taps, pH 8.6, before affinity-labelling as described in the Experimental section.

Parameter	Specific binding of [3 H]dexamethasone mesylate (fmol/mg of protein)	
	M_r 45000	M_r 66000
(a) pH		
7.4	325	
8.6	589	
(b) Length of incubation (h)		
0	123	122
6	169	182
12	589	262
(c) Concn. of [3 H]dexamethasone mesylate (nM)		
50	651	291
100	1053	229
150	1654	454
(d) Protein concn. (μ g)		
50	64	168
100	483	243
150	1654	229
(e) Hormonal status		
Intact	589	262
Adrenalectomized	167	250
(f) Effect of salt and detergent extraction (% extracted)		
Control	0.0	0.0
0.1 M-NaCl	3.8	23.7
0.6 M-NaCl	2.5	31.8
1.0 M-NaCl	0.0	0.0
0.1 % Triton X-100	55.3	27.5

the plasma membranes is approximately doubled when the labelling incubation is at pH 8.6, rather than at pH 7.4 (Table 2a). Table 2(b) summarizes the data for the specific binding recovered in the two major peaks after incubation with [3 H]dexamethasone mesylate for 0, 6 or 12 h. The dependence of affinity labelling on the concentration of affinity ligand is shown in Table 2(c), and the dependence on the concentration of plasma membrane is shown in Table 2(d). Therefore, affinity-labelling incubations were for 12 h in the presence of 100 nM-[3 H]dexamethasone mesylate and 150 μ g of plasma-membrane protein at pH 8.6. We next examined the affinity labelling of rat liver plasma membranes from animals subjected to various hormonal regimes before death. Table 2(e) shows that adrenalectomy of the animal resulted in a marked decrease in the M_r -45000 entity here, but did not affect the labelling of the M_r -66000 entity. To ensure that serum components which may be contaminating the plasma-membrane preparation were not being affinity-labelled, $(\text{NH}_4)_2\text{SO}_4$ -precipitated serum was prepared from the same animals and affinity-labelled

under conditions identical with those used to affinity-label the plasma membrane. Figs. 4(a) and 4(b) are autoradiographs of SDS/polyacrylamide gels. Fig. 4(a) shows the labelling of rat liver plasma membranes, and Fig. 4(b) shows that there is a heavily labelled band at M_r 66000, but no labelling which corresponds to the M_r -45000 entity.

The salt- and detergent-extractability of the binding sites for [3 H]dexamethasone mesylate was also examined. Table 2(f) shows that the M_r -45000 entity was resistant to salt extraction, whereas the M_r -66000 entity was partially extracted by 0.1 M- and 0.6 M-NaCl. Peptides of both M_r values were extracted by Triton X-100.

DISCUSSION

We have shown that two classes of dexamethasone-binding sites are present on male-rat liver plasma membranes. Membrane binding sites for glucocorticoids have been reported in liver [6], but only for natural steroids; in contrast with the study presented here, dexamethasone-binding sites were not found. In the light of our investigations, the explanation for the failure to detect dexamethasone-binding sites in those experiments is that optimal binding conditions were not used. Dexamethasone-binding sites have been reported in plasma membranes from skeletal muscle [8], but again, in that system no binding occurred at 0 °C, in contrast with our findings. In this case, the explanation may be the tissue difference. The presence of more than one class of ligand-binding site has been demonstrated in other systems. Bresson *et al.* [10] reported a biphasic Scatchard plot for the binding of oestradiol to rat pituitary membranes, and multiple binding sites for thyroid hormones have also been reported on rat liver plasma membranes [25].

Since the dexamethasone-binding studies provided evidence for peptide-binding sites having affinities consistent with their binding of glucocorticoids in physiologically relevant conditions, we proceeded to identify the binding sites with the affinity-labelling analogue of dexamethasone, dexamethasone mesylate. Two sites were specifically labelled by the affinity reagent, one of M_r 66000 and one of M_r 45000. The M_r -66000 band did not correspond to the binding sites identified in the binding studies described above. Its quantity was not altered after adrenalectomy, and it was partially extracted with NaCl. Furthermore, it was present in high amounts in serum. In fact, it is known that dexamethasone mesylate covalently labels compounds not bound by dexamethasone itself. Our calculations of efficiency of the dexamethasone mesylate labelling indicated that more than was being identified in the binding studies was being labelled by dexamethasone mesylate. Simons [24] has shown that serum albumin is labelled with dexamethasone mesylate. The M_r -66000 entity may be serum albumin.

On the other hand, the M_r -45000 entity has the same characteristics as the dexamethasone-binding sites: it is decreased upon adrenalectomy and is not extracted by NaCl. Its concentration in the plasma membrane is such that it may correspond to the lower-affinity plasma-membrane binding site.

To date, our methods of detection have not allowed identification of the lower-capacity higher-affinity entity. One candidate for this site is the glucocorticoid receptor, which has an M_r of approx. 90000 [26]. The affinity of

dexamethasone for the membrane sites was lower than that of dexamethasone for intracellular liver receptor sites and, although the steroid specificity was generally the same, triamcinolone acetonide competes effectively with dexamethasone for the cytosolic glucocorticoid receptor, whereas triamcinolone acetonide was not a good competitor for dexamethasone binding to the plasma-membrane binding sites. These findings and the M_r values of the affinity-labelled entities suggest that the membrane sites are not the glucocorticoid receptor. However, the possibility exists that the membrane environment has altered both the specificity and affinity of dexamethasone binding, and that a cleavage product of the glucocorticoid receptor is present on the membrane.

The possibility of redistribution from the serum and from the cytosol to the plasma membrane in both the binding studies and the affinity-labelling studies must be considered. In the binding studies, we chose dexamethasone as the glucocorticoid ligand to avoid binding to serum binding globulins, which may have been contaminating the plasma-membrane preparation. Measurements of lactate dehydrogenase indicated that there was little cytosolic contamination of the plasma-membrane preparation. Furthermore, the extraction studies, which showed that concentrations of NaCl up to 1.0 M did not extract binding sites, and that the detergent Triton X-100 extracted all of the binding sites, indicated that the binding sites are integral membrane proteins. In the affinity-labelling studies, we have shown, using conditions identical with those used to affinity-label the plasma membrane, that there is no labelled peptide corresponding to the M_r -45000 entity in the serum. Therefore, we conclude that the M_r -45000 entity is a genuine component of the plasma membrane.

Corticosteroid-binding globulin (CBG) binding sites have been identified on plasma membranes of glucocorticoid-responsive tissues. Specific binding of CBG has been detected on membranes prepared from human prostates [27]. Specific CBG-binding sites have been demonstrated in membranes prepared from rat kidney, and cross-linking techniques have been used to establish that a CBG-specific membrane binding protein has an M_r of 16000. Cellular uptake of CBG-cortisol complexes is suggested to be the result of receptor-mediated endocytosis, and it is proposed further that uptake and delivery of CBG-bound steroids to nuclear receptors would prevent loss of the hormonal signal by metabolism [28]. Although dexamethasone does not itself bind to CBG, it is possible that the membrane environment has altered the binding properties and that CBG in the membrane binds dexamethasone and dexamethasone mesylate.

In conclusion, binding studies and affinity-labelling techniques have allowed us to identify an M_r -45000 peptide on rat liver plasma membranes which binds glucocorticoids. This is the first identification of a plasma-membrane binding protein for steroids. The role of this peptide in transport of the steroid into the cells and its possible relationship to CBG and/or the CBG-binding protein requires investigation.

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