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Specific Cytoplasmic Glucocorticoid Hormone Receptors in Hepatoma Tissue Culture Cells

JOHN D. BAXTER* AND GORDON M. TOMKINS*

Laboratory of Molecular Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **Kinetic and equilibrium studies are presented for the reversible binding of [^{3}H]dexamethasone by "specific" macromolecular receptors in the cytoplasmic fraction of cultured rat hepatoma cells. As in the case of the nuclear receptors in the same cells, the binding affinities of various steroids for the cytoplasmic receptors are closely correlated with the activities of these compounds as inducers of both tyrosine aminotransferase (EC 2.6.1.5) and cell adhesiveness. This suggests that the binding reaction is important for the biological effects of the hormones. Steroid-binding activity is inhibited by various proteases, mercurials, and 1 M KCl, but not by DNase or RNase. The receptors sediment in sucrose gradients in 0.5 M KCl near 4S, and at lower ionic strength near 7S; some of their physical properties are altered upon binding steroid. Bound dexamethasone can be recovered from the receptors as the unaltered steroid.**

Hepatoma tissue culture cells contain "specific" glucocorticoid hormone receptors, which seem to mediate the hormonal induction of tyrosine aminotransferase (EC 2.6.1.5; ref. 1). Studies of intact cells indicated that these receptors occur in both the nucleus and, to a lesser extent, in the cytoplasm. The present report describes the properties of the cell-free binding reaction between dexamethasone and the "specific" cytoplasmic receptors, and discusses the relationship between this reaction and the biological activity of the steroids.

MATERIALS AND METHODS

Many of the reagents used and the techniques of growing and harvesting hepatoma tissue culture cells have already been described (1). Radioactive steroids were prepared in aqueous solution, and the benzene in which they had been dissolved was removed by evaporation. The [^{3}H]dexamethasone (9–12 Ci/mmol) migrated on thin-layer chromatograms (1) with authentic nonradioactive dexamethasone. Nonradioactive steroid solutions were prepared in ethanol. The concentration of ethanol in the reaction mixtures did not alter the steroid-binding reaction.

To prepare cytoplasmic extracts, we resuspended cells, after harvesting, in phosphate-buffered saline (1) (0–4°C) and centrifuged at 600 × *g* for 4 min; all subsequent operations were performed at 0–4°C. The cell pellet was resuspended in 1–2 volumes of 20 mM *N*-tris-(hydroxymethyl)methylglycine (Tricine), 2 mM $CaCl_2$, 1 mM $MgCl_2$, (pH 8.0), homogenized as described (1), and the mixture was centrifuged at 140,000 × *g* for 1 hr. The supernatant medium, "cytoplasmic extract," was stored at 0–4°C and used for experiments within 48 hr.

The binding reaction was performed at 0–4°C by incubation of aliquots of cytoplasmic extract in the homogenization buffer (ordinarily in a total volume of 0.4 ml) with [^{3}H]dexamethasone, either alone or in the presence of competing nonradioactive dexamethasone (5×10^{-6} to 5×10^{-5} M). After the incubation period (90 min unless stated otherwise), a 50-μl suspension of activated charcoal (100 mg/ml of Norit A, Fisher) was added to each 0.4 ml of the reaction mixture. (The charcoal was treated with 6 N HCl, washed, neutralized, and finally washed with water prior to use.) The charcoal-containing mixture was agitated on a Vortex mixer for 20 sec, then centrifuged at 600 × *g* for 2 min. The supernatant medium was transferred to other centrifuge tubes and centrifuged at 12,000 × *g* for 10 min, and aliquots were assayed for radioactivity (counting efficiency 31–33%) and protein (1).

RESULTS

Assay of specific dexamethasone binding by cytoplasmic extracts

The cell-free dexamethasone binding reaction was studied by an assay which depends on the fact that activated charcoal can adsorb steroids (see also refs. 2 and 3). Thus, when a solution of radioactive steroid is treated with charcoal, which is then removed by centrifugation, only a small fraction of the radioactivity remains in the supernatant. However, when an equal concentration of the steroid is first allowed to react with a cytoplasmic extract, and the mixture is then treated with charcoal, considerably more radioactivity remains unsedimented (which indicates the binding of the steroid by cytoplasmic components). If the cytoplasmic extract is incubated in the presence of the same concentration of radioactive steroid plus a much higher concentration of nonradioactive steroid (to compete with radioactive compound for a limited number of binding sites), the amount of radioactivity remaining unsedimented after charcoal treatment decreases toward the value observed when no cytoplasmic extract is used. The difference between the amount of [^{3}H]dexamethasone resisting charcoal treatment in the competitor-free and competitor-containing incubations is referred to as the "specifically" bound steroid. This value has been shown by gel filtration experiments to correspond to the amount of a dexamethasone bound to high-affinity macromolecular receptors

* Present address: University of California, San Francisco, Department of Biochemistry and Biophysics, San Francisco, Calif. 94122.

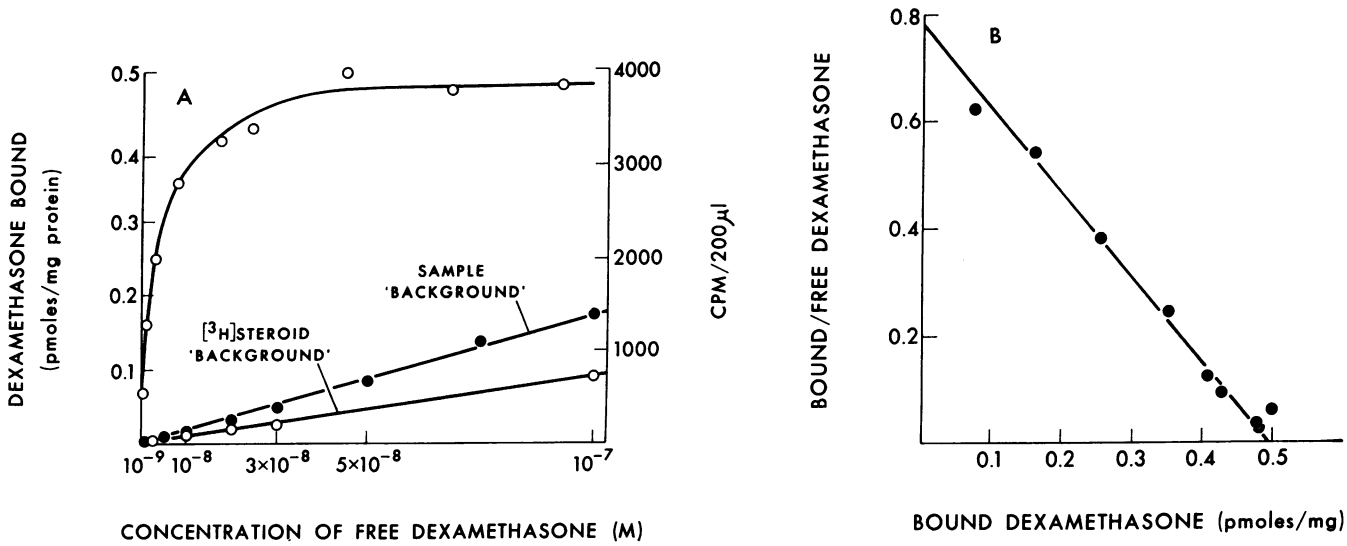

FIG. 1. (*A*) Specific cytoplasmic binding of [^{3}H]dexamethasone. 2-hr incubations were performed in triplicate at 0–4°C. The tubes contained, in a volume of 0.4 ml, 5.4 mg/ml of cytoplasmic protein and various concentrations of [^{3}H]dexamethasone (9 Ci/mmol) in the absence or presence of "competing" (1.25×10^{-5} M) nonradioactive dexamethasone. Other incubations were performed with [^{3}H]dexamethasone in buffer alone ([^{3}H]*steroid "background"*). After incubation, the mixtures were treated with charcoal as described, and 200-μl aliquots were assayed for radioactivity remaining in the supernatant medium. The amount of specifically bound dexamethasone (*top curve*) was determined by subtracting the radioactivity that resisted charcoal adsorption in the competitor-containing incubation (*sample "background"*) from the comparable value obtained in the corresponding competitor-free incubation. The data, represented either as cpm per 200-μl aliquot, or as pmol of steroid bound/mg of cytoplasmic protein, are plotted as a function of the free-steroid concentration at equilibrium. The free steroid was determined by subtracting the bound steroid from the total steroid present in the incubation mixture.

FIG. 1. (*B*) Scatchard plot of the specific-binding data shown in Fig. 1*A*.

(manuscript in preparation). Obviously, the assay would not detect bound radioactivity that dissociates during the charcoal treatment or that, itself, adsorbs to the charcoal.

The assay was performed so that: the amount of steroid bound was linearly related to the receptor concentration (between 2 and 20 mg of cytoplasmic protein/ml); the charcoal was not saturated with steroid, even when the competitive nonradioactive hormone was present; the mixing time and the amount of charcoal used allowed for adequate binding of only free steroids; and the charcoal did not diminish the protein content of the sample.

In Fig. 1*A*, the amount of dexamethasone specifically bound by cytoplasmic extracts is plotted as a function of the free-steroid concentration in the incubation medium. The [^{3}H]steroid "background" is the amount of free steroid not removed by the charcoal (*see legend*) and the sample "background" is the amount of radioactivity remaining after charcoal treatment in incubations containing the competing nonradioactive steroid. The latter presumably reflects both free steroid, not removed by charcoal, and steroid "nonspecifically" associated with other molecules in the cytoplasmic extract. The specific binding data are plotted in Fig. 1*B* by the Scatchard technique (4) and yield a single straight line, indicating that only a single class of specific receptor sites is detected by the assay. The dissociation constant for the reaction: dexamethasone + receptor $\rightleftharpoons$ dexamethasone–receptor complex is 3.0×10^{-9} M (SD = 0.9×10^{-9} M as determined in seven experiments at several con-

TABLE 1. *Comparison of the dexamethasone concentrations required for specific binding and for induction of tyrosine aminotransferase and cell adhesiveness*

Response measured	Dexamethasone concentration (M) required for half-maximal response	Dexamethasone concentration (M) required for maximal response	Lowest dexamethasone concentration (M) required for response
Specific binding by cell-free cytoplasmic extracts	3.1×10^{-9} (SD = 0.8×10^{-9})	$1\text{–}4 \times 10^{-8}$*	$<10^{-11}$
Specific binding by hepatoma tissue culture cells at 37°C	about 9×10^{-9}	about 5×10^{-8}	$<5 \times 10^{-10}$
Induction of tyrosine aminotransferase	8×10^{-9}	$5 \times 10^{-8} - 10^{-7}$	about 2×10^{-9}
Induction of cell adhesiveness	4×10^{-9}	$2\text{–}5 \times 10^{-8}$	about 2×10^{-9}

Cell-free binding of [^{3}H]dexamethasone was measured at 0–4°C. Data for binding of [^{3}H]dexamethasone by whole cells at 37°C are taken from a previous report (1). Data for the induction of tyrosine aminotransferase and of cell adhesiveness at 37°C were taken from Samuels and Tomkins (5) and Ballard and Tomkins (6, 7), respectively.

* This value represents the range of seven experiments.

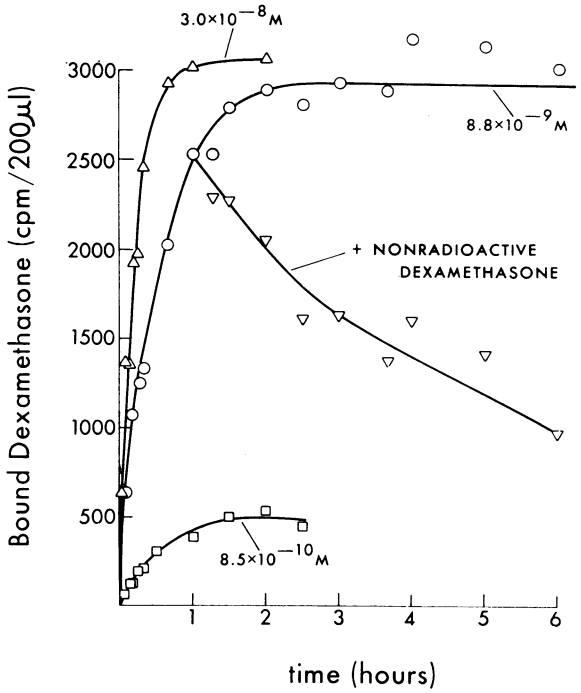

FIG. 2. Kinetics of dexamethasone association and dissociation with the specific cytoplasmic receptors. [^{3}H]Dexamethasone (9 Ci/mmol) in the absence and presence of competing (10^{-5} M) nonradioactive dexamethasone was added at zero time to samples of cytoplasmic extract (final concentration of 8.8 mg protein/ml) to yield the concentrations indicated. Aliquots of 0.4 ml were mixed with charcoal at the indicated times and assayed for specific binding. The initial free [^{3}H]dexamethasone concentrations, shown in the figure, decreased to 7.0×10^{-10} M, 7.8×10^{-9} M, and 2.8×10^{-8} M, respectively, during the first 20 min. Concentrations of free [^{3}H]dexamethasone were determined by subtracting the bound dexamethasone from the total present in the mixture. As indicated, at the end of 1 hr, nonradioactive dexamethasone was added to a portion of one of the incubations to a final concentration of 6×10^{-5} M (▽—▽, nonradioactive dexamethasone added).

centrations of cytoplasmic extract and receptor). For this experiment, the concentration of receptor sites was 2.6×10^{-9} M. If we assume 5×10^{-7} mg of protein per cell, there are about 1.5×10^5 receptor sites per cell.

The relationship between specific dexamethasone binding and induction

Table 1 illustrates the similarity between the dexamethasone concentrations required for half-maximal and for maximal biological activity, and for specific dexamethasone binding by either whole cells (1) or by cell-free extracts. These correlations suggest that binding may be a necessary event in the induction.

Kinetics and reversibility of specific cytoplasmic binding

Fig. 2 depicts the time course of association and dissociation at 4°C of the dexamethasone-receptor complex. The data show that the reaction is readily reversible, since adding an excess of nonradioactive steroid to the complex rapidly displaced receptor-bound radioactivity.

For the binding reaction: steroid + receptor $\rightleftharpoons$ steroid–receptor complex, second-order kinetics of binding should apply. Therefore, a plot, at early times, of time versus $\log_{10}$ (free-steroid concentration/free receptor-site concentration) should be linear (8). Fig. 3*A* shows that this prediction is verified. The mean association-rate constant (8) was determined in four experiments, at four steroid and two receptor-site concentrations, and the range was 2.6–5.5 $\times 10^6$ M^{-1} min^{-1}, with a mean of 4.1×10^6 M^{-1} min^{-1}. Furthermore, the rate of dissociation for the binding reaction should be first order and a plot of $\log_{10}$ (bound-steroid concentration) versus time should be linear, which also appears to be the case (Fig. 3*B*). The dissociation-rate constant of 2.9–3.1 $\times 10^{-3}$ min^{-1} was determined (8) from the time of half dissociation (221 min for the experiment shown in Fig. 2).

From the dissociation- and association-rate constants, the equilibrium (dissociation) constant was calculated to be 7.4×10^{-10} M (5.3×10^{-10}–1.9×10^{-9} M), as compared to 3.0×10^{-9} M determined from the equilibrium data presented above.

Effects of inducer analogs on specific dexamethasone binding

A number of steroids were previously classified (5) on the basis of their activity at 10^{-5} M as inducers of tyrosine aminotransferase or cell adhesiveness (6), and on their ability (at 10^{-5} M) to interfere with induction by 10^{-7} M cortisol. These steroids also influence the specific nuclear binding of dexamethasone or cortisol in the same way (1).

Table 2 correlates the effects of a larger number of analogs on the specific cell-free cytoplasmic binding of [^{3}H]dexamethasone with their biological actions. At the concentrations used, the "optimal" inducers totally prevented specific binding by 10^{-8} M dexamethasone, whereas "sub-optimal inducers" allowed 0–10%, "anti-inducers" allowed 11–31%, and "inactive" steroids allowed 39–105% of the binding. Evidently, the biological activity of the steroids correlates very well with their ability to compete with dexamethasone for specific binding [presumably reflecting their own affinity for the specific receptors (manuscript in preparation)]. These studies are also consistent with the hypothesis (5) that various steroids exert their biological effects not only by reacting with the receptors, but also by additional factors (perhaps by determining the proportion of the receptors in an "active" conformation).

Properties of the specific dexamethasone receptors

Binding activity was totally destroyed by incubating cytoplasmic extracts with any of the following proteolytic enzymes: trypsin (500 μg/ml), elastase (porcine pancreas, 75 μg/ml), papain (1 mg/ml), α-chymotrypsin (75 μg/ml), or pronase (B grade, 100 μg/ml). Similar treatments with collagenase (*Clostridium histolyticum*, 75 μg/ml), RNase (bovine pancreas, 50 μg/ml), DNase (bovine pancreas, 75 μg/ml), DNase (porcine spleen, 50 μg/ml), neuraminidase (*C. perfringens*, 50 μg/ml), lysozyme (chicken egg-white, 100 μg/ml), or lipase (porcine pancreas, 1 mg/ml) had no effect on binding activity. Therefore, the receptors are probably proteins.

Binding activity is also inhibited by $HgCl_2$ (10^{-4} M), *p*-chloromercuriphenyl sulfonic acid (10^{-4} M), *p*-chloromercuribenzoic acid (2×10^{-4} M), Triton X-100 (0.05%), and 1 M KCl.

In homogenizing buffer at low ionic strength, the receptors sediment in sucrose gradients (9) at 6–8 S (Fig. 4). However, when a cytoplasmic extract is incubated with [^{3}H]dexa-

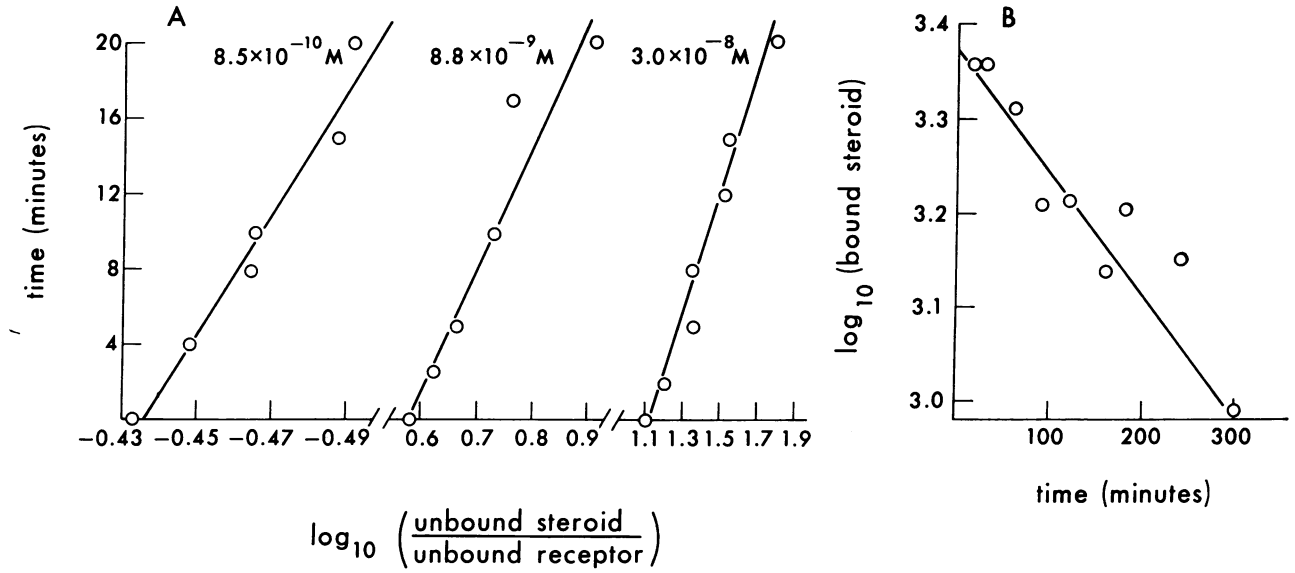

FIG. 3. Analysis of the kinetic data. *A*. From data shown in Fig. 2, time is plotted (for the first 20 min, *see text*) as a function of $\log_{10}$ (free dexamethasone concentration/free receptor-site concentration). The unbound steroid (free-dexamethasone concentration) was determined by subtracting the total of the bound steroid from the total dexamethasone concentration. The unbound receptor-site concentration was determined by subtracting the bound concentration of dexamethasone from the total receptor-site concentration. The latter was assumed to equal the amount of specifically bound dexamethasone in the reaction with 3×10^{-8} M [^{3}H]dexamethasone (at which concentration the specific receptors are nearly saturated, see Table 1). *B*. The dissociation data (Fig. 2) was recalculated as $\log_{10}$ (bound-steroid concentration) and plotted as a function of the time after adding the "chase" of nonradioactive dexamethasone.

TABLE 2. *Effect of various steroids on (a) the cell-free binding of dexamethasone by the specific cytoplasmic receptors and (b) on induction of tyrosine aminotransferase and cell adhesiveness*

Steroid (or analog)	Biological activity (5)	[^{3}H]dexamethasone (10^{-8} M) bound in the presence of 10^{-5} M analog* (% of control)	Induction by 10^{-5} M of analog†		Induction by 10^{-7} M cortisol in the presence of 10^{-5} M analog‡	
			TAT	adhesiveness	TAT	adhesiveness
SC14266§	Inactive	105 (97–109)	0	0	100	100
Tetrahydrocortisol	Inactive	101 (97–102)	0	...	100	...
Epicortisol	Inactive	99 (97–101)	0	8	100	100
20β-Hydroxycortisol	Inactive	92 (88–96)	0	...	100	...
Androstenedione	Inactive	79 (76–80)	0	...	100	...
20α-Hydroxycortisol	Inactive	39 (36–41)	0	...	100	...
Cortisone	Anti-inducer	31 (31–31)	0	...	48	...
Testosterone	Anti-inducer	27 (25–28)	0	0	66	87
17β-Estradiol	Anti-inducer	25 (25–26)	0	...	57	...
17α-Methyltestosterone	Anti-inducer	11 (10–12)	0	0	12	0
5α-Dihydrocortisol	Sub-optimal inducer	10 (9–11)	34	38	68	58
11β-Hydroxyprogesterone	Sub-optimal inducer	0 (−0.3–1.3)	33	72	59	...
17α-Hydroxyprogesterone	Sub-optimal inducer	2 (0.7–2.1)	5	0	16	24
Corticosterone	Optimal inducer	0 (−0.6 to −0.1)	100	84	>100	>100
Cortisol	Optimal inducer	0 (+0.1–0.9)	100	100	>100	>100
Dexamethasone	Optimal inducer	0 (−0.5–0.4)	100	100	>100	>100

* Aliquots of cytoplasmic extract were incubated in a total volume of 0.4 ml with 10^{-8} M [^{3}H]dexamethasone (9 Ci/mmol) and 10^{-5} M nonradioactive analog for 2 hr, and radioactivity that resisted charcoal treatment was measured. "Background" radioactivity (see Fig. 1*A*) was subtracted from each value and the difference is shown as the % of the value (minus "background") obtained on incubation with [^{3}H]dexamethasone in the absence of competitor (*control*). The "100% value" was 10,300 cpm/ml [(11,235) − (935) cpm/ml]. The mean of three incubations is reported; the range of these is shown in parentheses.

† The data for tyrosine aminotransferase (5) and for cell-adhesiveness (6) induction are reported as the % of the induction with 10^{-5} M cortisol obtained with 10^{-5} M analog (TAT = tyrosine aminotransferase).

‡ The data (5, 6) are reported as the % of induction by 10^{-7} M cortisol alone, compared to the induction by 10^{-7} M cortisol in the presence of 10^{-5} M analog.

§ Data for induction by the spironolactone analog, SC14266 (Searle), were obtained from P. L. Ballard (personal communication).

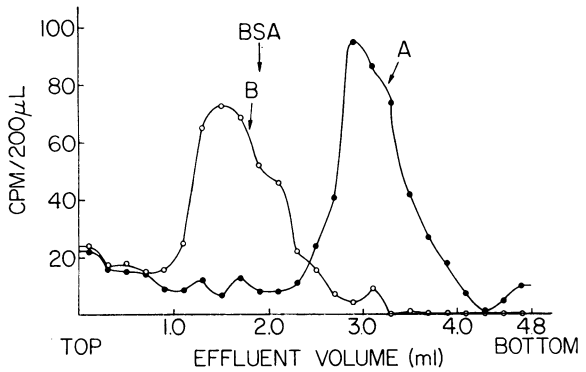

FIG. 4. Sedimentation of the specific receptor in sucrose gradients. Aliquots of cytoplasmic extract (29 mg of protein/ml) were incubated for 90 min with 2×10^{-8} M [^{3}H]dexamethasone (9 Ci/mmol), then KCl was added to a portion to a final concentration of 0.5 M. 0.3-ml aliquots of the samples, with and without 0.5 M KCl, were layered onto 4.5-ml, 5–20%, sucrose gradients (9) in the buffer used for the homogenizations. The gradient that received the KCl-containing sample also contained 0.5 M KCl. A third gradient received, instead of cytoplasmic extract, 0.3 ml of 50 mg/ml of bovine plasma albumin (Pentex biochemicals). The gradients were centrifuged at 56,700 rpm in a Beckman SW65.1 rotor at 4°C for 11 hr. After centrifugation, 0.2-ml fractions were collected, made up to 0.4 ml with homogenizing buffer, and assayed for radioactivity that resisted charcoal treatment. The latter is plotted as a function of the effluent volume. Other experiments showed that these concentrations of sucrose do not affect the charcoal assay. *A*, (●), gradient without added KCl. *B*, (○), gradient with added KCl. The migration of bovine plasma albumin (indicated as *BSA* on the figure) was assayed by measuring the absorbance at 280 nm of fractions collected from the gradient.

methasone and then made up to 0.5 M KCl, the specific receptors sediment nearer to 4 S (Fig. 4). Other, usually minor, peaks that sediment between 2 and 10 S are also seen occasionally. None of these peaks is observed when the binding reaction is performed in the presence of excess nonradioactive steroid, a result indicating that they represent the sedimentation of specifically bound dexamethasone.

In crude extracts, the receptors are much more stable when complexed with steroid than in the free state. Furthermore, there is a rapid loss of binding activity when steroid-free cytoplasmic extracts are incubated with various materials, including cell nuclei, glass beads, Sephadex, and cellulose casing (Visking). However, if the cytoplasmic extract is first incubated with [^{3}H]dexamethasone and then treated similarly, the rate of inactivation is considerably slower.

Extraction and chromatography of bound radioactivity

Dexamethasone and cortisol bound to the specific nuclear receptors can be recovered unaltered (1). Furthermore, we were unable to demonstrate metabolic conversion of dexamethasone by intact cells, although cortisol is converted to metabolites that include dihydrocortisol and tetrahydrocortisol (1). Radioactivity associated with the specific cytoplasmic receptors in cell-free binding experiments was also examined. After incubation of cytoplasmic extracts with 2×10^{-8} M [^{3}H]dexamethasone (12 Ci/mmol) in the absence and presence of excess nonradioactive dexamethasone, 81,000 and 9,900 dpm/ml, respectively, resisted charcoal treatment. The radioactivity that resisted charcoal treatment (88% of which was specifically bound) was extracted with dichloromethane from the sample incubated without competing nonradioactive steroid, and was subjected to thin-layer chromatography in solvent systems described previously (1). Virtually all of it migrated with authentic nonradioactive dexamethasone.

DISCUSSION

The present studies show that each hepatoma tissue culture cell contains about 150,000 molecules of a specific protein that reversibly binds adrenal steroid hormones. The steroid–receptor complexes sediment more rapidly at low ionic strength than at increased salt concentrations, and the binding reaction is inhibited by reagents that interact with protein-SH groups.

The data strengthen our earlier impression (1) that these specific receptors in some way mediate the actions of glucocorticoid hormones, since there is a good correlation between the association with the receptors and the biological activity of the steroids. Thus, "optimal" and "sub-optimal" inducers bind more strongly with the receptors (as judged by displacement of radioactive dexamethasone) than do compounds previously found to be biologically inactive. Likewise, "anti-inducers" interfere with the formation of the dexamethasone–receptor complex. The quantitative relationships between inducer–receptor complex formation and biological activity are also consistent with a role for the specific receptors in hormone action. Such correlations do not exist for other steroid interactions described previously, such as the "nonspecific" association (1), steroid binding by P2, a cytoplasmic macromolecule in hepatoma tissue culture cells (10), or for steroid binding by rat transcortin (manuscript in preparation). Although steroid binding by transcortin can be measured by the charcoal assay (manuscript in preparation), the reaction with P2 is not "specific" by the displacement criteria used in the present communication.

When intact cells are incubated with radioactive steroid, the majority of the specifically bound hormone is localized in the nucleus (1). Isolated nuclei, however, are much less able to associate specifically with radioactive steroid (1). These observations lead us to believe that the cytoplasmic receptor described here is somehow involved in promoting the nuclear localization of specifically bound steroid. If this is the case, then the glucocorticoid-receptor system strongly resembles other steroid-receptor systems (11–15), in which the initial cell–steroid interaction occurs in the cytoplasm, and is followed by a transfer of bound steroid to the nucleus.

If the nucleus is indeed the site of the biological actions of the steroids, we must still identify the exact nuclear reaction or reactions modulated by the hormone–receptor complex. Previous work in our laboratory, as well as on numerous other eukaryotic systems (16), has suggested that specific gene expression is controlled by labile inhibitors of messenger RNA translation. The relation between the nuclear localization of the steroid and this control of messenger function is not as yet clear.

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