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PHARMACOLOGICAL CHARACTERIZATION OF TYPE II GLUCOCORTICOID BINDING SITES IN AtT20 PITUITARY CELL CULTURE

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Summary—Recent evidence indicates that at least two functional glucocorticoid receptors (Type I and Type II) are present in many tissues. It has also become increasingly recognized that, as in other systems, stimulus–response relationships for steroid hormones are often nonlinear. Thus, precise pharmacological parameters are required to establish a functional relationship(s) between binding site and response characteristics. We therefore pharmacologically characterized a glucocorticoid binding site present in AtT20 mouse pituitary cells, a cell line extensively used in studying Type II glucocorticoid receptor function. By several different criteria, glucocorticoids were shown to bind to a single class of binding sites, which, in comparison to available literature, correspond to classical Type II glucocorticoid receptors. No evidence for Type I adrenal steroid binding sites was observed, under the experimental conditions used. Unambiguous K_b values for both glucocorticoid agonists and antagonists were therefore calculated. These parameters should prove of use in elucidating the relationships between glucocorticoid receptor activation and different responses in both AtT20 cells and other glucocorticoid responsive tissues.

INTRODUCTION

In endocrine systems a close agreement between binding of a hormone (i.e. K_b) to a specific site and the induction of a biological response (i.e. EC_{50}) has generally been accepted as evidence that these two events are causally related. However, it has become increasingly apparent that stimulus–response relationships for steroid hormones are often nonlinear, i.e. for a given hormone, the EC_{50} of the measured response is not the same as its binding site affinity (K_b). For example, differences in EC_{50} and E_{max} values for glucocorticoids have been reported for both induction of different enzymes in the same cell line [1, 2] and the same enzyme in different cell lines [2, 3]. Similarly, the EC_{50} values for glucocorticoid inhibition of ACTH release in primary pituitary cell culture are markedly time-dependent [4]. Thus, as widely recognized in other fields e.g. [5], classification of steroid binding sites as physiologically relevant receptors requires accurate pharmacological characterization of both steroid binding and steroid efficacy.

The presence of multiple binding sites for glucocorticoids further complicates the identification of receptor linked responses. Glucocorticoids bind to at least two classes of intracellular sites, Type I (mineralocorticoid) and Type II (glucocorticoid) (reviewed in [6]). Activation of these sites by steroid binding is generally believed to initiate the biological effects of these hormones. Both classes of binding sites appear to be present in most, but not all, tissues, although in different proportions. Subtypes of both sites have been suggested e.g. [7–9]. Selective binding of glucocorticoids to transcortin is also well documented to complicate interpretation of glucocorticoid binding site studies e.g. [10, 11].

In general, identification of glucocorticoid binding sites has relied on limited descriptive characterization, e.g. the relative binding affinity of ligands (RBA, commonly defined as the percentage difference between IC_{50} values derived from competition studies against a fixed concentration of radiolabeled ligand, where the IC_{50} of the corresponding unlabeled ligand = 100%). RBAs can only be used to characterize a specific binding site if care is taken to prevent binding of radiolabeled and cold ligands to other sites present in the preparation studied. For example, use of the selective Type II ligand, RU26988, to selectively mask Type II binding sites has allowed partial characterization of putative Type I receptor binding sites e.g. [12, 13]. It is unclear, however, to what extent previously reported Type II binding site characteristics are influenced by the copresence of Type I

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receptors, since selective ligands for both sites have only recently become available. Accurate pharmacological characterization of both sites is required to devise appropriate strategies for studying the physiological roles of glucocorticoid receptor subtypes.

The mouse pituitary cell line, AtT20, has been extensively utilized as a model system for Type II glucocorticoid receptor function e.g. glucocorticoid regulation of ACTH secretion and POMC gene expression [14]. Partial characterization of glucocorticoid binding sites, utilizing nonselective ligands, suggests that only Type II binding sites are present in this cell line [15, 16]. However, Type I receptors have been reported in other pituitary preparations [12]. Glucocorticoid regulation of ACTH secretion in AtT20 cells, as in rat pituitary, has been suggested to occur through multiple mechanisms [14], raising the possibility of different glucocorticoid receptors mediating these effects. As a first step in elucidating the true relationships between glucocorticoid receptor activation and response, we therefore reexamined and extended the pharmacological characteristics of glucocorticoid binding sites in AtT20 cell cytosol.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM, 4500 mg/l glucose) was obtained from Gibco (Grand Island, N.Y.); fetal calf and bovine sera were obtained from J. R. Scientific (Woodland, Calif.); [6,7-(n)^{3}H]dexamethasone (39–50 Ci/mmol) and [1,2,6,7-(n)^{3}H]corticosterone (112 Ci/mmol) were obtained from New England Nuclear (Boston, Mass) and [1,2-(n)^{3}H]aldosterone (75 Ci/mmol) was obtained from Amersham (Arlington Heights, Ill.). RU26988 (11β, 17β-dihydroxy-21-methyl-17α-pregna-1,4,6-trien-20-yn-3-one) and RU26752 (7α-(acetylthio)-17α-hydroxy-3-oxo-pregn-4-ene-21 carboxylic acid γ-lactone 7 acetate) were generously provided by Roussel-UCLAF (Romainville, France). Unlabeled corticosterone was obtained from Steraloids (Wilton, N.H.). All other compounds were from standard commercial sources.

Cell culture

Mouse AtT20 tumor cells were grown and subcultured on 15 cm plates, in DMEM containing 5% fetal calf and 10% bovine sera. Following removal of medium, cells were harvested by scraping into serum free DMEM (5 ml/plate), 4–5 days after subculturing (60–80% confluency). Cells were refed 1–2 days prior to harvesting. Following centrifugation (1000 *g* for 15 min at room temperature) the medium was replaced with cold TEGMD homogenization/incubation buffer (10 mM Tris, 1 mM EDTA, 20 mM molybdic acid, 5 mM dithiothreitol and 10% glycerin, pH 7.4) and centrifugation repeated.

Cytosolic binding studies

Cytosol from AtT20 cells was prepared and incubated as previously described [17]. In brief, pooled cell pellets were homogenized in cold TEGMD buffer and then centrifuged at 105,000 *g* for 60 min at 4°C. Aliquots of the supernatant/cytosol fraction (47 ± 11 μg cytosolic protein/100 μl final incubation volume) were incubated with various concentrations of radiolabeled steroids, with or without unlabeled competitors, for 18–22 h at 4°C.

Separation of bound from free steroid was achieved by passage through LH-20 Sephadex columns (1.25 ml). Triplicate aliquots (100 μl) from each incubate were assessed to minimize errors associated with intercolumn variation in recovery. Eluate fractions containing bound steroid were counted on a scintillation counter at 45% efficiency.

Specific binding was defined as the difference in radiolabeled steroid bound in the absence or presence of RU26988 (1.0 μM). Cytosolic protein content was determined by the method of Bradford [18], using bovine serum albumin as standard.

Initial experiments established that the 18–22 h incubation period used was sufficient to achieve and maintain equilibrium binding for all three radiolabeled steroids tested, and that binding was linear over the protein concentration range used.

Data analysis

In competition studies, IC_{50} values were obtained by fitting data to a single site binding model [19, 20]:

$$B = \frac{B_t}{1 + ([L]/IC_{50})^n}$$

where B_t = total number of binding sites labeled by the radioligand in the absence of competing drugs, B = specific binding at a given radioligand concentration, [L] = concentration of the competing ligand, IC_{50} is the concentration of competing ligand that inhibits 50% of the total specific binding and n = slope index (a parameter that describes the steepness of the curve).

Data from the concentration-dependent binding of radioligands were fit to a general form of the logistic function [21, 22]:

$$B = \frac{B_{max}}{1 + (K_d/[D])^n}$$

where [D] = radiolabeled ligand concentration, B_{max} = maximum binding capacity and K_d = [D] producing 50% B_{max}. Fitted parameter estimates were not significantly different from those obtained by Scatchard analysis.

A partial *F*-test [21] was used to determine the parallelism of [^{3}H]dexamethasone concentration–binding curves in the presence and absence of cold competitors. This was done by simultaneously fitting the set of curves to logistic functions with the slope indices either being allowed to vary, or being constrained to the same value.

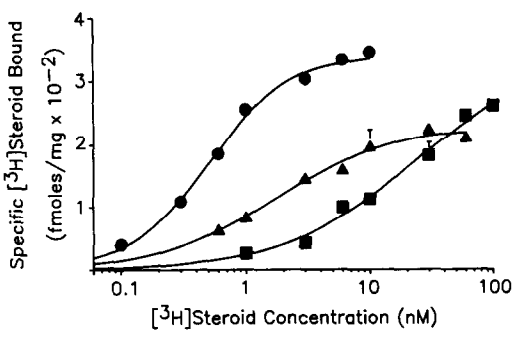

Fig. 1. Concentration-dependent binding of [^{3}H]steroids in AtT20 cell cytosol. Aliquots of AtT20 cell cytosol were prepared and incubated with increasing concentrations of [^{3}H]dexamethasone (●), [^{3}H]corticosterone (▲) or [^{3}H]aldosterone (■), for 18 h at 4°C, as described in Experimental. Nonspecific binding, defined as [^{3}H]steroid bound in the presence of 1 μM RU26988, was measured for each [^{3}H]steroid concentration, and subtracted from all data points. Each point is the mean ± SEM of triplicate data points from a single representative experiment. Curves shown are fits of the data to a logistic function.

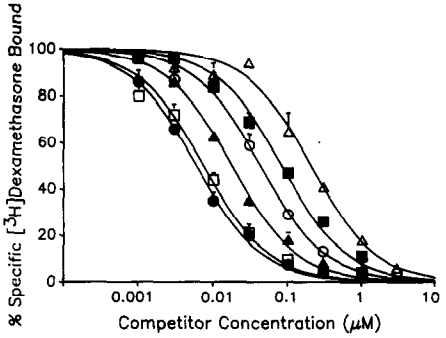

Fig. 2. Ligand specificity of [^{3}H]dexamethasone binding sites in AtT20 cell cytosol. Aliquots of AtT20 cell cytosol were prepared and incubated with [^{3}H]dexamethasone alone (3.0 ± 0.1 nM), or in the presence of increasing concentrations of cold competitors [(□), RU26988; (●), dexamethasone; (▲), corticosterone; (○), progesterone; (■) aldosterone and (△) RU26752], as described in Experimental. Nonspecific binding, defined as [^{3}H]dexamethasone binding in the presence of 1 μM RU26988, was subtracted from all data points. Each point represents the mean ± SEM from three separate experiments. Curves shown are fits of the data to a single site binding model, with the slope index constrained to 1.

Least squares regression analysis was used in all curve fitting procedures, with convergence set a 0.1%. Additional analyses and statistical tests were done on the PROPHET computer system. All values are given as mean ± SEM, with significance set at $P < 0.05$.

RESULTS

Specific binding of radiolabeled dexamethasone and corticosterone was concentration-dependent and saturable (Fig. 1). Specific [^{3}H]aldosterone binding was also concentration-dependent, but saturation could not be determined due to the high ratio of nonspecific binding sites at high (> 100 nM) [^{3}H]aldosterone concentrations. The magnitude of specific [^{3}H]dexamethasone binding was remarkably consistent between assays and characterized by a fitted B_{max} of 367 ± 9 fmol/mg ($n = 5$). However, preliminary experiments ($n = 2$) suggested that a small decrease in binding might occur at saturating (> 10 μM) concentrations of this steroid (e.g. Fig. 3). Fitted B_{max} values for specific binding of both [^{3}H]aldosterone and [^{3}H]corticosterone were lower than [^{3}H]dexamethasone (Fig. 1), and subject to pronounced interassay variability (data not shown). Slopes derived from the concentration-binding curves for all three radiolabeled steroids were not significantly different from 1. Fitted K_d values for [^{3}H]corticosterone and [^{3}H]aldosterone were significantly greater than [^{3}H]dexamethasone ($P < 0.05$, 1.63 ± 0.27 vs 10.79 ± 4.92 vs 0.60 ± 0.12 nM respectively, ANOVA followed by Newman–Keuls multiple range test, $n = 3$).

The characteristics of glucocorticoid binding sites were next assessed by displacement of a fixed concentration of [^{3}H]dexamethasone binding by increasing concentrations of cold competitors (Fig. 2). Each competitor tested completely inhibited specific [^{3}H]dexamethasone binding. Fitted IC_{50} values were 6.43 ± 1.00, 7.72 ± 0.85, 17.95 ± 1.05, 42.88 ± 5.80, 84.81 ± 5.53 and 203.57 ± 21.88 nM, for dexamethasone, RU26988, corticosterone, progesterone, aldosterone and RU26752, respectively. Except for dexamethasone and RU26988, IC_{50} values were significantly different from each other ($P < 0.05$, ANOVA followed by Newman–Keuls multiple range test, $n = 3$). For all steroids tested, the slopes of the fitted curves were not significantly different from one. Assuming a single site model, K_b values were therefore calculated from fitted IC_{50} values, using a K_d value of 0.6 nM for [^{3}H]dexamethasone (see Table 1).

To confirm the existence of a single type of glucocorticoid binding site, we studied the effects of a fixed concentration of cold steroids on binding over a wide range of [^{3}H]dexamethasone concentrations. Concentrations of cold ligands were selected to cause about

Table 1. Affinity of ligands for Type II glucocorticoid binding sites in AtT20 cell cytosol

Steroid	K_b^a (nM)	RBA[b]
Dexamethasone	1.11 ± 0.28	100
RU26988	1.15 ± 0.31	97
Corticosterone	3.27 ± 0.62	34
Progesterone	8.58 ± 3.45	13
Aldosterone	17.55 ± 3.95	6
RU26752	41.41 ± 12.79	3

[a] Pooled K_b values ($n = 5$) derived from displacement of [^{3}H]dexamethasone concentration-dependent binding curves (see Results; $n = 2$), or from fitted IC_{50} values ($n = 3$), according to the relationship $K_b = IC_{50}/(1 + [D]/K_d)$, using a K_d of 0.6 nM for [^{3}H]dexamethasone.

[b] Relative binding affinity $K_{bdex}/K_b \times 100\%$.

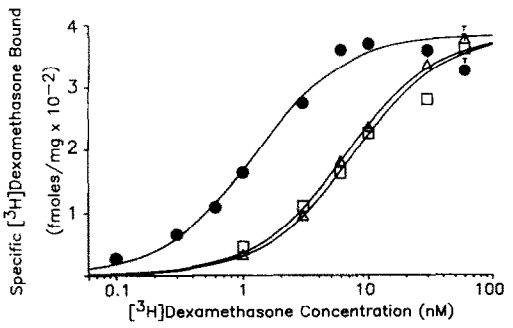

Fig. 3. Effect of Type I and Type II selective binding site antagonists on [^{3}H]dexamethasone concentration-dependent binding in AtT20 cell cytosol. Aliquots of AtT20 cytosol were prepared and incubated with increasing concentrations of [^{3}H]dexamethasone alone (●), or in the presence of RU26988 (□, 10 nM) or RU26752 (△, 400 nM), as described in Experimental. Nonspecific binding, defined as [^{3}H]dexamethasone bound in the presence of 1 μM RU26988, was assessed at each [^{3}H]dexamethasone concentration, and subtracted from all points. Each point represents the mean ± SEM of triplicate determinations from an individual experiment. The experiment was repeated with similar results.

a 10-fold shift in the [^{3}H]dexamethasone binding curve, as estimated from previously obtained IC_{50} values (10, 10, 30, 100, 100 and 400 nM; dexamethasone, RU26988, corticosterone, progesterone, aldosterone and RU26752, respectively). At these concentrations, all ligands caused a surmountable shift to the right in the [^{3}H]dexamethasone binding curve (Fig. 3 and data not shown). As observed in competition studies (Fig. 2), approximately 40 times as much RU26752 was required to cause inhibition of [^{3}H]dexamethasone binding equivalent to RU26988 (Fig. 3). Variance ratio testing between variable and common slope logistic fits to the data revealed no significant deviations from parallelism with any of the steroids tested. Therefore, competitor K_b values were calculated from the difference in fitted apparent K_d values for [^{3}H]dexamethasone obtained in the presence or absence of the test steroid. These K_b values were similar to those calculated from IC_{50} determinations, and the overall binding affinity constants obtained from these combined studies are reported in Table 1. Derived affinity constants for radiolabeled- and their corresponding unlabeled-steroid were not significantly different (t-test, $n \geq 3$).

DISCUSSION

The rank order of steroid binding to AtT20 cell cytosol observed presently is typical of Type II binding sites, confirming and extending previous studies [15, 16, 23]. The observation that all cold steroids tested completely inhibited binding by a fixed concentration of [^{3}H]dexamethasone, with slope indices not significantly different from one, suggests that [^{3}H]dexamethasone labels only one set of binding sites in AtT20 cytosol. Similarly, if [^{3}H]dexamethasone bound to multiple sites, addition of fixed concentrations of cold competitors should have produced complex effects on the [^{3}H]dexamethasone concentration–binding curve, which was not observed. The binding affinities for [^{3}H]dexamethasone and [^{3}H]corticosterone determined presently from binding at apparent equilibrium are in agreement with those determined from the ratios of dissociation and association rate constants [24], but approximately 5 times lower than those previously reported in AtT20 cells [16, 23]. These differences, and the variable binding obtained with [^{3}H]corticosterone and [^{3}H]aldosterone, probably reflect minor methodological differences, e.g. the differential dissociation of [^{3}H]ligands during the separation protocol used [16, 25].

Accumulated evidence suggests that only classical Type II glucocorticoid receptors are present in AtT20 cell cytosol. Our study gives no evidence for multiple glucocorticoid binding sites in this cell line. However, in other tissues, several groups have suggested multiple forms of Type II receptors (commonly referred to as Type II (Type 1A) and 1B). The ligand binding properties of these sites appear similar [9]. Type 1B glucocorticoid receptors have been suggested to mediate the anti-inflammatory effects of steroids by inhibition of arachidonic acid release [26]. Preliminary evidence indicates that dexamethasone pretreatment does not inhibit [^{3}H]arachidonic acid release from prelabeled stores in AtT20 cells (Gannon, McEwen and Roberts, unpublished observations). Biochemical evidence also indicates that AtT20 cells have only one form of Type II receptors [27, 28].

Type I glucocorticoid binding sites were not present in AtT20 cell cytosol, under the incubation conditions used presently. Type I glucocorticoid binding sites, present in preparations of rat pituitary [12] and brain [13, 17, 29], can be identified by high affinity binding of aldosterone, corticosterone and dexamethasone, measured in the presence of RU26988 to eliminate binding of ligands to Type II receptors (K_d around 0.5–2 nM for all ligands [17]). The lack of high affinity binding by [^{3}H]aldosterone, obtained presently in the presence of RU26988, suggests that Type I receptors are not present in AtT20 cell cytosol. This was confirmed by the lack of effect of low concentrations of RU26752, a selective Type I ligand, on dexamethasone binding. Further supporting the absence of Type I binding sites in AtT20 cytosol, nonspecific binding of [^{3}H]steroids was equivalent in the presence of either corticosterone (2.5 μM), a mixed Type I and Type II ligand, or RU26988 (1 μM), a selective Type II ligand (data not shown).

It should be noted that the prototypical selective Type I ligand RU26752 had reasonable affinity for the Type II binding site (Table 1), albeit at concentrations at least an order of magnitude higher than its estimated affinity at Type I binding sites [29]. Higher

binding of RU26752, relative to aldosterone, has been interpreted as evidence for multiple forms of Type I receptors in rat kidney [7]. However, Type II receptors are also present in kidney [8, 30]. Our study indicates that, at the concentration of aldosterone and RU26752 used by Agarwal and Kalimi (100 nM) [7], significant occupancy of Type II binding sites by both ligands would occur. Thus the affinity of RU26752 for Type II receptor sites, should be carefully considered in experiments designed to identify glucocorticoid receptor subtypes.

Endogenous contaminants do not appear to interfere with the determination of steroid binding site parameters determined in this study. Significant steroid contamination from the serum based medium would be predicted to decrease the apparent affinity of [^{3}H]steroids for their binding sites, due to competition with exogenous [^{3}H]ligands. Pituitary cells also contain a transcortin, or transcortin-like molecule, which has preferential affinity for corticosterone [10, 11, 31]. Removal of exogeneously added steroid by this protein, if significant, would also tend to shift concentration-binding curves to the right of their true location, since the free steroid concentration would be underestimated. However, K_b values for radiolabeled or cold steroid obtained presently were not significantly different. In addition, slope indices of fitted curves, derived from several different experimental manipulations, gave no indication for complex binding site profiles. Thus it appears that the cytosolic isolation procedure used in these studies adequately removed both serum-based contaminants and transcortin-like molecules.

The absence of binding sites with Type I characteristics in AtT20 cell cytosol suggests that the complex transcriptional effects of glucocorticoids on POMC gene expression [14] are mediated through Type II receptor activation. However, additional glucocorticoid binding sites have been reported in AtT20 [32], and other [33–35], cell membranes. The function of these sites, if any, is unknown. In addition, potential nonreceptor mediated effects of steroids, such as lipid fluidity changes [36], must also be considered as potential mediators of glucocorticoid action.

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