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SHORT COMMUNICATION

BIOPOTENCY OF CORTICOSTERONE AND DEXAMETHASONE IN CAUSING GLUCOCORTICOID RECEPTOR DOWNREGULATION

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Summary—The biopotencies of dexamethasone and corticosterone in causing glucocorticoid receptor downregulation in the AtT-20 cell were assessed and compared to their affinity for the isolated, cytosolic glucocorticoid receptor. Dexamethasone depleted receptor by 50% at a concentration of 4.6 nM. Its K_d for the receptor was 9.1 nM. The comparable values for corticosterone were 520 and 18.8 nM respectively. These results suggest that receptor depletion is a receptor mediated process; at some point the steroid must be bound to receptor in order to cause depletion. Further, the discrepancies between the two values for corticosterone support the hypothesis that it is the transformed receptor that is depleted.

INTRODUCTION

Glucocorticoids downregulate the number of their own receptors in target tissues. This has been shown most convincingly in cloned cell models like the AtT-20 [1, 2], HeLa S3 (3) and GH1 [4] cells, but also has been demonstrated in lymphocytes of intact human volunteers [5] and in rat brain [6]. This process occurs in the presence of protein synthesis inhibitors like cycloheximide and is not associated with a change in receptor binding affinity. Placement of "receptor-depleted" cells into steroid-free media allows the cells to replete their complement of glucocorticoid receptors over a several day period [1].

Since a tissue's response to hormone depends upon its receptor content [7], it is important to elucidate the mechanism of this receptor-regulating process. As yet, however, the exact steps are unknown. Indeed, it is not established that this is a receptor-mediated action. In this paper we report the results of our investigation into the relationship between the binding of glucocorticoids to the glucocorticoid receptor of the AtT-20 cell and their potency at causing downregulation. The goal is to determine whether this process is receptor-mediated and, in this manner, cast light on the subcellular mechanism of receptor regulation.

EXPERIMENTAL

Cells

AtT-20/D-1 cells were grown in Dulbecco's modified Eagle's minimum essential media [DMEM] (GIBCO, Grand Island, NY) supplemented with dextrose (3.5 g/l), sodium bicarbonate (3.7 g/l), 5% fetal bovine serum (Irving Scientific, Santa Anna, CA), penicillin (5000 U/l and streptomycin (5000 U/l). Cells were seeded into tissue culture flasks at a concentration of 300,000/ml. Final incubate volumes ranged from 10 to 150 ml. All were incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air.

Steroid

The sources, dilutions, and storage of the steroids have been described [8].

Buffers

Buffer I; 10 mM Tris–HCl, 154 mM sodium chloride, pH 7.4. Buffer II; 10 mM Tris–HCl, 1 mM EDTA, 6 mM 1-thioglycerol, pH 7.4 Buffer III; Buffer II made 20 mM sodium molybdate.

Steroid-cell binding

Incubations were started by the addition of the appropriate steroid in ethanol. The cells were then grown for 2 h or several days. To determine receptor content, cells had to be washed free of nonradioactive steroid. To accomplish this, 100–150 ml aliquots of cell suspensions were gently centrifuged (800 *g*, 5 min, 5°C), the supernatants discarded and the cell pellets resuspended in 75 ml of fresh, steroid-free media. After being allowed to stand at 37°C for 30 min this wash step was repeated. Ninety minutes later the cells were again collected, washed with ice-cold Buffer I, and then suspended in 1–2 ml ice-cold Buffer II, and ruptured through the use of a metal dounce (5 strokes). A cytosol was prepared by centrifugation in a Beckman (Palo Alto, CA) microfuge B (5 min, 4°C, 8740 *g*). The pellet was discarded and aliquots of the cytosol were incubated with 10 nM tritiated triamcinolone acetonide. Since the K_d of triamcinolone acetonide binding to the AtT-20 cell glucocorticoid receptor is 3.4 nM [8] this 10 nM concentration nearly saturates the receptor. After 2 h unbound steroid was removed with charcoal dextran (2% Norite A charcoal plus 0.5% Dextran T-70, 10 mM Tris, 1 mM EDTA, pH 7.5). Samples were agitated for 5 min and centrifuged (800 *g*, 5 min, 5°C). The supernatants were transfered to miniscintillation vials. Four ml of Ready-solve (Beckman, Palo Alto, CA) was the scintillant.

Displaceable binding

Displaceably-bound tritiated ligand was the measure of receptor content. To derive this value, each incubation with tritiated glucocorticoid was paired with an identical tube that contained a 1000-fold excess of the nonradioactive steroid. The amount of radioactivity bound by the latter was subtracted from the former to yield displaceable binding [9].

Other assays and procedures

Scatchard analysis was used to determine the number and affinity of binding sites [10]. Specifically, cytosol was incubated with concentrations of tritiated dexamethasone that ranged from 1.25 to 40 nM. In the case of corticosterone nonspecific binding was too high to allow the accurate use

of this direct Scatchard analysis. Consequently, a competitive technique had to be used [11]. In this approach, cells were incubated with tritiated dexamethasone at concentrations which ranged from 1.25 to 20 nM in the absence or presence of competing concentrations of nonradioactive corticosterone. The concentrations of corticosterone used were 4.2–25 nM. Protein content was determined by the method of Lowry *et al.*[12].

RESULTS AND DISCUSSION

Glucocorticoids deplete the AtT-20 cell of its glucocorticoid receptor [1, 2]. We have shown that depletion is moderately fast; in the presence of 20 nM tritiated dexamethasone the half-time of receptor depletion (i.e. the time to deplete 50% of the initially-bound receptor) is 30 h [13]. Importantly, this receptor-regulating process does not deplete the cell of receptor totally. A plateau is reached. After 4–5 days of exposure, binding is reduced to 20–28% of the initial value [2] and this does not change further even if the incubation is continued for another 2 weeks [13]. Thus, overall, glucocorticoids cause their target cells to establish a new receptor equilibrium rapidly and this receptor level is stable until agonists are removed.

The goal of these studies was to determine the biopotency of glucocorticoids in causing receptor downregulation. In previous studies [1, 2], receptor depletion was demonstrated using cells that were incubated chronically with tritiated glucocorticoids. Preliminary trials indicated that this approach was not feasible for the current protocol because high concentrations of agonist were going to be needed. In the approach ultimately adopted, cells were incubated first with various concentrations of nonradioactive glucocorticoids to cause depletion and then the amount of receptor remaining was determined after removal of nonradioactive agonist. With this approach, the cells had to be washed thoroughly with steroid-free media to remove both bound and free nonradioactive agonist before exposure to tritiated ligand. To determine the efficacy of the wash procedure, cells that had been incubated with either 10^{-5} M corticosterone or 10^{-6} M dexamethasone for 2 h at 37°C were washed in steroid-free media as described in the Experimental section and then their cytosolic receptor content determined and compared to that of a steroid-free control. The results showed that the binding of the corticosterone-treated cells was 83.5 ± 9.0% (mean ± SEM; $n = 6$) of the control while the dexamethasone-treated sample bound 79.4 ± 8.6% ($n = 5$) as much as control. Clearly, even after being incubated with very high concentrations of agonist for times sufficient to allow binding to come to completion [14], the wash procedure was able to remove nearly all of the nonradioactive steroid.

The potencies of dexamethasone and corticosterone at causing receptor depletion are shown in Fig. 1. Both steroids cause a concentration-dependent decrease in receptor number. Dexamethasone is more potent and the two depletion curves are roughly parallel, suggesting a similar mechanism of action. The concentrations of agonist needed to deplete the cell of 50% of the amount of control receptor are corticosterone 520 nM and dexamethasone 4.6 nM.

The binding affinities of the two agonists for the cytosolic glucocorticoid receptor of the AtT-20 cell were investigated by Scatchard analysis. The K_d for dexamethasone is 9.1 ± 1.4 nM (mean ± SEM; $n = 7$). It was not possible to use the same analysis to calculate a dissociation constant for corticosterone. Nonspecific binding was too high and, thus, the amount of determined displaceable binding was erratic. A competitive technique had to be used instead [11]. This value (K_i) is comparable to the dissociation constant and is 18.8 ± 3.8 nM ($n = 3$). These two values of receptor affinity are comparable to those reported using an intact AtT-20 cell assay [14]. Thus, it appears that these values are both reproducible and truly reflective of the affinity the agonists have for the receptor in the cell.

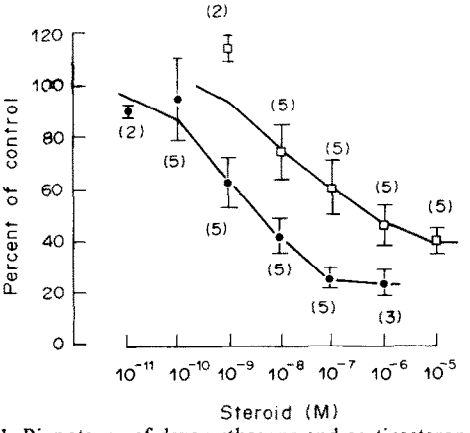

Fig. 1. Biopotency of dexamethasone and corticosterone in causing receptor depletion. AtT-20 cells were seeded at approx. 300,000/ml in media supplemented with the indicated concentration of glucocorticoid. In each case a nonsteroid-treated flask served as control. After 5 days, cells were gathered, washed to remove all steroid and cytosol prepared. The amount of tritiated triamcinolone acetonide which was displaceably-bound/mg cytosol protein was compared to that found for the simultaneously-run control and expressed as a percentage. The values shown here are the mean ± SEM of the number of determinations indicated in the parentheses. Dexamethasone (●). Corticosterone (□).

The facts that (1) the *order* of glucocorticoid potency in causing receptor depletion is the same as their *order* of binding preference for the glucocorticoid receptor and (2) the concentration of dexamethasone needed to deplete the cell of its receptor is on the same *order* as its affinity for the receptor, suggest that depletion is a receptor-mediated process.

The curves in Fig. 1 suggest that the process of depletion does not go to completion. The effect appears to plateau at steroid concentrations around 10^{-6} to 10^{-5} M. The fact that there is a "floor" value for the extent of depletion of glucocorticoid receptors, has also been reported by McEwen's group, who noted that there was a limit to how far the glucocorticoid receptor level could be lowered by chronic corticosterone administration to intact rats [15]. Interestingly, they found that this "floor" value represented about 40% of the total receptor content, similar to that seen here with corticosterone in the cloned cell. Certainly, it would have been desirable in these experiments to use even higher concentrations of agonist to prove whether a plateau had been reached, however, this was not feasible, for at these higher concentrations the complete removal of nonradioactive steroid by the wash procedure could not be assured.

If, indeed, the two agonists are approaching a plateau of effect, it would appear that the end points of depletion for the two steroids are not identical. Dexamethasone depletes the cell of about 80% of its receptor whereas corticosterone depletes the cell of 60%. Several possibilities could account for this difference. First, it could be that corticosterone is only a partial agonist in the AtT-20 cell. We have already shown that corticosterone can suppress AtT-20 cell ACTH secretion to the same extent as dexamethasone [8]. Hence, in at least some aspects, corticosterone is a full agonist. A second, and more likely, possibility that would account for at least part of this difference is that corticosterone and dexamethasone differ in their ability to transform and translocate the glucocorticoid receptor. It has been reported that dexamethasone–receptor complexes have twice the ability to bind to the nucleus as those of corticosterone [14]. Further, more recently, Harrison and Miyabe have reported

that corticosterone is only about 70% as efficient as dexamethasone in causing transformation of the glucocorticoid receptor [16]. Thus it may be that this difference between the end points of receptor depletion reflect, at least partially, the differences between the two steroids ability to transform and then cause nuclear translocation of the glucocorticoid receptor. This would explain the large difference found between the concentrations of corticosterone needed to cause depletion and saturate receptor binding. This formulation is consistent with the report of McIntyre and Samuels who used the GH1 pituitary cell and found that it was the transformed glucocorticoid receptor which underwent agonist-induced down regulation [4].

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