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SUBMICROMOLAR FREE CALCIUM MODULATES DEXAMETHASONE BINDING TO THE GLUCOCORTICOID RECEPTOR

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Calcium decreases the affinity of dexamethasone for the glucocorticoid receptor in cytosol from cultured rat hepatoma cells. The rate of association is decreased three-fold; the rate of dissociation is unaffected. Calcium is effective within the range of concentrations at which free cytoplasmic Ca^{2+} exerts its second messenger functions in living cells. This implies that calcium may act as a physiological modulator of glucocorticoid hormone action at the receptor level.

INTRODUCTION

The glucocorticoid receptor is one of the few proteins known to control gene expression in higher organisms by directly interacting with DNA (1). This interaction requires prior binding of the steroid hormone to the receptor within the cytoplasm of target cells (2). Hormone binding to the receptor can be studied in vitro by incubating [^{3}H]-labelled steroids with cytosol from glucocorticoid-sensitive rat hepatoma (HTC)[4] cells (3). The glucocorticoid response of intact cells does not always fit predictions based on the receptor-steroid binding parameters determined in cytosol (2). Regulatory factors that can no longer operate in cell-free experiments might control the glucocorticoid receptor under physiologic conditions.

When studying the kinetics of dexamethasone interaction with the HTC receptor we found that binding constants were not independent of receptor

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4. Abbreviations : Dexamethasone, 9α-fluoro-16α-methyl-1Iβ,17,21-trihydroxy-1,4-pregnadiene- 3,20-dione; EGTA, ethylene glycol bis (β-amino-ethyl-ether)-N,N,N',N'- tetraacetic acid; HTC, hepatoma tissue culture.

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(i.e. cytosol) concentration. This appeared to be due to the presence of thermostable factor(s) such as the one(s) described in other glucocorticoid-sensitive cells (4). It has been proposed that phosphorylation of the glucocorticoid receptor controls its binding properties (4). The involvement of calmodulin, an ubiquitous thermostable protein that influences protein phosphorylation in a calcium-dependent way (5), was therefore a likely possibility. This led us to investigate the influence of calcium on dexamethasone binding to the glucocorticoid receptor of HTC cells.

MATERIALS AND METHODS

Frozen pellets of HTC cells grown and harvested as described previously (3) were disrupted (tight-fitting Dounce homogeniser, 70 strokes) in buffer (3-5 x 10^7 cells ml^{-1}) containing 20 mM N-[tris-(hydroxymethyl) methyl]-glycine (Tricine, Calbiochem, San Diego, CA) and 10 mM Na_2MoO_4, pH 7.4 at 22° C. Particles were removed by centrifugation at 15,000 r.p.m. for 20 min (Sorvall SS-34 rotor) and then at 49,000 r.p.m. for 60 min (Spinco 50Ti rotor). The final supernatant (cytosol) was frozen at -80° C. Cytosol aliquots (0.1 ml) were incubated at 0° C in the homogenisation buffer, unless indicated otherwise (final volume 0.4 ml), in the presence of [^{3}H] dexamethasone (46 Ci/mmole, Radiochemical Centre, Amersham, UK). At the end of the incubation period, 0.2 ml of an aqueous charcoal suspension (50 mg ml^{-1}) containing dextran (5 mg ml^{-1}) was added. Incubations were agitated for 10 sec and centrifuged to remove free steroid. Radioactivity in 0.25 ml of the supernatant was counted in picofluor (Packard, Warrenville, IL) using a Berthold (Wildbad, FRG) scintillation counter (efficiency 58 %) to determine macromolecule-bound steroid. Receptor binding was calculated (2) by subtracting nonspecific binding determined in parallel incubations containing 0.2 mM $HgCl_2$. Identical results were obtained using 10 µM non-labelled dexamethasone instead of $HgCl_2$. In some experiments charcoal was replaced by 0.8 ml of a hydroxylapatite suspension (Biogel HT, Biorad, Richmond, CA, 10 g dl^{-1}). The mixture was centrifuged, and the pellet was washed three times with buffer and counted for radioactivity.

RESULTS AND DISCUSSION

Dexamethasone binding was examined in the presence or absence of EGTA, a compound that preferentially chelates Ca^{2+}. When determined 30 min after addition of the steroid, binding was dramatically increased by EGTA. This time-interval reflects the initial rate of binding, as equilibrium was not reached before 16 to 24 h under such incubation conditions. EGTA did not act by chelating heavy metals since binding was significantly depressed by adding $CaCl_2$ to normal cytosol or by adding it in excess over EGTA; addition of $MgCl_2$ had no effect (Fig. 1). These experiments suggest that calcium decreases the rate of binding of dexamethasone to the glucocorticoid receptor.

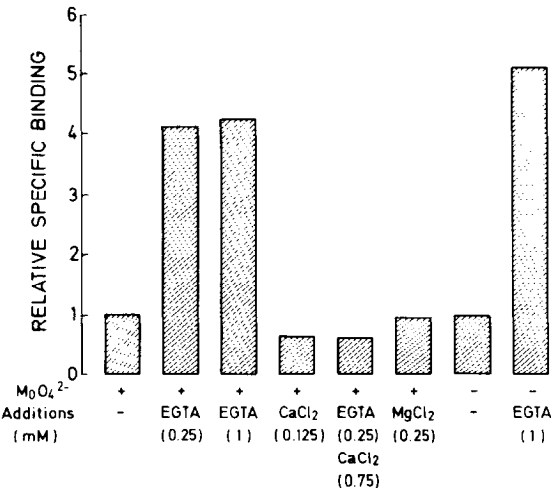


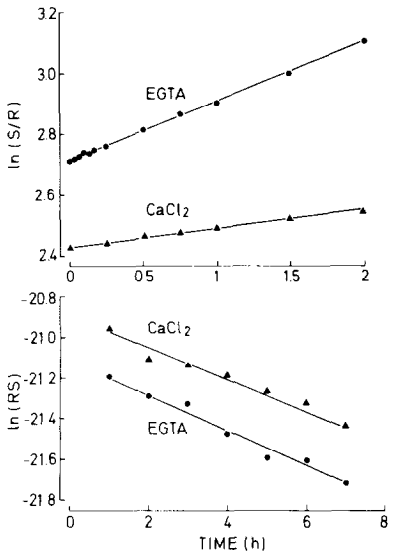
Fig. 1. Effect of ions on the initial rate of dexamethasone binding to the glucocorticoid receptor. Aliquots of cytosol were incubated in duplicate in the presence of 8 nM [^{3}H] dexamethasone without or with the additions indicated on the Figure. Molybdate-free incubations contained cytosol from cells homogenised in buffer devoid of this salt. After 30 min of incubation, bound and free steroid were separated using the charcoal assay and binding was determined as described in the Methods. The data have been normalised for the specific binding seen in control incubations, where mean total and nonspecific binding was 1385 and 235 dpm, respectively. The various treatments did not affect nonspecific binding.

Earlier work indicated that each receptor site binds noncovalently and reversibly one steroid molecule without interactions between sites (2). Thus, the rate of binding is governed by ligand concentration and by the rate constants for association (k_1) and for dissociation (k_2). In kinetic experiments, we determined whether calcium acted by modifying one or both of these constants. The data, shown in Fig. 2, have been plotted assuming that

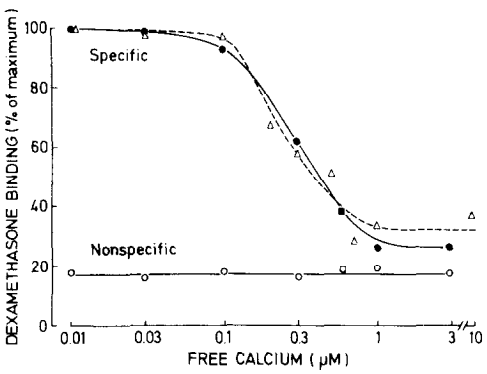
$$\frac{d(RS)}{dt} = k_1 (R)(S) - k_2 (RS)$$

where the symbols between parentheses denote concentrations as follows : RS, receptor-steroid complex; R, free receptor; S, free steroid. We found that dissociation rates in the presence of EGTA or calcium did not significantly differ and conformed in both cases to a first-order process, with $k_2 = 1.35 \times 10^{-3}$ min^{-1} (mean of 3 experiments).

In contrast, the association process was clearly inhibited by calcium. Rate constants for association calculated by assuming that the latter is second-order and that k_2 (RS) is negligible at early time-intervals were 4.1×10^5 and 1.6×10^5 M^{-1}.min^{-1} with EGTA and calcium, respectively. Thus,



(2)



(3)

Fig. 2. Rate of dexamethasone binding and dissociation in presence of calcium or EGTA. HTC cytosol was preincubated in triplicate with 1 mM $CaCl_2$ (▲) or 1 mM EGTA (●). After 30 min, binding was initiated by addition of 8 nM [^{3}H]dexamethasone. Specific binding was determined as a function of time (upper panel), as described in the Methods. After 16 h, a negligible volume of nonlabelled dexamethasone was added to the incubations (final concentration 12.5 μM), after which specific binding was determined as a function of time (lower panel). S, free steroid; R, free receptor; RS, receptor-steroid complex. Regression lines do not coincide at zero time because incubations contained different receptor concentrations.

Fig. 3. Dependence of initial rate of dexamethasone binding on free calcium ion concentration. Incubations were performed in triplicate as described in Fig. 1. Known levels of free Ca^{2+} were obtained using EGTA-$CaCl_2$ buffers ($CaCl_2$ was Titrisol, Merck, Darmstadt, FRG) at a 2.5 mM final concentration (12) and assuming an apparent combined association constant CA EGTA = 2.98 x 10^7 M^{-1} (ref. 13).

After 30 min, specific and nonspecific binding was determined by the charcoal (●,○) or hydroxylapatite assay (△); in the latter experiments molybdate-free cytosol and buffers were used. 100 % Specific binding corresponded to 5910 d.p.m. in the pellet. Nonspecific binding was 1300 d.p.m. With the charcoal assay, the corresponding values were 2090 and 395 d.p.m. in the supernatant. The squares refer to data obtained with cytosol incubated in absence of the calcium-EGTA buffers and using the charcoal assay.

calcium reduces the affinity of the receptor for dexamethasone by decreasing the rate constant for association by a factor of three.

If the control of glucocorticoid hormone binding exerted by calcium has a physiologic significance it should take place at free Ca^{2+} concentrations that occur within the cell, i.e. 3-4 orders of magnitude lower than the millimolar concentrations prevailing outside the cell (6). This question was explored by determining initial rates of dexamethasone binding in incubations where free Ca^{2+} ion concentrations were kept constant by calcium-EGTA

buffers. The data (Fig. 3) show that calcium acts in the submicromolar range. The rate of association of dexamethasone with the receptor was actually modulated by free Ca^{2+} between 0.1 and 1 μM, with a half-maximal effect seen at ∿ 0.3 μM. At free Ca^{2+} concentrations above that value, binding was inhibited; at lower concentrations, binding was stimulated. Nonspecific binding was unaffected (Fig. 3).

Calcium has been reported to cause aggregation of the glucocorticoid receptor (7). In our assay bound steroid was separated from free steroid by adsorption of the latter to charcoal and subsequent centrifugation, followed by quantification of bound steroid in the supernatant. Thus, precipitation of calcium-induced receptor aggregates and loss of receptor with the charcoal pellet might have accounted for our observations. This was ruled out. First, the receptor aggregation reported was seen at millimolar calcium concentrations. Second, even millimolar calcium did not affect receptor site concentrations under our assay conditions (not shown). Third, the results obtained with the charcoal assay could be duplicated using the hydroxyl-apatite assay (8) in which bound and free steroid are separated by precipitation of the receptor followed by quantification of bound steroid in the pellet (Fig. 3).

HTC cytosol preparation and incubation were performed in the presence of sodium molybdate because this salt stabilises the glucocorticoid receptor of HTC cells (for at least 72 h at 0° C, not shown) as has been reported in other tissues (9). As molybdate not only protects the glucocorticoid receptor but also prevents it from undergoing transformation to the DNA-binding state (10), perhaps receptor sensitivity to calcium was induced by molybdate. This too was ruled out as EGTA still stimulated dexamethasone binding in cytosol prepared and incubated in the absence of molybdate (Fig. 1). Molybdate-free conditions were also used for the experiment with the hydroxylapatite assay in which results identical to those with molybdate were obtained (Fig. 3).

We conclude from these observations that the binding properties of the glucocorticoid receptor are modified by fluctuations of free Ca^{2+} concentrations within the intracellular physiologic range. Control by such Ca^{2+} concentrations of many biochemical reactions is mediated by calmodulin, a protein that binds calcium with an apparent equilibrium constant of 10^6-10^7 M^{-1} (ref. 5). Phenothiazine drugs such as trifluoperazine inhibit the effects of the calmodulin-calcium complex (5). If calmodulin were involved in the regulation of the glucocorticoid receptor by calcium, trifluoperazine should prevent micromolar free Ca^{2+} from depressing the rate of dexamethasone binding to HTC cytosol. Instead, trifluoperazine and several other functional analogues inhibited dexamethasone binding. The data (Van Bohemen and Rousseau, in preparation) suggest that these calmodulin inhibitors directly interfere with steroid binding to the receptor. Thus, results with trifluoperazine were inconclusive. Free cytosolic Ca^{2+} may act as a second messenger in response to several hormones and extracellular stimuli (11). Whether or not calmodulin is involved in the calcium-mediated control of glucocorticoid receptor activity, our findings point to a novel mechanism for the coupling of these hormonal and extracellular regulatory signals to glucocorticoid hormone action.

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