GLUCOCORTICOID RECEPTOR-MEDIATED STIMULATION OF 5'-NUCLEOTIDASE IN HUMAN LYMPHOBLASTOID IM-9 CELLS

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1. Introduction

Lymphoid tissue is a major target for glucocorticoids. In liver, these hormones act through an induction of enzymes mediated by the nuclear binding of a cytoplasmic receptor protein. In lymphocytes, glucocorticoids inhibit cell proliferation and membrane transport processes, but the biochemical basis of these changes remains unknown (reviewed [1]). In humans, circulating lymphocytes and permanent lines derived thereof provide an easily accessible source of cells for receptor studies [2,3]. The lymphoblastoid IM-9 cell line has been extensively used for investigating the insulin receptor [2]. Although IM-9 cells are not growthinhibited by glucocorticoids (unpublished) they are sensitive to these hormones. Cortisol was reported to increase the insulin-binding capacity of IM-9 cells [4], a finding confirmed in our laboratory.

We report here that IM-9 cells do contain a typical glucocorticoid receptor but no receptor for other steroid hormones. In addition, we show that dexamethasone, a synthetic glucocorticoid, stimulates specifically 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5), an enzyme associated with the plasma membrane in human lymphocytes [5], and that the stimulation is mediated by the glucocorticoid receptor.

2. Materials and methods

All cell washings and homogenization, and centrifugation steps were at 0°C.

2.1. *Cell cultures*

IM-9 cells were grown at 37°C as spinner cultures (density up to 2.5×10^6 cells/ml) in RPMI medium containing 10% fetal calf serum, 50 units penicillin/ml and 50 units streptomycin/ml (all from Gibco Bio-Cult, Paisley). In serum-free medium experiments, serum was replaced by an equal volume of 10 g/l bovine serum albumin solution in phosphate-buffered saline (PBS) containing 150 mM NaCl, 25 mM KCl, 8 mM $Na_2HPO_4 \cdot 2\ H_2O$, 1.5 mM KH_2PO_4 (pH 7.4).

2.2. *Binding studies*

For cell-free binding, cells were resuspended in serum-free medium for 45 min at 37°C, washed in PBS, and stored as pellets at −80°C. The pellets were added with 20 mM *N*-[tris-(hydroxymethyl)methyl]-glycine (Tricine, Calbiochem, San Diego, CA), 10 mM $Na_2MoO_4 \cdot 2\ H_2O$ (pH 7.4) (2.5×10^8 cells/ml) and homogenized with the tight pestle of a Dounce homogenizer. The homogenates were centrifuged for 20 min at 16 000 rev./min (Sorvall RC2-B centrifuge, rotor SS-34). The supernate was centrifuged for 60 min at 49 000 rev./min (Beckman L2-65B centrifuge, rotor 50Ti). Portions (0.1 ml) of the resulting supernate (cytosol fraction) were incubated at 0°C in the presence of [^{3}H]dexamethasone with or without 12 μM nonradioactive dexamethasone, following which specific binding was determined by the charcoal assay [6].

Binding to intact cells was done as in [7] by incubating 4.8×10^6 cells in 0.4 ml aliquots of serum-free medium in the presence of 1,2-[^{3}H]dexamethasone (spec. radioact. 23 Ci/mmol, Radiochemical Centre, Amersham) at up to 100 nM. Parallel incubations contained in addition 12 μM unlabelled dexamethasone for determination of nonspecific binding. After equilibrium had been reached (3 h at 37°C) cells were washed 3 times with 3.5 ml ice-cold PBS by centrifugation at

4000 rev./min for 5 min in a Martin-Christ UJIS centrifuge. The pellet was resuspended in 0.7 ml 1.5 mM $MgCl_2$ solution, lysed by freeze-thawing, and centrifuged at 10 000 rev./min for 2 min in a Beckman microfuge B. Supernate-bound and particulate-bound steroid was determined by counting the radioactivity associated with the supernatant and the pellet fractions, respectively [8].

2.3. *Biochemical determinations*

Cells were washed by centrifugation with Puck's buffer containing 137 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 5 mM glucose (pH 7.45). The last pellets were kept frozen at −80°C. After thawing, pellets were homogenized either in a 0.1% (w/v) Triton X-100 solution by 10 s–sonication at 60 W (Branson Sonifier), or in 0.25 M sucrose, 3 mM imidazole–HCl (pH 7.4) by 10–15 strokes of the tight-fitting pestle of a Dounce homogenizer. 5′-Nucleotidase was assayed at pH 7.5 by two different methods based on the determination of either the P_i formed from adenosine 5′-monophosphate disodium salt [9], or the radiolabelled adenine liberated from [2-^{3}H]adenosine 5′-monophosphate, ammonium salt ([10], under the conditions in [11]). Incubations were for 60 min at 37°C. Identical specific activities were obtained by both methods. The following constituents were assayed by published procedures: alkaline phosphodiesterase I (EC 3.1.4.1), alkaline phosphatase (EC 3.1.3.1), glutamine synthetase (EC 6.3.1.2), glucose 6-phosphatase (EC 3.1.3.9) [9], tyrosine aminotransferase (EC 2.6.1.5) [12], nucleoside diphosphatase (EC 3.6.1) [13], protein [14].

3. Results and discussion

3.1. *Identification of the glucocorticoid receptor*

Cell-free binding experiments showed that the cytosol fraction from IM-9 cells contains a constituent that binds [^{3}H]dexamethasone in a saturable way, i.e., becomes undetectable in the presence of 100–1000-fold excess nonradioactive dexamethasone. The kinetic parameters of specific dexamethasone binding are given in table 1. The rate of association followed second-order kinetics. At 1 nM dexamethasone, equilibrium was reached after 18 h. Binding was completely reversible and dissociation was a first-order process with $t_{1/2} \sim 8$ h. The steroid concentration-dependence of equilibrium binding yielded data compatible with a single class of noninteracting sites which bind dexamethasone with high affinity (fig.1). The Hill coefficient was 0.99 ± 0.07 (SD). The equilibrium constant was similar to the ratio of rate constants (table 1).

Specific binding of [^{3}H]dexamethasone was inhibited when the cytosol fraction was preincubated with 50 μg/ml of either of trypsin, chymotrypsin, pronase, 6 mM *N*-ethylmaleimide or 12 mM iodo-

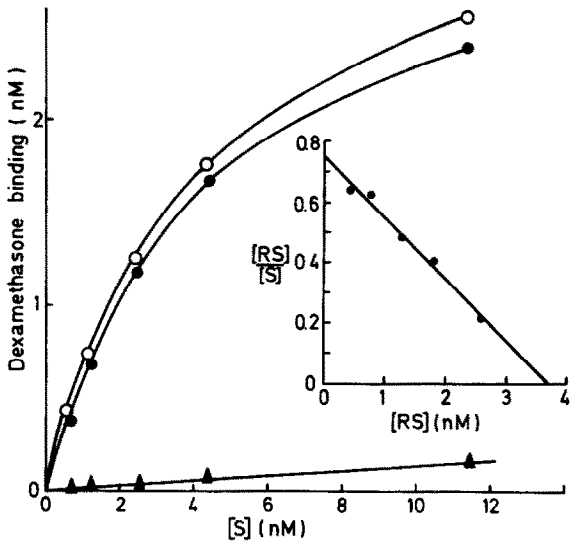

Fig.1. Concentration-dependence of [^{3}H]dexamethasone binding at equilibrium, in the cytosol fraction from IM-9 cells. (○) Total; (▲) nonspecific; (●) specific binding. Incubations were for 20 h at 0°C. RS and S refer to specifically-bound and free steroid, respectively.

Table 1
Kinetic parameters of [^{3}H]dexamethasone binding to glucocorticoid receptor in the cytosol fraction from IM-9 cells

k_{on} ($M^{-1} \cdot min^{-1}$)	k_{off} (min^{-1})	k_{off}/k_{on} (nM)	K_d (nM)	R_o (pmol/mg protein)	Sites/cell
2.6×10^5	1.5×10^{-3}	5.8	5.0 ± 1.5	1.23 ± 0.45	23 000

Mean ± SD of 2 or 11 (K_d, R_o) expt. at 0°C. k_{on} and k_{off} refer to the rate constants determined from the time kinetics. K_d and R_o refer to the equilibrium dissociation constant and concentration of receptor sites determined from Scatchard plots

acetamide, whereas 50 μg/ml DNase I or RNase I had no effect. Binding was abolished at pH <6.5 and >8.5 or after heating the cytosol fraction at 45°C.

In IM-9 cell cytosol fraction, a variety of nonradioactive steroids competitively inhibited [^{3}H]dexamethasone binding, in keeping with their affinity for the rat hepatic glucocorticoid receptor [1,6]. The order of potency was:

dexamethasone > corticosterone > cortisol > 11β-hydroxyprogesterone > 11-deoxycorticosterone > progesterone > 11-deoxycortisol > aldosterone > testosterone > estradiol.

Cortisol had a K_d (40 nM) which is near the value of free circulating concentration of cortisol in man.

Finally, upon incubation of intact cells with [^{3}H]-dexamethasone at 37°C, ~50% of the specifically bound steroid was found associated with the particulate fraction, as is the case when glucocorticoids are bound to the glucocorticoid receptor in intact lymphocytes [1,8]. Thus, the kinetic, chemical and steric properties of the specific dexamethasone binding sites are consistent with their being borne by the glucocorticoid receptor described in other cells [6,8].

An important point in view of using the IM-9 cell line as a model system for the human glucocorticoid receptor is to determine whether it contains receptors for any of the 4 other classes of steroid hormones as is the case of certain cloned cell lines [15]. The cytosol fraction was incubated for 20 h with the ^{3}H-labelled natural mineralocorticoid, progestogen, androgen or estrogen at a concentration 30-fold greater than the published K_d-values for their respective receptor. There was very little specific binding of these steroids except progesterone (fig.2). In all cases, Scatchard analysis of results obtained at several steroid concentrations showed that the binding, extrapolated for infinite concentration of free steroid, corresponded to the concentration of sites defined with [^{3}H]dexamethasone. Thus, the binding of progesterone and of the sex steroids reflects their interaction with the glucocorticoid receptor for which they are known antagonists [6]. It is concluded that IM-9 cells possess only one type of steroid receptor and which is specific for glucocorticoids.

3.2. *Glucocorticoid-stimulation of 5'-nucleotidase activity*

We found that IM-9 cells contain a typical (K_m, pH optimum, association with membranes) 5'-nucleotidase, an enzyme of pericellular membranes in several

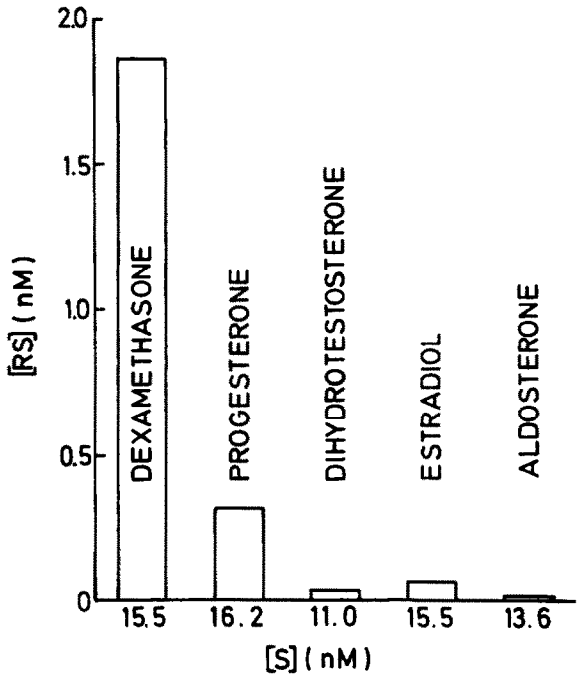

Fig.2. Specific binding (RS) of the ^{3}H-labelled steroid hormones to the cytosol fraction from IM-9 cells. Incubations were at 0°C for 20 h in the presence of the indicated concentrations (S) of free steroid.

cell types [16] and in human lymphocytes [5]. When the cells were incubated with 0.5 μM dexamethasone for 48 h, 5'-nucleotidase activity was stimulated ~2.5-fold, from 4.6 ± 0.7–11.3 ± 1.1 SD (n = 13) mU/mg protein. Results were identical whether the activity was determined by assaying the phosphate released or the [^{3}H]adenine produced from [^{3}H]AMP.

Several experimental results suggest that this effect of dexamethasone is mediated by the glucocorticoid receptor and not by a direct action of high steroid concentrations on the cell membrane.

(i) The stimulation required >10 h exposure to the steroid to occur. This lag period is reminiscent of that of the glucocorticoid receptor-mediated stimulation in rat hepatoma cells of alkaline phosphodiesterase I [11], another plasma membrane enzyme [16] which is lacking in IM-9 cells.

(ii) 5-Nucleotidase was stimulated by as little as 3 nM dexamethasone. The dose–response curve was superimposable to that of receptor occupancy by dexamethasone in intact cells at 37°C (fig.3).

(iii) The effect of different steroids (10 μM, 48 h)

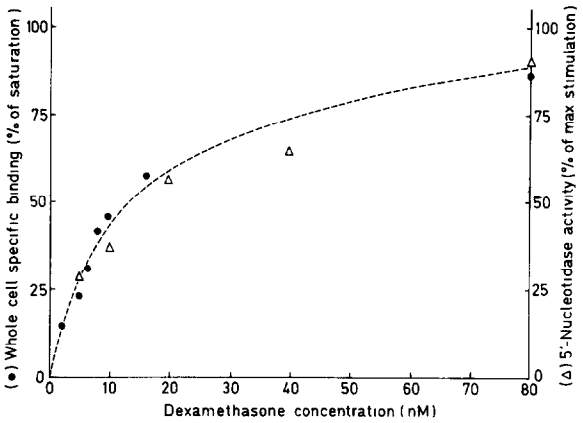

Fig.3. Concentration-dependence of dexamethasone binding and 5'-nucleotidase stimulation in intact IM-9 cells. Cultures were incubated at 37°C in serum-free medium, for 3 h with labeled (•) or for 48 h with unlabeled (Δ) dexamethasone. Specific binding ranged from 2800–11 200 dpm/aliquot. Nonspecific binding ranged from 285–1900 dpm/aliquot. Basal 5'-nucleotidase activity was 2.8 mU/mg protein. After maximal stimulation 5'-nucleotidase activity was 12 mU/mg protein.

on 5'-nucleotidase of IM-9 cells corresponds to what is expected from their interaction with the glucocorticoid receptor of these cells and from their agonist or antagonist properties in other systems [1,6]. Normalised potencies were as follows: dexamethasone 100, corticosterone 88, 11β-hydroxyprogesterone 21, aldosterone 14, 11-deoxycorticosterone, progesterone, 11-deoxycortisol and dihydrotestosterone, 0.

The stimulation of 5'-nucleotidase by glucocorticoids is specific in that none of the following enzyme activities were altered following dexamethasone treatment (0.5 μM, 48 h) of IM-9 cells: tyrosine aminotransferase, glutamine synthetase, glucose 6-phosphatase or a nonspecific phosphatase acting on glucose 6-phosphate, nucleose (ADP)-diphosphatase and alkaline phosphatase.

We have shown that cells from the permanent human lymphoblastoid IM-9 line possess a typical glucocorticoid receptor. A glucocorticoid response can be quantitatively measured which is the specific stimulation of the plasma membrane enzyme 5'-nucleotidase. This model is of interest for studying the glucocorticoid receptor and steroid structure–activity relationships, as well as the hormonal control of membrane enzymes and biogenesis, in the human species.

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