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INFLUENCE OF ENZYME TREATMENT ON DEXAMETHASONE BINDING IN ISOLATED THYMOCYTES

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SUMMARY

Incubation of mouse thymocytes in the presence of phospholipase A_2 or neuraminidase induces a decrease in their steroid binding capacity, due to a diminution of the number of binding sites rather than to a change of receptor affinity. The same decrease was also observed in cell free extracts of enzyme treated cells.

INTRODUCTION

It is currently accepted that steroid hormones diffuse freely through the cell membrane and bind to macromolecular receptors in the cytoplasm of target tissue cells; this interaction being the first step of hormone action[1–3]. However, several authors have postulated that the plasma membrane could play a role in the recognition and transport of the steroid into the cell. This hypothesis is supported by several kinds of experimental evidences.

First, kinetic experiments showed that the association of the steroid to its binding sites was more rapid in cell free preparations than in intact organs or isolated cells[4, 5]. Then, several groups have reported the existence of specific steroid binding sites at the outer surface of target cells[6] or in isolated plasma membrane fraction[7]. And finally, reagents which alter the functionnal properties or the chemical composition of the cell membrane may impair steroid receptor association. For example, SH blocking agents such as iodoacetamide or parachloromercuribenzoate decreased cellular uptake of steroids in isolated hepatocytes or uterine horns[4, 5]. Moreover, Harrisson *et al.*[8, 9, 10] presented evidence that treatment of A_t T 20/D-1 pituitary cells by phospholipase A_2 or neuraminidase abolished their steroid binding capacity.

In order to investigate further the effect of membrane alterations on steroid binding we studied the influence of phospholipase A_2 and neuraminidase on dexamethasone binding in isolated thymus cells, a well known glucocorticoid target tissue[11–15].

MATERIAL AND METHODS

1. *Animals and reagents*

Female C_{57} BL/6 mice, 6–8 weeks old were adrenalectomized under pentobarbitone anesthesia 4–5 days before each experiment and given 1% saline as drinking water. [^{3}H]-dexamethasone (^{3}HDM), 23–29 Ci/mmol was obtained from the Radiochemical Center (Amersham, U.K.). [^{3}H]-uridine, 25 Ci/mmol was purchased from C.E.A. (France). Unlabelled dexamethasone was obtained from Sigma, conventional reagents were Baker A grade. Phospholipase A_2 (EC 3.1.1.4, pig pancreas) was purchased from Boehringer and neuraminidase (EC 3.2.1.18, *Clostridium perfringens*) was obtained from Sigma.

2. *Methods*

The procedure used for isolation of thymocytes has been described elsewhere[16–18]. The cell suspension recovered from the thymus contained more than 95% of small lymphocytes and the viability of these thymocytes estimated by trypan blue exclusion was over 97%. Usually, these cell suspensions were adjusted to contain 10^7 cells/ml in minimal essential medium (MEM GIBCO) supplemented with sodium pyruvate (1 mM), glutamine (2 mM), penicillin-streptomycin (100 units/ml) and 1% V/V non essential aminoacids (GIBCO Ref. 114).

Determination of steroid binding in whole cells. 0.3 ml of cell suspension were distributed in 1.5 ml conical plastic vials containing 0.3 ml of ^{3}HDM solution (the final concentration of radioactive steroid was in the range 6×10^{-9} to 10^{-7} M). These samples were incubated for 20 min at 37°C in 95% air 5% CO_2 atmosphere under continuous shaking. At the end of the incubation period the samples were centrifuged for 15 s in an Eppendorf 3200 centrifuge, supernants discarded and the cells resuspended in 0.3 ml of ice cold radioactivity free medium A containing: $Na^+ = 133$, $K^+ = 6$, $Ca^{++} = 1$, $Mg^{++} = 1$, $Cl^- = 134$, $H_2PO_4^- = 6$, Tris–HCl = 5, and glucose = 5 all in mM, pH 7.4.

0.2 ml of cell suspension was then filtered through Whatmann GF/A filters and washed with

3 × 10 ml of ice cold buffer. The radioactivity collected on the filters was counted by liquid scintillation spectrometry. In each experiment the cells were incubated with ^{3}HDM ± 5×10^{-5} non radioactive dexamethasone. The residual binding in the presence of non radioactive dexamethasone represents non specific binding. The specific binding of the steroid is the difference between the total binding of ^{3}HDM and this non specific binding. Non radioactive and tritiated dexamethasone were dissolved in ethanol, but the final concentration of ethanol in the samples was less than 0.5%, a concentration which does not affect the binding.

Determination of steroid binding in cell free extracts. Cells pooled from 10 thymuses were disrupted by ultra-sonic treatment (60 s 20 Khz in ice cold medium A). Homogenates thus prepared were centrifuged at 105,000 *g* for 1 h and 0.3 ml aliquots of cytosol were added to 0.3 ml of ^{3}HDM solution (final concentration 10^{-7} M) with or without unlabelled dexamethasone in 1.5 ml plastic vials. After 120 min incubation at 0–4°C (this time has been shown to be sufficient to reach equilibrium), the entire incubate was passed through 3.6 ml G 50 Sephadex in 5 ml pipets to separate the residual free steroid from that protein bound. The columns were equilibrated and eluted with 0.1 M tris–3 mM $CaCl_2$, pH 7.4. All this procedure was carried out in a cold room at 4°C. The protein bound steroid was recovered in the void volume[19]. Aliquots of the void volume were taken for radioassay by liquid scintillation spectrometry and for determination of protein content by the method of Warburg and Christian[20].

Enzyme treatment. 0.6 ml of cell suspension were preincubated in the presence of enzyme at 37°C in 95% air 5% CO_2. Enzyme concentrations and length of incubation periods will be given for each experiment in the results section. At the end of the incubation, the samples were centrifuged for 15 s in an Eppendorf 3200 centrifuge, supernatants discarded and the pellets washed twice using MEM solution and assayed for ^{3}HDM binding as described above. In each experiment control samples were preincubated in the same conditions but without enzyme. After 3 h incubation at 37°C in the presence of 50 μg/6 × 10^6 cells phospholipase A_2 or 60 min incubation in the presence of 75 μg/6 × 10^6 cells neuraminidase, the viability of the cells remains over 85%.

[^{3}H]-*Uridine uptake and incorporation measurements.* After preincubation at 37°C in the presence or absence of enzymes, the samples were washed twice using MEM maintained at 37°C, resuspended in the same medium and then received 1 μCi of [^{3}H]-Uridine. After 60 min incubation at 37°C in 95% air 5% CO_2 the cells were centrifuged at 10,000 *g* for 15 s, washed twice and resuspended in ice cold buffer A. 0.2 ml of the cell suspension were dissolved overnight in 1 ml Soluene (Packard) and counted by liquid scintillation spectrometry to give uptake counts. Acid precipitable radioactivity (incorporation) is measured by adding ice cold 5% trichloracetic acid to 0.4 ml of cell suspension. The precipitates were collected by filtration on Whatmann GF/A filters, washed twice by 2 × 10 ml of 5% trichloracetic acid and then counted by liquid scintillation spectrometry.

RESULTS

Figure 1 shows the specific binding of 5×10^{-8} M ^{3}HDM in thymocyte suspensions as a function of the time of preincubation in the presence of either 20 μg/6 × 10^6 cells phospholipase A_2 or 30 μg/6 × 10^6 cells of neuraminidase. The specific binding of the steroid decreases with prolonged incubation to about 40–50% of control level. Incubation with the tracer was for 20 min at 37°C. The specific binding of 5×10^{-8} M ^{3}HDM (20 min 37°C) was also assayed as a function of enzyme concentration after 2 h preincubation (37°C) with phospholipase

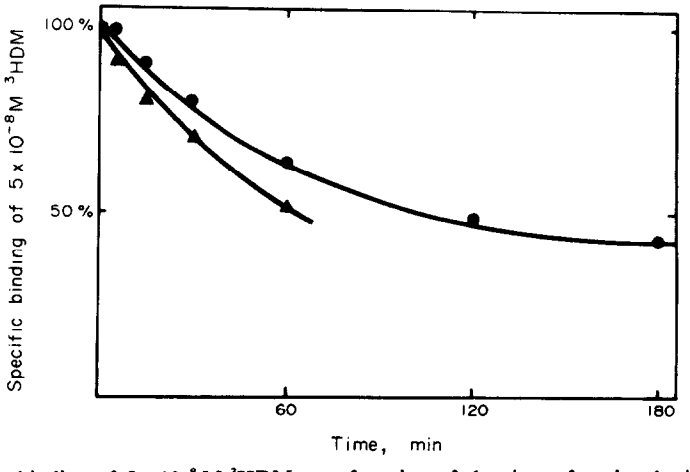

Fig. 1. Specific binding of 5×10^{-8} M ^{3}HDM as a function of the time of preincubation with either 20 μg/10^6 cells phospholipase A_2 (●) or 30 μg/10^6 cells neuraminidase (▲) $n = 3$. Incubation with the tracer was for 20 min at 37°C.

A_2 or 30 min incubation with neuraminidase. This binding decreases with increasing enzyme concentrations (Fig. 2). The following preincubation conditions were used in the subsequent experiments with phospholipase A_2: 20 μg/6 $\times$ 10^6 cells of enzyme and 2 h incubation at 37°C. The effect of phospholipase A_2 treatment on binding of various concentrations of ^{3}HDM is shown in Fig. 3. Over a wide range of concentrations (6×10^{-9}–10^{-7} M) the specific binding of the steroid was always lower in treated samples than in control samples. The binding in the presence of an excess of non labelled dexamethasone (i.e. the non specific binding) is nearly identical in the two groups of samples and represents about 25% of the total binding. Scatchard plots[21] derived from these binding curves (Fig. 3) show that the decrease of steroid binding after enzyme treatment corresponds to a 50% fall in the number of binding sites, without substantial changes in receptor affinity. In both groups of samples, enzyme treated and control, K_D values were calculated from the slopes of the Scatchard plots to be $K_{D\,37°C} = 4.4 \times 10^{-8}$ M ($n = 5$).

We investigated also the effect of enzyme preincubation on the rate of spontaneous steroid complex dissociation. Both control and enzyme treated samples were incubated with 5×10^{-8} M ^{3}HDM for 20 min at 37°C. At the end of the incubation, the cells were centrifuged, the pellets resuspended in steroid free MEM, maintained at 37°C, and either filtered immediately (T_0) as described above or after various periods of incubation at 37°C. As shown in Fig. 4, the level of binding at zero time (T_0) is lower in enzyme treated

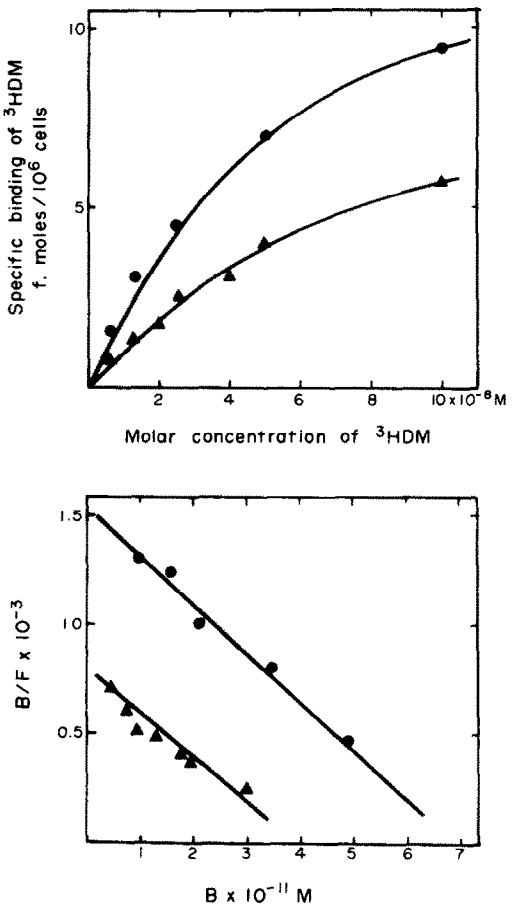

Fig. 3. Top: Specific binding of ^{3}HDM (6×10^{-9} to 10^{-7} M, 20 min 37°C) after 2 h preincubation at 37°C in the absence (●) or presence (▲) of 20 μg/6 $\times$ 10^6 cells phospholipase A_2. Bottom: Scatchard plots of ^{3}HDM binding in control (●) and enzyme treated (▲) samples ($n = 5$).

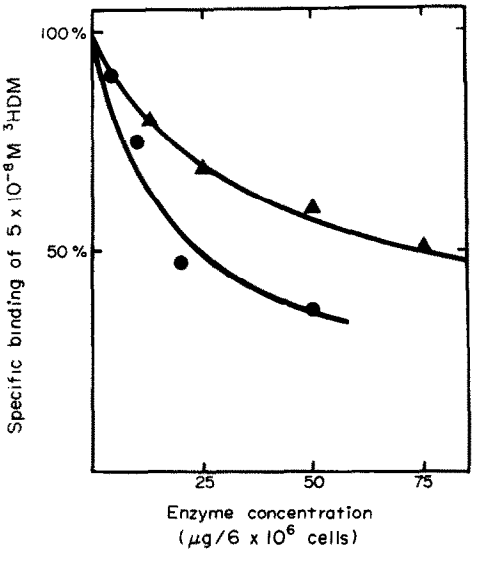

Fig. 2. Specific binding of 5×10^{-8} M ^{3}HDM after 2 h preincubation at 37°C in the presence of various concentrations of phospholipase A_2 (●) or 30 min incubation in the presence of neuraminidase (▲). Control samples (100%) were preincubated during the same period of time in the absence of enzyme ($n = 4$).

than in control samples, but the kinetic of dissociation of ^{3}HDM from its binding sites is nearly identical in the two groups. The dissociation is complete after 2 h at 37°C.

In another experiment (not shown) we demonstrated that the presence of 20 μg/6 $\times$ 10^6 cells of phospholipase A_2 during the dissociation period does not affect this off rate. We also investigated the influence of preincubation of cells (2 h 37°C) with phospholipase A_2 on their cytosolic receptor content. Fig. 5 shows that on the basis of protein content the number of cytoplasmic receptors in enzyme treated samples represent about 50% of that in control. No broad variation could be demonstrated between the protein content of enzyme treated and control cytosolic extracts.

Utilisation of macromolecular precursors has been widely accepted to be representative of cell metabolism. We therefore studied both uptake and incorporation of [^{3}H]-Uridine in enzyme treated and control samples. As shown in Fig. 6 the enzyme treatment affected these two parameters to the same extent leading to a 40% decrease of precursor utilisation.

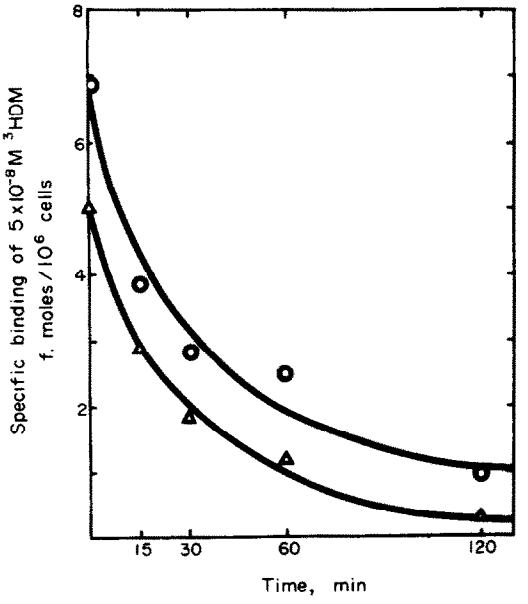

Fig. 4. Dissociation curves of ^{3}HDM binding at 37°C in steroid free medium. (○) control thymocytes, (△) phospholipase treated thymocytes.

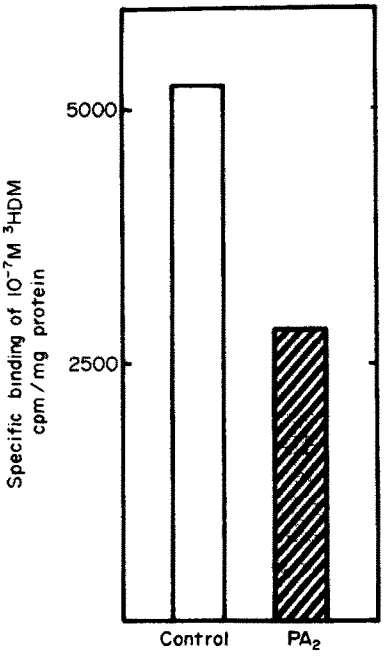

Fig. 5. Specific binding of 10^{-7} M ^{3}HDM in cytosol preparations from control (□) and phospholipase A_2 treated (▨) thymocytes ($n = 3$).

DISCUSSION

Incubation of mouse thymocytes in the presence of enzymes which are known to alter cell membrane induces a decrease of their binding capacity. This diminution correspond to a 50% lowering of receptor concentration without significant change in receptor affinity. We therefore presented results similar to those obtained by Harrisson *et al.*[8] except that using enzyme concentrations equivalent and even higher than those used by these authors, we failed to totally abolish steroid binding.

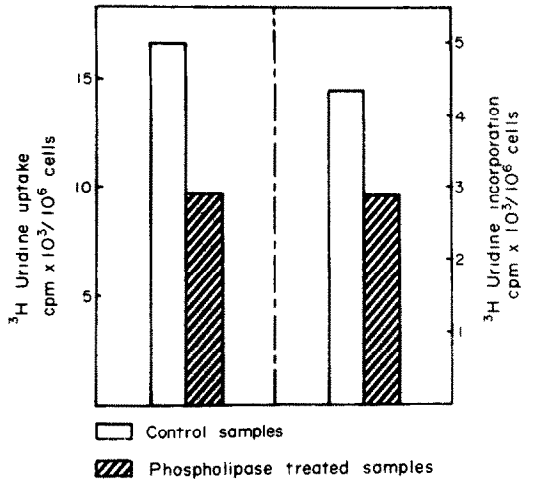

Fig. 6. ^{3}H Uridine uptake (left) and incorporation in trichloracetic acid precipitable fraction (right) in control (□) and phospholipase A_2 treated thymocytes (▨) ($n = 3$).

Central to an appreciation of the enzyme effects is the knowledge of the level of enzyme action. One hypothesis is that enzyme could act inside the cell and affect the cytosolic receptors. This hypothesis is supported by the recent work of Schulte *et al.*[22] who demonstrated that treatment of cytosolic extracts by phospholipase A decreases the binding of triamcinolone acetonide in fibroblast and thymocytes. However this work is in contradiction with that of Hermann *et al.*[23] and Fanestil *et al.*[24] who claimed the insensitivity of glucocorticoid receptors to non proteolytic enzymes. Moreover, Harrison *et al.*[8] demonstrated that phospholipase A_2 and neuraminidase do not modify steroid receptor interaction in cell free extracts of the A_t T 20/D-1 cell line.

Robinson and Wilkinson[25] demonstrated that lymphocyte treatment by various phospholipases produces an increase in cell membrane permeability and induces a leaching out of intracellular enzymes. It is therefore possible that during preincubation in the presence of enzyme, some receptor material is lost which accounts for subsequent decrease of steroid binding. The increase in permeability due to enzymes could otherwise have favoured the escape of factors essential to steroid receptor association. These low molecular weight substances could be a modulator like that postulated by Defer *et al.*[26], a nucleotide [27] or even cations.

Another possible explanation of our results would be the existence of a population of binding sites associated with the cell membrane, which are stripped from the lipid matrix during enzyme treatment or during cell disruption and cytosol preparation. This component could play a role in steroid uptake as suggested by Harrisson *et al.* (8) or by Jackson and Chalkley[28]. Wira and Munck however were unable to demonstrate the existence

of specific cortisol binding in rat thymocyte membrane fraction.

While the experimental evidences presented in this work does not explain conclusively the mechanism of enzyme induced decrease of steroid binding sites, it confirms that membrane integrity appears necessary to complete steroid–receptor interaction.

It is important to note that enzyme treatment also disturbs other unrelated mechanisms such as uridine uptake and RNA synthesis, suggesting that enzyme-induced membrane alteration is not restricted to the steroid pathway.

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