THE THYMOCYTE PLASMA MEMBRANE: THE LOCATION OF SPECIFIC GLUCOCORTICOID BINDING SITES

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KEY WORDS: cortisol; thymocytes; specific binding sites.

A passive role is ascribed to the plasma membrane in the classical scheme of action of steroid hormones on target cells. Recently studies of binding of steroid hormones with plasma membranes of target cells have been published [2, 3] and the investigators concerned regard the plasma membrane as the primary stage in the realization of the hormonal signal. However, during work with plasma membranes the possibility of their contamination with the cytosol fraction cannot be ruled out [4]. At the same time, it has been shown that proteins which, in the native cell, are located in the plasma membrane, may enter the cytosol fraction when the cells are homogenized. The coarser the homogenization, the more protein is found in the cytosol [6]. The procedure of isolation and purification of the cytosol which is generally adopted includes rigorous homogenization of the cells. It is thus possible that proteins specifically binding steroid hormones may pass from the membrane into the cytosol during isolation. Consequently, experiments to study the location of structures binding steroid hormones must be conducted on intact cells.

In modern molecular endocrinology it is now possible to determine the localization of receptors for biologically active substances with the aid of ligands, with high affinity for the receptor, immobilized on polymers [5, 8]. The aim of this investigation was to study the ability of cortisol (hydrocortisone, HC), immobilized on polyvinylpyrrolidone (PVP-HC), to reduce binding of ^{3}H-HC by thymocytes of adrenalectomized rats.

EXPERIMENTAL METHOD

Noninbred male albino rats weighing 120-150 g underwent bilateral adrenalectomy. On the 4th-5th day after the operation the animals were decapitated undersuperficial ether anesthesia. The thymocytes were isolated as described perviously [1]. A modified method of washing the cells was used to remove unbound and nonspecifically adsorbed ligand [7], after preliminary sedimentation of the cells on coverslips. The coverslips (18 × 18 mm) were placed on the bottom of glass bottles and covered with 10 ml of a suspension of thymocytes ($5 \cdot 10^6$ cells/ml). After sedimentation of the suspension for 30 min at room temperature the coverslips were turned over and the cells resuspended. To determine incorporation of ^{3}H-HC into the thymocytes, coverslips with cells adsorbed on them on both sides were placed in bottles containing a solution of ^{3}H-HC (37°C) in the presence or in the absence of PVP-HC. The cells were then washed off the coverslips in 3 volumes of Hanks' solution at 4°C for 2 min. The coverslips were then dried, heated, and placed in a scintillation flask, where the cells were solubilized with 0.1 ml of a 2% solution of sodium dodecylsulfate, after which 5 ml of toluene-alcohol scintillator was added. Radioactivity in the sample was counted on an SL-30 liquid scintillation counter (Intertechnique, France). The PVP-HC used in the work has a mol. wt. of 23,000 daltons and contained 4.2% of HC by weight. Antibodies against HC were taken from a commercial kit (Cortctk-125, from Sorin Biomedica, Italy).

EXPERIMENTAL RESULTS

After a first step the properties of native HC, as a highly specific ligand, and of HC immobilized on the polymer, were compared. This was done by competitive analysis of binding of ^{3}H-HC with antibodies in the presence of HC and of PVP-HC. As Fig. 1 shows, HC and PVP-HC were equally capable of displacing ^{3}H-HC from its complex with antibodies, evidence that their competitive power was identical.

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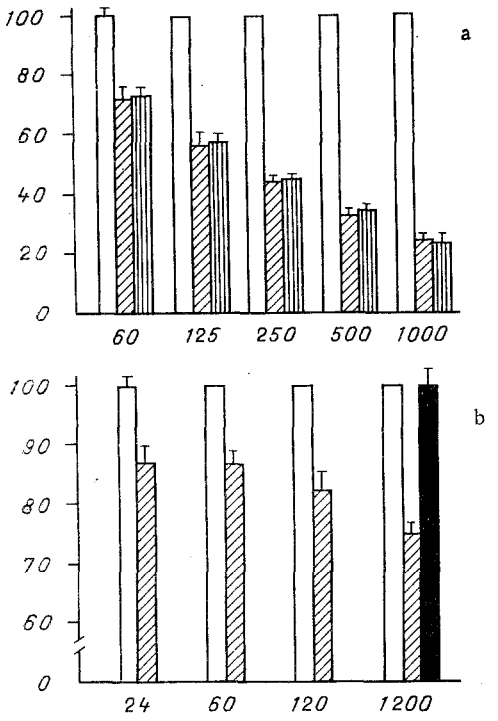

Fig. 1

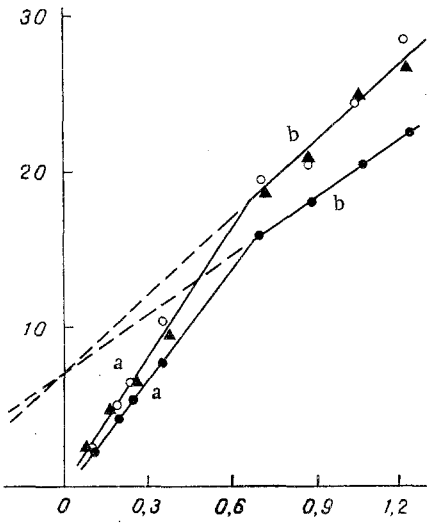

Fig. 2

Fig. 1. Dependence of binding of ^{3}H-HC by antibodies (a) and thymocytes of adrenalectomized rats (b). Abscissa, concentration of HC and PVP-HC (in nM); ordinate, $B/\Delta_0 \times 100\%$, where B_0 stands for binding of ^{3}H-HC in absence of unlabeled glucocorticoids and B for binding of ^{3}H-HC in presence of MC and PVP-HC. Unshaded columns, in absence of HC and PVP-HC: obliquely shaded column, in presence of HC; black oclumn, in presence of PVP. Concentration of ^{3}H-HC in medium 12 nM.

Fig. 2. Binding of ^{3}H-HC (filled circles) by thymocytes of adrenalectomized rats in presence of HC (triangles) and PVP-HC (empty circles). Abscissa, $1/C$ (nM^{-1}), where C stands for ^{3}H-HC concentration in incubation medium; ordinate, $1/B \times 10^3$, where B denotes quantity of ^{3}H-HC incorporated into thymocytes (in cpm/10^6 cells). Straight lines drawn by method of least squares. Concentrations of HC and PVP-HC 100 nM.

The next step was to study the ability of PVP-HC to penetrate into viable thymocytes. PVO-HC incorporating fluorescein (PVP-HC$_{fl}$ for 1 h at 37°C, and without subsequent washing, was examined in a fluorescence microscope. Against the yellow-green background of fluorescence it was possible to see black spots with clear boundaries, which, in transmitted light, were seen to be cells. This indicates that PVP-HC did not penetrate into the thymocytes during this period. In the next series of experiments binding of ^{3}H-HC by thymocytes was determined in the presence of PVP-HC. It will be clear from Fig. 1 that a twofold excess of PVP-HC leads to significant reduction of binding ($P = 0.05$) compared with the control (binding in the absence of PVP-HC). The polymer itself had no such effect. By subtracting from the total ^{3}H-HC accumulated by the cells the quantity accumulated in the presence of a 100-fold excess of PVP-HC (which reflects specific binding), and expressing the reduction of binding in the presence of a twofold excess as a ratio of this specific binding, it was found that in the latter case about half of the specific binding sites of HC by thymocytes were blocked. The character of the reduction of ^{3}H-HC binding by the cells with a further increase in PVP-HC concentration is evidence of possible saturation of the binding process.

It was then necessary to discover whether the observed reduction of ^{3}H-HC binding by the thymocytes was the result of competition for the same binding sites on the plasma membrane or whether PVP-HC somehow or other affected the properties of the membrane itself and indirectly changed the properties of HC binding sites.

To study this problem inhibitor analysis was carried out: In the presence of a constant concentration of HC or PVP-HC (100 nM), binding of ^{3}H-HC was studied in different concentra-

tions — from 0.86 to 12 nM. The results are shown graphically in Fig. 2 in modified Lineweaver–Burk plots. Binding of ^{3}H-HC by thymocytes in these plots was described, both in the absence and in the presence of competitors, by two straight lines: One straight line (Fig. 2a) characterizes the unsaturable component, nonspecific binding; whereas the other straight line (Fig. 2b) reflects binding of ^{3}H-HC with the saturable component of the cells, i.e., specific binding. It will be clear from Fig. 2 that the extrapolated regions of the straight lines, reflecting binding of ^{3}H-HC by the saturable system without competitors and in the presence of 100 nM HC or PVP-HC, intersect on the ordinate at the same point, in agreement with the case of competitive inhibition.

Hence it follows that specific binding sites for HC on rat thymocytes are accessible for PVP-HC which does not penetrate into the cell, which means that the binding sites for the glucocorticoid are locatd in the plasma membrane.

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MECHANISM OF STIMULATION OF ANTIBODY-FORMING ABILITY OF BONE MARROW CELLS OF MICE IMMUNIZED WITH STAPHYLOCOCCI

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The writers showed previously that splenocytes and bone marrow cells (BMC) of mice immunized with staphylococci are able to enhance the immunoreactivity of intact syngeneic recipients of these cells to homologous antigen [3, 4]. It has also been shown that the stimulating effect of immune splenocytes is due to immunologic memory cells present in the transplant [2]. There is information in the literature on the ability of memory B cells to migrate from peripheral lymphoid organs into bone marrow tissue [10, 13]. Meanwhile the regulatory role of bone marrow in immunogenesis is well known [5, 8].

The aim of this investigation was to study the mechanism of formation of the ability of BMC of mice immunized with staphylococci. The research tasks included a study of the antibody-forming ability of bone narrow of primed animals and the effect of irradiation of BMC *in vitro* on their stimulating activity, and a study of the role of the thymus and spleen in the formation of this activity.

EXPERIMENTAL METHOD

Experiments were carried out on CBA and BALB/c mice and also on mice with congenital absence of the thymus (nude). Staphylococcal corpuscular antigen (SCA) was obtained as described previously [4]. The donors of BMC were immunized intravenously with SCA in a dose of $5 \cdot 10^9$ bacterial cells. After 5 days the animals were killed by cervical dislocation, bone marrow was taken from their femora, and a cell suspension with a density of $4 \cdot 10^7$ cells/ml was prepared by phosphate-salt buffer (pH 7.2). Mice of the experimental group were given an intra-

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