Vol. 103, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1981 Pages 682-689

IDENTIFICATION OF SPECIFIC HIGH AFFINITY SITES FOR THE ESTRADIOL RECEPTOR IN THE ERYTHROCYTE CYTOSKELETON

Giovanni Alfredo Puca and Vincenzo Sica

Istituto di Patologia generale, First Medical School, University of Naples, Via S. Andrea delle Dame, 2, 80138 Naples, Italy

Received September 4,1981

SUMMARY. The possible relationship of the soluble, "cytosolic" estradiol receptor with complex membranous and cytoskeletal structures of the cell matrix has been studied using a model erythrocyte system. Extraction of erythrocyte ghosts with a nonionic detergent (Triton X-100) under conditions that yield a cytoskeletal matrix reveals the presence of a limited number (less than 100) of specific sites having high affinity (k_d 10^{-9} M) for the estradiol-receptor complex. The interaction between the estradiol receptor and the cytoskeleton is critically dependent on temperature and it is improved by 25 mM KCl or NaCl and by 2.5 mM $MgCl_2$. The data suggest that the estradiol receptor, which has been generally considered to be freely "soluble" in the cytoplasm, may actually be physiologically associated in an integral manner with a complex cytoskeletal network in the cell cytoplasm.

INTRODUCTION. Steroid hormone receptors have generally been considered as being water-soluble macromolecules, floating in the cytoplasm of their target cells, waiting for the arrival of a specific hormone molecule. Formation of the hormone-receptor complex presumably leads to putative conformational changes which determine the transfer of the receptor to the nuclear compartment. This mechanism has become the "central dogma" for the action of steroid hormones, and most experimental data have been interpreted on the basis of this model. Many aspects of this thesis have not been elucidated or addressed during the more than 10 years since this model was first proposed (see Ref. 1).

Recently much data have been published which question the validity of this central dogma of the mechanism of steroid action, and serious doubts must be raised concerning at least one of its major points, i.e., the presence in the cytosol (of target cells) of the receptor as an unrestrained, freely water-soluble molecule (2-5).

Increasing awareness of the intricate and complex nature of the ultrastruc-

0006-291X/81/220682-08$01.00/0

tural organization of the cytoplasm and the high degree of integration of the various cytoplasmic elements suggests that steroid hormone receptors may be closely related to a highly organized microscopic network of structural elements (6). This network forms a morphological and functional unit of fundamental importance in cellular functions (7-15).

The present studies provide evidence that the estrogen receptor of calf uterus interacts with high affinity and in a specific and saturable manner with components of the erythrocyte cytoskeletal system; the latter may generally be considered as a simplified model system for exploring cytoskeletal properties of more complex eucaryotic cells.

MATERIALS AND METHODS. 17β-Estradiol-2,4,6,7-t_4 (84 to 110 Ci/mmol) was from Amersham. Cow blood erythrocyte ghosts and cytoskeleton were prepared as described by Bennett (16,17); these were resuspended with phosphate buffer at a protein concentration of 3 to 10 mg/ml, diluted 1:1 (v:v) with 99% glycerol and stored at -70° until used.

Calf uterine cytosol was prepared as previously described (18) except that only phosphate buffer (7.5 mM phosphate buffer, pH 7.5) instead of Tris-HCl EDTA and dithiothreitol buffer was used. The assay of specific binding activity of cytosol was performed by the Dextran-coated charcoal method (19). The binding of the [^{3}H]estradiol-receptor complex to erythrocyte ghosts and cytoskeletons was performed in duplicate. Calf uterine cytosol was preincubated for 10 min at 20° in a total volume of 0.4 ml of phosphate buffer with 2.5 pmoles of [^{3}H]estradiol, 12.5 µmoles KCl and 1.25 µmoles $MgCl_2$. The reaction was started by adding 0.1 ml of cytoskeleton or ghost suspension (0.25 to 2 mg of protein) and followed either for 2 hours at 20° or for 30 min at 30°. Parallel tests were performed in the presence of 1×10^{-6} M unlabeled estradiol. The reaction was stopped by adding 10 ml of ice-cold phosphate buffer. Samples were centrifuged at 30,000 x g for 20 min at 4° and the pellets were washed three times. The final pellet was resuspended with 1 ml of phosphate buffer and assayed for radioactivity. The values obtained in the presence of unlabeled hormone were subtracted from the total.

RESULTS. Specific binding sites for radioactive estradiol can be detected in erythrocyte membranes, but only in the presence of calf uterine cytosol. These sites are only detected after extraction of the membranes with detergent (Fig. 1). The maximal number of binding sites appears by treatment of erythrocyte membranes with 1% Triton X-100 and does not increase with higher detergent concentrations. This extraction procedure is used operationally to prepare the erythrocyte "cytoskeleton" (20). The tritiated hormone does not associate with the ghosts or cytoskeleton if the uterine cytosol is omitted, if the

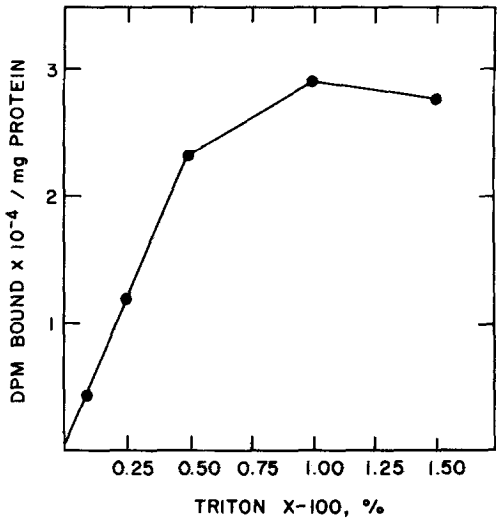

Fig. 1. Effect of extracting erythrocyte ghosts with Triton X-100 on the appearance of specific binding of [^{3}H]estradiol-receptor complex. Erythrocyte ghosts were extracted for 15 min at 0° with the indicated concentrations of Triton X-100. After extraction, the ghosts were centrifuged and washed four times with phosphate buffer. The pellets were resuspended at a final protein concentration of 12 mg per ml with phosphate buffer. Aliquots of 0.1 ml were then incubated for two hours at 20° with 0.2 ml of calf uterine cytosol, previously labeled with [^{3}H]estradiol.

cytosol is inactivated by heating (30 min at 60°), if a cytosol from a non-target tissue (e.g., liver, thymus) is used or if similar amounts of other proteins (e.g., albumin, ovalbumin, immunoglobulins) with no specific receptor sites for estradiol are used. This association must therefore be considered to be a receptor-mediated interaction with components of the erythrocyte cytoskeleton.

Binding of the estradiol receptor to the cytoskeleton is time and temperature dependent (Fig. 2). Temperatures higher than 30° cannot be used easily because of the inherent instability of the estradiol-receptor complex. Preincubation of calf uterine cytosol with the hormone for 2 hours at 20° does not change the subsequent binding of the [^{3}H]estradiol-receptor complex to the cytoskeleton at 0° (dotted line, Fig. 2).

The interaction is affected in important ways by KCl or NaCl. Binding increases about three-fold in the presence of 25 mM KCl; higher concentrations decrease the binding but not to values below those seen without KCl, even at ionic strengths higher than 1 M (Fig. 3A). In the presence of 25 mM KCl, Ca^{++}

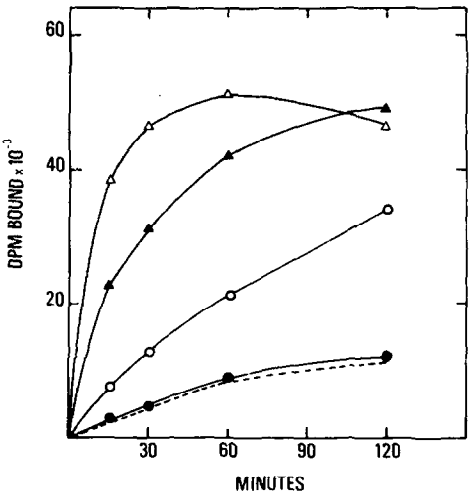

Fig. 2. Time course of binding of estradiol-receptor complex to erythrocyte cytoskeleton. Erythrocyte cytoskeleton was incubated with 0.1 ml of calf uterine cytosol. Incubations were carried out at 0° (●), 10° (○), 20° (▲) and 30° (Δ). At the indicated times the reaction was stopped by adding cold phosphate buffer and the binding was measured. The dotted line represents the binding at 4° after preincubation of the estradiol-receptor complex for 2 hr at 20°.

(up to 5 mM) has no stimulatory effect, while 5 mM Mn^{++} slightly increases the binding. Mg^{++} stimulates the binding substantially, with an optimal concentration at 2.5 mM (Fig. 3B).

The binding of the [^{3}H]estradiol-receptor complex is linearly related to the concentration of cytoskeleton in the range of 100 to 1000 µg protein per ml, provided the concentration of estradiol receptor is not limiting (not shown). However, when the cytoskeleton concentration is kept constant while that of the receptor is increased, the interaction appears to be saturable and of high affinity (Fig. 4A). Scatchard analyses of such saturation experiments suggest the presence of two classes of binding sites differing markedly in their affinity and quantity (Fig. 4A). The dissociation constant of the higher affinity component is about 10^{-9} M. Mg^{++} increases substantially the number of these high affinity sites without modifying the dissociation constant (Fig. 4B). From the data described in figure 4B, and independently determining that 1 mg of cytoskeletal protein corresponds to about 5 x 10^9 erythrocytes, the cytoskeleton from one erythrocyte appears to bind a maximum of 50 to 100

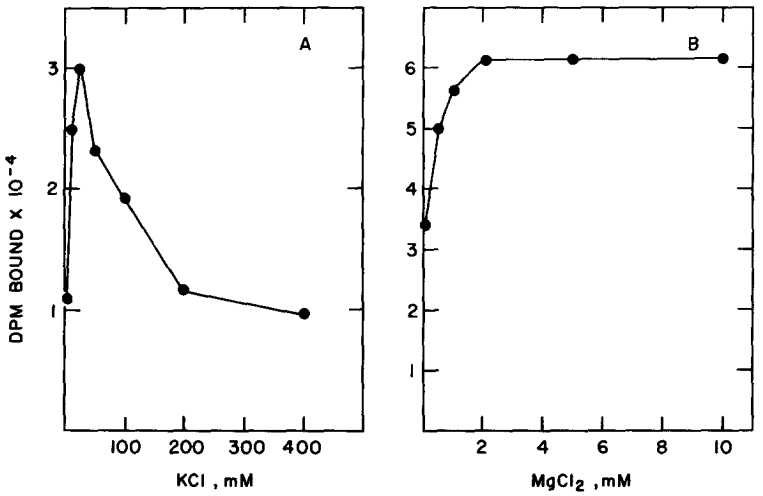

Fig. 3. Effect of increasing concentrations of KCl and $MgCl_2$ on the binding of [^{3}H]estradiol-receptor complex to erythrocyte cytoskeleton. A. Erythrocyte cytoskeleton was incubated for 2 hr at 20° with 0.2 ml of calf uterine cytosol with the indicated concentration of KCl. B. Erythrocyte cytoskeleton and calf uterine cytosol were incubated in phosphate buffer containing 25 mM KCl and the indicated concentration of $MgCl_2$.

molecules of estradiol-receptor complex, assuming that one mole of estradiol is bound per mole of receptor.

When the various forms of the estradiol receptor are prepared (18) and studied separately, the native 8 S form of the receptor binds with great affinity to the cytoskeleton, while those forms obtained after controlled proteolysis by the Ca^{++}-dependent uterine protease or by trypsin (18) have lost the ability to bind to the cytoskeleton despite the fact that the binding of estradiol to these receptor forms is unaltered (data not shown).

DISCUSSION. The calf uterine estradiol-receptor complex displays almost no specific binding to erythrocyte ghosts. However, when many of the proteins and most of the lipid bilayer are removed with the nonionic detergent, Triton X-100, a discreet and finite amount of binding is found associated with the Triton shells. The inability of the erythrocyte ghosts to bind the estradiol receptor might be due to an internal localization of the putative binding sites, to factors related to steric hindrance or to their occupancy by other molecules which can be removed by the detergent. It is certain, however, that

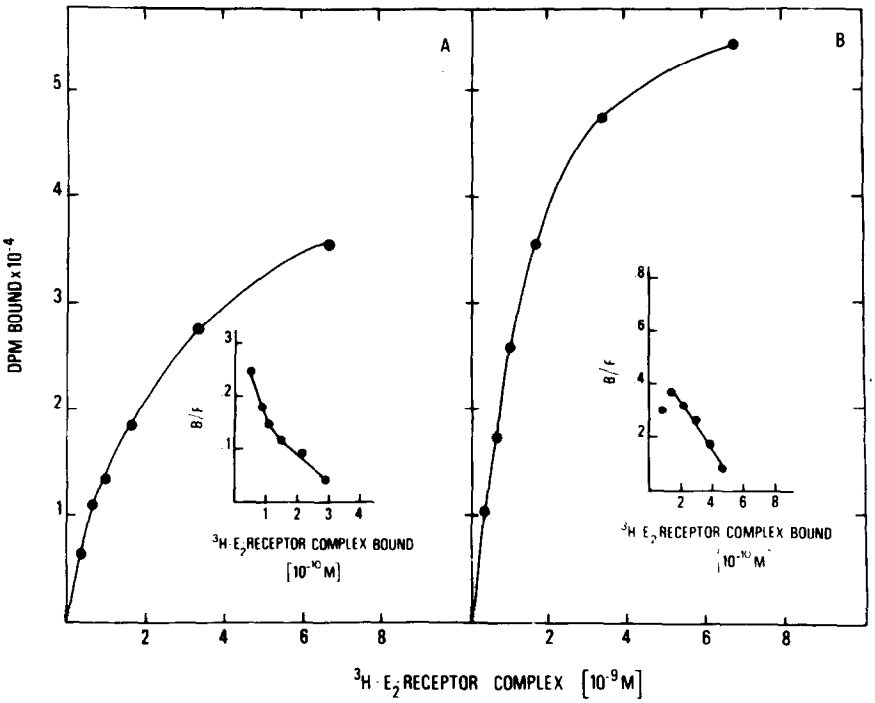

Fig. 4. Effect of increasing the concentration of [^{3}H]estradiol-receptor complex on the binding of erythrocyte cytoskeleton. Erythrocyte cytoskeleton was incubated for 2 hr at 20° with different amounts of calf uterine cytosol. The binding was carried out in the presence of 25 mM KCl alone (A) or in the presence of 25 mM KCl and 2 mM $MgCl_2$ (B). Inserts: Scatchard plots of the data.

these sites are very strongly associated with the cytoskeleton of the erythrocyte membrane. The strong dependence of estradiol receptor binding on temperature also suggests the requirement for an "open" cytoskeletal organization. This temperature effect is not simply a manifestation of a necessary modification or activation of the receptor (21) since prior heating of the cytosol does not facilitate the subsequent binding to cytoskeletons at low temperature (Fig. 2). The temperature requirement might reflect the need for increased fluidity of the residual membrane components, it might induce necessary conformational changes, or it might facilitate access of the receptor to its binding site. Alternatively, low temperatures may actively disrupt necessary organizational arrangements of structural proteins essential for binding.

Saturation analysis of the receptor-cytoskeletal interaction reveals the

presence of a very limited number (less than 100) of high affinity binding sites per erythrocyte ghost. This excludes the possibility that one of the major protein components of the cytoskeleton (e.g., band 3, spectrin, actin, ankyrin, band 4.1) may be responsible for the binding. Although ionic strength influences the receptor-cytoskeletal interaction in a nonspecific manner, the cation-dependent enhancement of binding appears to be selective for Mg^{++}.

These data provide evidence for the existence of specific and high affinity binding components in the cytoskeletal matrix of the erythrocyte membrane. Similar systems may exist in target cells, which could in part explain data apparently contradictory with the general model of action of steroid hormones (2-5, 22-31). Results to be published elsewhere demonstrate that high affinity cytoskeletal binding sites very similar to those described here also exist in estrogen target cells. Thus, it is conceivable that the traditional water-soluble nature of the native, 8 S estradiol receptor found in the cytosol is primarily an artefact of tissue homogenization and preparation (3-5), and that the receptors normally exist in situ as part of a complex cytoskeletal matrix (24) that constitutes a highly organized microtrabecular network of the cell cytoplasm. Similar approaches have recently demonstrated analogous erythrocyte cytoskeletal interactions for the hormone-dependent adenylate cyclase system (32-34).

ACKNOWLEDGEMENTS. The authors thank Drs. Naji Sahyoun and Vann Bennett for their helpful advice. This work was supported by the Progetto Finalizzato: Controllo della Crescita Neoplastica, by a grant from the Wellcome Research Laboratories, RTP, North Carolina and the National Institute of Health Contract No. NO1-CB-64074.

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