ACTIVATION OF ESTRADIOL–RECEPTOR COMPLEX BY ATP IN VITRO

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1. Introduction

Upon their entry into target cells, steroid hormones initially interact with cytoplasmic receptors to form hormone–receptor complexes which subsequently translocate onto sites on nuclear chromatin [1]. However, upon their extraction from target cells at low temperatures, receptors can be complexed with hormones to form complexes which are said to be in non-activated form with little affinity for isolated nuclei. The receptor complexes in such preparations can be activated by incubation at elevated temperature [2], high ionic strength [3,4], dilution of cytosol or gel filtration [5] and some other treatments [6,7]. Receptors activated by one of the above methods acquire an increased affinity for isolated nuclei [8,9], DNA [10,11], ATP–Sepharose [12] and altered mobility on DEAE-Sephadex [13]. An ATP-dependent activation of glucocorticoid receptors has been reported [14,15]. We report here activation of estrogen receptor at low temperatures (0–4°C) involving an incubation with 5–10 mM ATP. The extent of receptor activation was measured by determining the binding of estradiol–receptor complex to isolated nuclei or DNA-cellulose. Because of the convenience and availability, hen oviducts were used for nuclear uptake studies whereas rat uterus estradiol receptor was utilized for sucrose gradient analysis and DNA-cellulose chromatography. Estradiol receptors from hen oviducts have been reported [16,17].

2. Materials and methods

2.1. *Preparation of estradiol–receptor complex*

Immature (14–18 day old) female albino rats were obtained from Maguran Farms (Troy, MI). Rats were killed by cervical dislocation and the uterine horns were removed. The tissue was stripped of fat and homogenized (5 uteri/ml) in buffer containing 40 mM Tris–HCl, 12 mM thioglycerol, 10% glycerol (pH 8.0). The homogenate was centrifuged at 150 000 × *g* for 90 min. Aliquots of the resultant cytosol were incubated with 15 nM [^{3}H]estradiol for 2 h at 4°C to form the complexes.. Hen oviduct cytosol was prepared as in [18] and was complexed with labeled estradiol as above.

2.2. *Nuclear binding assay*

Nuclei were isolated from hen oviduct tissue as in [19]. Nuclear uptake assay consisted of incubation of hormone–receptor complexes with different [ATP] at 4°C, followed by the addition of a nuclear aliquot containing ~50 μg DNA [8,20]. The nuclear binding assays were performed as in [8,14,15].

2.3. *DNA-cellulose chromatography*

Samples containing rat uterine estradiol–receptor complexes were chromatographed over separate identical 2 ml DNA-cellulose columns. DNA-cellulose was prepared according to [21] and the affinity chromatography procedures were performed essentially as in [15]. Aliquots containing receptor-complexes with and without ATP treatment were also layered over 5–20% linear sucrose gradients for analysis on sedimentation rate.

3. Results and discussion

Fig.1 shows the effect of ATP treatment on the nuclear uptake of [^{3}H]estradiol–receptor complex. Portions of hen oviduct cytosol were incubated with 12 nM [^{3}H]estradiol for 2 h at 4°C to form hormone–receptor complexes. The preparations were then incubated for 1 h at 4°C with varying [ATP].

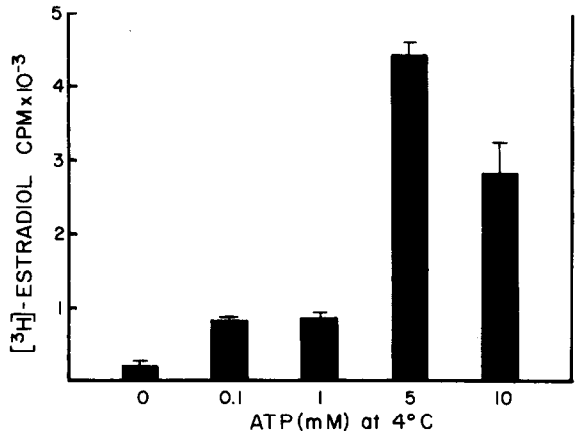

Fig.1. Effect of ATP on the nuclear binding of [^{3}H]estradiol–receptor complex. Freshly excised oviducts from White Leghorn hens were rinsed with cold 0.9% NaCl, transferred to 2 vol. (w/v) buffer containing 40 mM Tris–HCl, 12 mM thioglycerol and 10% glycerol (v/v) (pH 8.0) and homogenized first in a Waring blender and then with a tissumizer (Tekmar model SDT). The homogenate was centrifuged at 12 000 × *g* for 10 min followed by 150 000 × *g* for 90 min. Portions of this clear supernatant (cytosol) were incubated with 12 nM [2,3,6,7-^{3}H(*N*)]estradiol (98.5 Ci/mmol, New England Nuclear) for 2 h at 4°C in 0.5 ml total vol. The complexes thus formed were treated with different [ATP] for 60 min at 4°C. The mixtures were then incubated with aliquots of hen oviduct nuclei containing 50–100 μg DNA and the nuclear binding assays were performed as in [8,19]. The results are expressed as the mean ± SE of triplicate determinations in a single experiment.

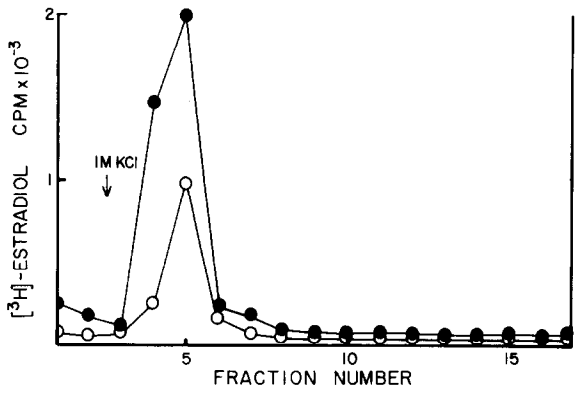

Fig.2. Measurement of activation of estradiol–receptor complex by DNA-cellulose affinity chromatography. Rat uterine cytosol preparations were obtained by the procedures in fig.1. Five uteri/ml homogenization buffer were used. The cytosol was labeled with 15 nM [^{3}H]estradiol for 90 min at 4°C in 0.5 ml total vol. Samples were then treated with 10 mM ATP or 10 mM Tris–HCl (pH 8.0) for 60 min at 4°C. The entire samples (1 ml) were then mixed with 2 ml DNA-cellulose preparation [11,21] equilibrated with 10 mM Tris–HCl, 12 mM thioglycerol and 0.01 M KCl buffer containing 20% glycerol (pH 8.0). The mixtures were continually mixed in a slurry form for 30 min over an Ames aliquot mixer. The slurry was then poured into columns. The columns were washed with the equilibration buffer and 1.2 ml fractions were collected. The [^{3}H]estradiol–receptor complexes were eluted using the same buffer but containing 1 M KCl. Small aliquots (0.5 ml) from each fraction were used to measure radioactivity as in [18,27]. (○) Control; (●) 10 mM ATP.

Aliquots of freshly prepared hen oviduct nuclei were then added to the complexes and nuclear binding assays performed. There was a small increase in the nuclear uptake of estradiol–receptor complex pretreated with 1 mM ATP whereas the maximal activation occurred with 5–10 mM nucleotide. A comparable activation occurred when estradiol–receptor complexes were warmed at 23°C for 1 h and then tested for their nuclear uptake (not shown).

The results of numerous studies have suggested presence of a DNA binding site for steroid hormone-receptors [4,11,21–23]. It has been further shown that the binding to DNA-cellulose is a property of an activated receptor [11,21,24]. Fig.2 illustrates results of chromatography of rat uterine cytosol preparations containing estradiol–receptor complexes. Aliquots of the uterine cytosol containing estradiol–receptor complexes incubated with and without 10 mM ATP were chromatographed over separate identical 2 ml DNA-cellulose columns. About 9% of the total receptor–hormone complexes present in the control sample were retained on DNA-cellulose column. A partial activation of cytosol receptor may occur during processing and fractionation procedures in a time-dependent manner [6]. However, ATP pretreatment increased the DNA binding by 3-fold causing 26% of the original complexes to be retained on the affinity resin.

A hormone-dependent activation of steroid receptors has been reported in cell-free preparations [22]. Several studies have suggested that receptor activation involves structural or conformational changes in the receptor molecule which result in the increased affinity of receptor to polyanionic resins presumably due to emergence of positively charged groups at the surface of receptor protein [2]. Mammalian estrogen receptor has been reported to dimerize upon activation as determined by sucrose density centrifugation analysis [25]. Fig.3 illustrates the sedimentation pro-

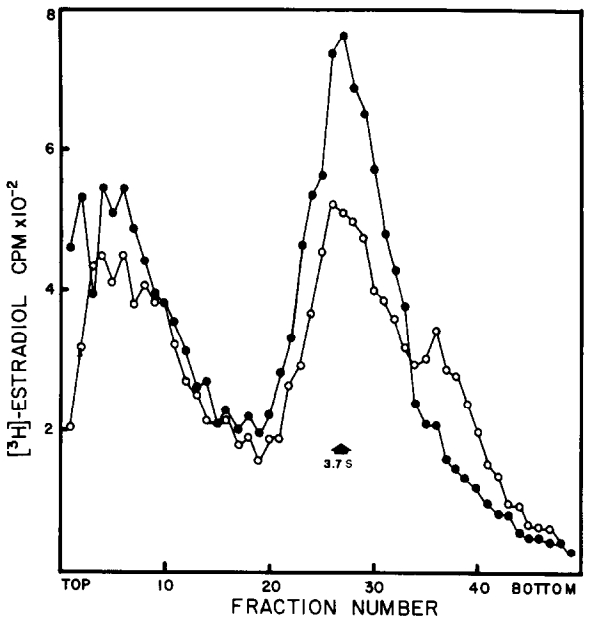

Fig.3. Sucrose gradient analysis of [^{3}H]estradiol–receptor complex. Rat uterine cytosol containing [^{3}H]estradiol–receptor complex was treated with 10 mM ATP at 4°C as in fig.1,2. Linear 5–20% sucrose gradients (4.4 ml) were prepared in buffer containing 10 mM Tris, 12 mM thioglycerol and 0.3 M KCl using a Beckman Gradient Former. Samples (0.2 ml) containing estradiol–receptor complexes treated with and without ATP were layered on separate gradients. Receptor samples were diluted with buffer to lower the glycerol concentration. The gradients were centrifuged at 150 000 × *g* on a 50.1 SW rotor in Beckman L-75 ultracentrifuge for 16 h. The tubes were pierced at the bottom and 0.1 ml fractions collected. [^{14}C]Ovalbumin (3.7 S) was used as marker protein and layered on a separate gradient. (•) Control; (○) 10 mM ATP.

files of estrogen–receptor complex from rat uterine cytosol preparations activated by treatment with 10 mM ATP at 4°C. The majority of [^{3}H]estradiol–receptor complex sedimented as a 4 S entity over 5–20% linear sucrose gradients. ATP treatment at 4°C generated another peak of radioactivity corresponding to 5 S value characteristic of an activated estradiol–receptor complex. Such a change in sedimentation rate of non-activated receptor following activation was also observed by warming the cytosol preparations at 23°C for 40 min (not shown).

The effect of ATP on estradiol–receptor activation appears to be selective. Other nucleotides like ADP, AMP, cAMP and adenylimidodiphosphate were unable to mimic ATP effects (not shown). Our preliminary studies indicate that other nucleoside triphosphates may also enhance receptor activation. At this point the mechanism of receptor activation is not clear. However, the inability of ATP analog, adenylimidodiphosphate, to affect activation suggests that the hydrolysis of terminal phosphates of nucleoside triphosphates may be necessary for receptor activation. In this regard a phosphorylation–dephosphorylation mechanism has been suggested [26] to determine the level of active receptor capable of steroid binding. Whether receptor activation and its subsequent translocation onto nuclear sites also requires phosphorylation–dephosphorylation mechanisms is not clear. Alternately, ATP may be providing energy for the cytoplasmic receptor to translocate into the nucleus [18]. Other possibilities include the binding of the nucleotide with receptor in an allosteric manner that alters receptor conformation exposing sites that are required for binding to polyanionic resins or nuclear sites [2].

These studies offer a convenient procedure to study receptor activation at low temperature where the alterations in the metabolism and activities of cytosol constituents are at minimum. The method appears to be independent of requirement for divalent cations (not shown). They also reveal further information on the role of ATP binding by steroid receptors reported in [12,18,27,28].

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