ATP-dependent activation of glucocorticoid receptor from rat liver cytosol

Virinder K. MOUDGIL and Judith K. JOHN

Department of Biological Sciences, Oakland University, Rochester, MI 48063, U.S.A.

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The glucocorticoid–receptor complex from freshly prepared rat liver cytosol is in a non-activated form, with very little affinity to bind to isolated nuclei. When such preparations were incubated with 5–10 mM-ATP at 4°C, the receptor complex acquired the properties of an ‘activated’ transformed form, which readily bound to nuclei, ATP–Sephadex, phosphocellulose and DNA–cellulose. This transformation was comparable with the activation achieved by warming the steroid–receptor complex at 23°C. The effect of ATP was specific, as it was more effective than ADP, whereas AMP had no such effect on activation. The process of receptor activation was sensitive to the presence of 10 mM-sodium molybdate; the latter blocked activation by both ATP and heat. Bivalent cations had no observable effect on the receptor activation at low temperature, but they decreased the extent of activation by ATP. The steroid-binding properties of glucocorticoid receptor remained intact under the above conditions. However, a significant increase in steroid binding occurred when ATP was preincubated with cytosol receptor before the addition of [³H]triamcinolone acetonide. ATP also stabilized the glucocorticoid–receptor complexes at 23°C. These results suggest a role for ATP in receptor function and offer a convenient method of studying the activation process of glucocorticoid receptor under mild assay conditions.

Steroid hormones are known to interact initially with their target cells to form steroid–receptor complexes at physiological temperatures. The complexes so formed are located in the cytoplasm, from where they translocate into the nucleus (King & Mainwaring, 1974; Munck & Foley, 1979). At low temperature (0–4°C) the glucocorticoids interact with the target cell to bind their intracellular receptors, or with cytosols from such target-cell preparations to form ‘non-activated’ complexes that do not bind to nuclei (Munck *et al.*, 1972). The cytosol steroid–receptor complexes exist in a non-activated form and can be activated by warming at 23–37°C in the absence of nuclei. Such preparations containing activated receptor complexes have been shown to develop increased affinity for isolated nuclei or DNA (Baxter *et al.*, 1972; Beato *et al.*, 1973; Milgrom *et al.*, 1973; Atger & Milgrom, 1976; Goidl *et al.*, 1977; Cidlowski & Munck, 1978), and altered mobility on phosphocellulose (Parchman & Litwack, 1977) and DEAE-cellulose (Fevre *et al.*, 1979) columns. In addition to the incubation at elevated temperatures, activation of glucocorticoid–receptor complex can also be brought about by high ionic strength, gel filtration,

dilution of cytosol, alteration in pH and other treatments (Beato *et al.*, 1973; Cidlowski & Munck, 1978; Moudgil & John, 1979). The conditions mentioned above for receptor activation can affect the properties of the complex, because of changes in concentration and metabolism of various assay constituents or because of the labile nature of the glucocorticoid receptor. The rate of activation is extremely low at low temperature, ionic strength and neutral pH in concentrated cytosol (John & Moudgil, 1979). We demonstrate in the present paper a new method of activation of rat liver glucocorticoid–receptor complex at low temperature (0–4°C), involving an incubation with 5–10 mM-ATP. The indication that ATP may be involved in some aspect of receptor activation came from our studies on the interaction of glucocorticoid–receptor complex with immobilized ATP (Moudgil & John, 1979). It was observed that the freshly prepared rat liver cytosol contained a receptor which, when complexed with [³H]triamcinolone acetonide, had very little affinity for ATP–Sephadex. The complex acquired this ability to bind to ATP–Sephadex on heating at 23°C for various time periods. This increased ATP binding on activation of the steroid–receptor

complex has been shown to parallel the nuclear uptake of progesterone-receptor complex (Buller *et al.*, 1975).

Materials and methods

All reagents were of analytical grade. Nucleotides, activated charcoal, monothioglycerol, unlabelled triamcinolone acetonide and cortisol were purchased from Sigma; [6,7(n)-³H]triamcinolone acetonide (30–50 Ci/mmol) was from New England Nuclear and was repurified by t.l.c. by using methylene chloride/methanol (24:1, v/v). Sodium molybdate was purchased from Merck. All buffers were pH 8.0 at 23°C.

Preparation of glucocorticoid receptor

Male albino rats of Yale strain (150–200 g) were obtained from Maguran Farms, Troy, MI, U.S.A. The rats were bilaterally adrenalectomized and kept on salt water (0.9% NaCl) and Lab Chow (Ralston Purina Co., St. Louis, MO, U.S.A.) for 3 days and killed by cervical dislocation. The whole liver was quickly excised, wiped of adhering blood and rinsed in cold 0.9% NaCl. The tissue was minced and homogenized in 2 vol. (w/v) of homogenization buffer [40 mM-Tris/HCl, 50 mM-monothioglycerol, 10% (v/v) glycerol and 0.01 mM-KCl, pH 8.0] with a Tissuemizer (Tekmar model SDT). The homogenate was first centrifuged at 12000 g for 10 min and then at 150000 g for 60 min. The resultant clear supernatant was termed 'cytosol' and used immediately for various assays.

Activation of cytosol glucocorticoid-receptor complex

The freshly prepared rat liver cytosol was incubated with 10 mM-[³H]triamcinolone acetonide for 4 h at 4°C to form glucocorticoid-receptor complexes. After this, the mixtures were treated with different concentrations of ATP and incubated at 4°C for 40 min. Parallel samples in triplicate were incubated at 23°C with and without ATP to compare the extent of activation of glucocorticoid-receptor complexes. The control samples contained steroid-receptor complex in 10 mM-Tris/HCl/50 mM-monothioglycerol, pH 8.0, and no ATP. The non-specific binding was monitored by including triplicate samples pretreated with excess (100-fold) unlabelled cortisol or triamcinolone acetonide before the addition of labelled hormone.

Nuclear binding assay

The procedures for determining the nuclear binding of glucocorticoid-receptor complexes were as described previously (Buller *et al.*, 1975; Lohmar & Toft, 1975). The cytosol [³H]triamcinolone acetonide-receptor complex after treatment at 4°C

with ATP or at 23°C was incubated with rat liver nuclei prepared by the methods reported by Spelsberg *et al.* (1974), and each assay tube contained 50–100 µg of DNA (Burton, 1956). After gentle shaking for 1 h at 4°C, the nuclei were washed twice with 3–5 ml of buffer (0.01 M-Tris/HCl, 0.001 M-MgCl₂ and 10% glycerol, pH 7.5) and centrifuged at 1000 g for 10 min. Each nuclear pellet was resuspended in 2 ml of scintillation fluid [Bio-Sol (Beckman)/Spectrafluor (Amersham)/toluene, 2:1:15, by vol.] and transferred to a vial containing 3 ml of the same fluid for measurement of radioactivity.

Measurement of activation by binding to ATP-Sepharose

The procedure used to measure the binding of activated glucocorticoid-receptor complex was essentially as described by Moudgil & Toft (1975). The freshly prepared cytosol was incubated with [³H]triamcinolone acetonide for 4 h at 4°C to form glucocorticoid-receptor complexes. These preparations containing the complex were then treated with or without ATP at 4°C. Samples were also heat-activated by incubation of receptor complex at 23°C for 40 min and served as markers for comparing with the activation by ATP. After an initial activation, the receptor complexes were treated with charcoal suspension (1% Norit A and 0.05% Dextran T-70 made in 10 mM-Tris/HCl/50 mM-monothioglycerol, pH 8.0) to remove the free nucleotides. About 2% of the added nucleotides remained after this treatment, as tested by phosphate analysis (King, 1932). In order to avoid any dilution of the complexes, the charcoal suspension was first centrifuged (1000 g, 5 min) and the complexes were treated with the pelleted charcoal. After this, the samples were chromatographed on ATP-Sepharose columns as described by Moudgil & Toft (1975).

Binding to DNA-cellulose

DNA-cellulose was prepared by the method of Alberts & Herrick (1971). Calf thymus DNA (Sigma, type II) was linked to Cellex-410 (Bio-Rad) and our preparations contained 1.5 mg of DNA/g of cellulose. The affinity resin was stored as a slurry and hydrated in 10 mM-Tris/HCl/50 mM-monothioglycerol buffer, pH 8.0, for 2 days before use. The extent of DNA binding was measured by using batch assay. Equal portions of DNA-cellulose suspension equilibrated in 10 mM-Tris/HCl/50 mM-monothioglycerol buffer plus 0.01 M-KCl, pH 8.0, were transferred to a series of tubes and the contents were centrifuged (1000 g, 5 min). The receptor samples were incubated with DNA-cellulose at 4°C with continuous gentle shaking. The tubes with affinity resin were washed with equilibration buffer

and receptor complexes disrupted with 10mM-Tris/HCl/50mM-monothioglycerol buffer containing 1 M-KCl.

Phosphocellulose (Whatman P 11) was precycled with 0.5M-NaOH and with 0.5M-HCl and then equilibrated with 10mM-Tris/HCl/50mM-monothioglycerol containing 0.01 M-KCl, pH 8.0, for 2 days. Equilibrated phosphocellulose was then transferred to 4ml columns (packed volume) and columns were re-equilibrated before use. The details of fractionation are provided in the Figure legends.

Steroid binding

Charcoal-adsorption assays were performed to monitor the total and non-specific steroid binding. Triplicate samples (0.1 ml) from receptor preparations were incubated with 10mM-[³H]triamcinolone acetonide for 4 h at 4°C in a total volume of 0.5ml. The data were corrected for non-specific binding, which was monitored by including excess of cortisol in the samples before the addition of labelled steroid. An equal volume of dextran-coated charcoal was added to each tube and the contents were mixed gently. After 5 min incubation at 4°C, tubes were centrifuged at 1000 g for 5 min and supernatants were used to measure radioactivity (Moudgil & Toft, 1975, 1977).

Statistical evaluation of the results

The results were expressed (where appropriate) as

the mean ± S.E.M. of triplicate determinations in a single experiment. Each experiment was repeated three to six times with identical results.

Results

When freshly prepared [³H]triamcinolone acetonide–receptor complex was incubated with different concentrations of ATP at 0–4°C for 40 min, it acquired the ability to bind to isolated nuclei (Table 1). The nuclear uptake was not apparent at nucleotide concentrations below 3–5 mM, whereas 10 mM-ATP caused the maximum nuclear uptake. The nuclear binding observed after ATP treatment was comparable with nuclear binding obtained by activation of [³H]triamcinolone–receptor complex by warming at 23°C. This ATP-dependent transformation of cytosol receptor complex to a nuclear-binding form was found to be sensitive to the presence of 10 mM-molybdate either during or before activation. Molybdate has been shown to inhibit the salt- or heat-activation of thymocyte glucocorticoid–receptor (Dahmer *et al.*, 1979; Leach *et al.*, 1979) and heat-activation of avian progesterone–receptor (Toft *et al.*, 1979). A small time-dependent increase in the nuclear binding of non-activated glucocorticoid–receptor complex was seen in cytosol samples incubated at 4°C without any additives. This could result from a partial activation of receptor complex

Table 1. Effect of nucleotides and sodium molybdate on the uptake of [³H]triamcinolone–receptor complex by rat liver nuclei

Freshly prepared cytosol from adrenalectomized rats was incubated with 10nM-[³H]triamcinolone acetonide for 2 h at 4°C in a total volume of 0.5 ml. The complexes so formed were then incubated with and without the additives (nucleotides or molybdate) for 40 min at 4°C. Wherever appropriate, samples in triplicate were also incubated at 23°C for 40 min for heat-activation. After these treatments, portions of rat liver nuclei (0.4 ml, 50–100 µg of DNA) were added to the reaction mixtures and nuclear-binding assays were performed as described in the Materials and methods section. The data are corrected for non-specific steroid binding obtain by pretreatment of receptor preparations with a 100-fold excess of cortisol and triamcinolone acetonide. ‘Incubation temperature’ is the temperature at which [³H]triamcindone–receptor complexes formed at 4°C were incubated before the nuclear-binding assays (also performed at 4°C).

Nucleotide* or effector	Incubation temperature	Nuclear-bound [³ H]triamcinolone–receptor (% of control)
None	4°C	9.5 ± 1.8
None (control)	23°C	100
ADP (10mM)	4°C	62.0 ± 0.5
AMP (10mM)	4°C	8.7 ± 0.3
ATP		
(1mM)	4°C	13.6 ± 0.5
(5mM)	4°C	59.2 ± 1.4
(10mM)	4°C	88.0 ± 3.0
	23°C	138.0 ± 7.2
Molybdate (10mM)	4°C	2.4 ± 0.6
Molybdate (10mM) + ATP (10mM)	23°C	2.2 ± 0.5
	4°C	3.4 ± 0.5

* Removed by treatment with dextran-coated charcoal suspension before incubation of receptor with nuclei.

because of its incubation with nuclei for an extended period (60–90 min), as has been reported by Fevre *et al.* (1979). ATP may be interacting with or affecting the receptor and not nuclei, since removal of ATP by charcoal treatment before incubation of receptor with nuclei did not prevent the increase in nuclear binding (Table 1). In addition, when preparations that were already activated (salt- or heat-activated) were incubated with nuclei at 4°C, the presence of ATP did not alter the nuclear uptake of receptor complex to any significant extent (results not shown).

The effect of ATP on receptor activation appears to be specific (Table 1). ATP was consistently more effective than ADP in transforming the cytosolic receptor to the nuclear-binding form, whereas AMP had little or no effect. The extent of receptor activation by AMP as shown in Table 1 is also contributed by a partial activation of receptor in the absence of AMP. Our preliminary studies indicate that other nucleoside triphosphates may also promote receptor activation. Further quantitative studies on the receptor activation by these nucleotides should reveal, if any, the order of their potency in effecting receptor activation. Although the increased nuclear binding in the presence of ATP could result from high initial steroid binding, our results indicate otherwise. Two identical groups of receptor preparations containing [³H]triam-

cinolone–receptor complexes were treated with ATP as described in Table 1: one group was utilized for nuclear-uptake assay, and the other was employed for determining steroid binding by the charcoal-adsorption assay. The steroid-binding properties of the glucocorticoid–receptor complexes under such conditions have been reported to remain unchanged over a 100-fold range of ATP concentration (0.1–10 mM; John & Moudgil, 1979).

Activation of steroid receptors is thought to involve alterations in their structure and properties which enable them to interact with nuclear constituents. The mammalian oestrogen receptor has been shown to dimerize after activation, and this can be detected by sedimentation analysis (Notides & Nielsen, 1975). However, no such structural changes have been observed in other receptor systems (Buller *et al.*, 1975). The results of Fig. 1 show the sedimentation profiles of cytosolic glucocorticoid–receptor complex under low- (0.01 M-KCl) and high- (0.3 M) salt conditions. Under conditions described in Table 1 for receptor activation by ATP, there is no detectable change in the sedimentation of [³H]triamcinolone acetonide–receptor complex. However, a shift in the rate of sedimentation of glucocorticoid–receptor complex after activation is not ruled out, because the resolution of steroid–receptor complex obtained in this experiment depended on too few fractions. This suggests that, although

Fig. 1. Effect of ATP on the sedimentation rate of [³H]triamcinolone–receptor complex

The complex was treated with 10 mM-ATP for 40 min at 4°C (●). The controls samples contained receptor complex incubated with 10 mM-Tris/HCl/50 mM-monothioglycerol, pH 8.0, for 40 min at 4°C (○). Samples (0.2 ml) were layered over separate 4.4 ml linear (5–20%, w/v) sucrose gradients containing 10 mM-Tris/HCl/50 mM-monothioglycerol with either 0.01 M-KCl (a) or 0.3 M-KCl (b) and centrifuged at 150 000 *g* in a Sorvall OTD-65 ultracentrifuge for 4 h in a TV-865 vertical rotor. The tubes were pierced at the bottom and 0.2 ml fractions collected. [¹⁴C]Ovalbumin (3.7S) was used as a marker protein and layered on a separate gradient. Receptor complex in both control and ATP-treated samples sedimented as approx. 8S in low-salt and approx. 4S in high-salt gradients.

alterations in the structure and properties of cytosol glucocorticoid–receptor complex after activation by ATP are possible, this may or may not result in alteration in the size of the receptor.

Bivalent cations have been shown to be effective in causing changes in steroid-receptor properties (Sherman *et al.*, 1974; Kalimi *et al.*, 1975; Kaiser & Edelman, 1977). We investigated whether bivalent cations alone would affect the receptor activation at low temperature or influence the activation by ATP. Bivalent cations were incubated with receptor complexes for 40 min at 4°C before the addition of nuclei to determine if they could enhance the nuclear binding. The results of Table 2 show that none of the bivalent cations used was able to increase the nuclear binding of receptor complexes at the concentration used. If, however, cations were added along with ATP during receptor activation, their

effect was inhibitory. Both Mg^{2+} and Ca^{2+} decreased the ATP-induced nuclear uptake considerably. A more dramatic effect was seen with Mn^{2+} , which decreased the nuclear binding to background values. Although Mg^{2+} , Ca^{2+} and Mn^{2+} failed to activate the receptor complex, they may be effective at other concentrations. The effects seen on the ATP-activated receptor complex could be explained on the basis of chelation effect of these ions. In their presence, ATP may become limiting and not be available in concentrations high enough to influence receptor activation. Work is needed to determine if higher concentrations of ATP would reverse this inhibitory action of cations. Secondly, it is possible that these ions may be enhancing certain enzymic reactions, which in turn slow down receptor activation directly or indirectly.

Other criteria of measurement of receptor

Table 2. Analysis of glucocorticoid–receptor-complex activation in the presence of bivalent cations

The [3H]triamcinolone–receptor complex was incubated with 2 mM- $CaCl_2$, - $MgCl_2$ or - $MnCl_2$ in both the presence and the absence of 10 mM-ATP. Triplicate samples were treated with sodium molybdate and used as the control for receptor activation. After this treatment, nuclear-binding assays were performed as described in the Materials and methods section to determine the extent of receptor activation. All the solutions containing cations were made in 10 mM-Tris/HCl/50 mM-monothioglycerol buffer and pH was re-adjusted to 8.0 with 0.1 M-NaOH. All the procedures were carried out at 4°C.

Nucleotide	Bivalent cations	$10^{-3} \times$ Nuclear-bound [3H]triamcinolone–receptor complex (c.p.m./mg of DNA)
None	None	1.2 ± 0.3
ATP (10 mM)	None	138.0 ± 7.1
None	Mg^{2+}	1.22 ± 0.3
None	Ca^{2+}	1.21 ± 0.2
None	Mn^{2+}	1.23 ± 0.1
ATP		
(10 mM)	Mg^{2+}	105.0 ± 2.0
(10 mM)	Ca^{2+}	76.0 ± 6.0
(10 mM)	Mn^{2+}	8.2 ± 7.0

Table 3. Measurement of receptor activation by ATP–Sepharose affinity chromatography

The freshly prepared cytosol was incubated for 4 h with 10 nM-[3H]triamcinolone acetone and divided into five equal portions. Samples were treated with 5 or 10 mM-ATP for 40 min at 4°C. One sample was heat-activated (23°C), which served as a standard, and other samples were treated with 10 mM-Tris/HCl/50 mM-monothioglycerol, pH 8.0, to be used as a control. Preparations were also treated with sodium molybdate at both 4°C and 23°C to account for background or contribution by non-activated [3H]triamcinolone–receptor complexes. The above samples were chromatographed on separate identical 2 ml ATP–Sepharose columns equilibrated with buffer containing 10 mM-Tris/HCl, 50 mM-monothioglycerol, 0.01 M-KCl and 20% glycerol. The complexes were eluted with the same buffer containing 1 M-KCl. Ten 2 ml fractions were collected, with a flow rate of 0.5 ml/min. Each value corresponds to the total radioactivity in the 1 M-KCl fractions and represents [3H]triamcinolone–receptor complexes.

Nucleotide or effector	Incubation temperature of steroid–receptor complex	$10^{-3} \times$ ATP–Sepharose-bound [3H]triamcinolone–receptor complex (c.p.m.)
None	4°C	4.10
None	23°C	31.50
ATP (5 mM)	4°C	25.00
ATP (10 mM)	4°C	38.87
Molybdate (10 mM) + ATP (10 mM)	4°C	1.80
Molybdate (10 mM)	23°C	1.50

activation were also used to determine the extent of activation of glucocorticoid–receptor complexes by ATP. It has been demonstrated that ATP binding is a property of activated steroid receptors (Miller & Toft, 1978), and therefore another measure of receptor activation. Table 3 summarizes results of activation by ATP of glucocorticoid–receptor complex and its subsequent binding to ATP–Sephadex. The cytosol complexes were treated with and without ATP at different experimental conditions. Before their chromatography on separate identical ATP–Sephadex columns, the free nucleotide was removed by charcoal treatment (see the Materials and methods section). The adsorbed receptor complexes were eluted from the affinity columns by using high-salt buffer. The radioactivity in the entire peak fractions was added up and expressed as total receptor binding to ATP–Sephadex. As with other methods, a small increase in receptor–complex binding to ATP–Sephadex occurred in the untreated sample, owing to partial activation. The receptor–complex binding to ATP–Sephadex increased 22-fold in the ATP-activated sample compared with molybdate-treated control samples. The extent of receptor activation was comparable with that by heat-activation.

Activated receptors have been shown to acquire the ability to bind to DNA–cellulose (Yamamoto & Alberts, 1972; Toft, 1973; Kalimi *et al.*, 1975; Milgrom *et al.*, 1976) and phosphocellulose (Atger & Milgrom, 1976; Schrader *et al.*, 1975). The binding of receptors to these resins involve ionic interactions, which can be disrupted by high-salt conditions. In the present studies we utilized these affinity media to measure the extent of receptor activation (Table 4). Receptor complexes were incubated with 5 mM- or 10 mM-ATP at 4°C, then charcoal-treated and allowed to mix with contents of

a series of tubes containing DNA–cellulose in a slurry form. The suspensions were then washed with 10 mM-Tris/HCl/50 mM-monothioglycerol buffer containing 0.01 M-KCl and the adsorbed complexes were eluted with the same buffer but containing 1 M-KCl, pH 8.0. An identical set of tubes was heat-activated to compare the extent of receptor activation by ATP. Sodium molybdate was included to prevent activation and served as a control. As seen in Table 4, a 7-fold increase in receptor–complex binding to DNA–cellulose was observed in presence of 5 mM-ATP at 4°C. Slightly greater binding to DNA was observed in heat-activated samples. The extent of receptor activation was lower with higher ATP concentration, probably because of either incomplete removal of free nucleotide or alteration of DNA-binding site(s) during the ATP activation by 10 mM-nucleotide. Under similar experimental conditions, binding of ATP-treated glucocorticoid–receptor complex to phosphocellulose was tested as a measure of receptor activation. Fig. 2 illustrates the results of both heat- and nucleotide-activation of glucocorticoid–receptor complex. A comparable binding to phosphocellulose by the receptor complex activated by elevated temperature or presence of ATP shows that the nucleotide can be effectively used as a modulator of the activation process.

A phosphorylation–dephosphorylation mechanism has been suggested to determine the amount of active receptor capable of steroid binding (Nielsen *et al.*, 1977). The unbound receptor in these studies was found to be sensitive to inactivation by phosphatases, whereas steroid-bound receptor remained unaffected. We observed that incubation of rat liver cytosol with ATP at concentrations shown in Fig. 3 before the addition of steroid increased the hormone-binding capacity of the receptor. A 2-fold increase in steroid binding was seen with 1.0 mM-

Table 4. Measurement of receptor activation by DNA–cellulose

Cytosol samples (1 ml) containing [³H]triamcinolone–receptor complexes were treated with ATP (5 or 10 mM), 10 mM-molybdate or 10 mM-Tris/HCl/50 mM-monothioglycerol buffer, pH 8.0. The samples were incubated for 40 min either at 4°C or at 23°C and then added to series of tubes containing an equal volume of DNA–cellulose. The mixtures were shaken at 4°C for 1 h, with gentle stirring every 10 min. The unadsorbed complexes were washed off by centrifugation (1000 g, 5 min), with 10 mM-Tris/HCl/50 mM-monothioglycerol/20% glycerol/0.01 M-KCl, pH 8.0. The receptor complexes were eluted by treating the affinity resin with the same buffer (2 ml) but containing 1 M-KCl. The suspension was shaken gently for 15 min at 4°C and centrifuged at 1000 g for 5 min; 0.5 ml samples were used for measurement of radioactivity.

Nucleotide or effector	Incubation temperature of steroid–receptor complex	10 ⁻³ × DNA–cellulose-bound [³ H]triamcinolone–receptor complex (c.p.m.)
None	4°C	1.05
ATP (5 mM)	4°C	7.20
ATP (10 mM)	4°C	2.40
None	23°C	8.30
ATP (10 mM)	23°C	5.20
Molybdate (10 mM)	23°C	1.10

Fig. 2. *Phosphocellulose chromatography of activated $[^3\text{H}]$ triamcinolone-receptor complex*

The cytosol preparation containing $[^3\text{H}]$ triamcinolone-receptor complex was activated by either warming the complex at 23°C (●) or by incubation with 5 mM-ATP for 40 min at 4°C (○). Non-activated hormone-receptor complexes (▲) served as a control. The free nucleotide from both groups was removed by treating the mixtures with dextran-coated-charcoal suspension. The supernatants (0.5 ml) obtained after centrifugation (1000 g, 5 min) of a charcoal-treated receptor suspension were layered over an equilibrated 4 ml phosphocellulose column. The columns were washed with the equilibration buffer (10 mM-Tris/HCl/50 mM-monothio-glycerol/0.01 M-KCl/20% glycerol) and the complex was eluted with the same buffer containing 1 M-KCl. Ten 1.5 ml fractions were collected for each washing with low or high salt, at a rate of 0.5 ml/min; 0.5 ml samples were used to determine the radioactivity. The first peak of radioactivity represents the unadsorbed steroid-receptor complex and free $[^3\text{H}]$ triamcinolone acetone.

ATP. When preparations that contained receptor already complexed to the steroid, in contrast with receptor alone, were incubated with ATP at various concentrations, no increase in steroid binding was observed. It is not clear whether ATP is involved in the phosphorylation of steroid-binding site(s) of the receptor or whether it is stabilizing the protein in the

Fig. 3. *Influence of ATP on the steroid-binding capacity of unoccupied glucocorticoid receptor*

Freshly prepared cytosol was treated with different concentrations of ATP for 20 min at 23°C , either before or after the addition of 10 nM- $[^3\text{H}]$ triamcinolone acetone. The unoccupied receptor was first treated with ATP for 20 min at 23°C and then complexes to $[^3\text{H}]$ triamcinolone for 4 h at 4°C (●). In the second case the cytosol receptor was first complexed with $[^3\text{H}]$ triamcinolone for 4 h at 4°C and then kept with ATP at 23°C for 20 min (○). The extent of steroid binding was determined by using charcoal assays as described in the Materials and methods section. The data are corrected for non-specific binding.

absence of steroid. Another indication that ATP may be stabilizing the receptor comes from our results shown in Fig. 4. When $[^3\text{H}]$ triamcinolone acetone-receptor complexes were incubated at 23°C for different time periods, the half-life of the complex in control samples was about 8 h, whereas ATP-treated samples still retained 80% of the complexes. Therefore ATP treatment alters the half-life of the steroid-receptor complex, and the dissociation is either decreased or increased, depending on the nucleotide concentration. Lower concentrations of ATP (<1 mM) enhanced the rate of dissociation of the complex, whereas concentrations above 1 mM-ATP slowed this process. The findings are in agreement with the inhibitory and stimulatory effects of different concentrations of ATP on the rate of activation of progesterone-receptor complex (Toft *et al.*, 1977). The stabilizing effects of nucleotides on the specific glucocorticoid binding, and the re-activation of completely inactivated receptor, have also been reported (Sando *et al.*, 1979a,b).

Fig. 4. Effect of ATP on the stability of $[^3\text{H}]$ triamcinolone-receptor complex

Samples (0.1 ml) of $(\text{NH}_4)_2\text{SO}_4$ -fractionated $[^3\text{H}]$ triamcinolone-receptor complex were incubated with 0.1 mM-ATP (Δ) or 5 mM-ATP ($\bullet$) for different times at 23°C. The control samples ($\circ$) contained only 10 mM-Tris/HCl/50 mM-monothioglycerol buffer. At the end of each incubation period shown, an equal volume (0.5 ml) of charcoal suspension was added to assay mixtures, which were then centrifuged at 1000 g for 10 min. The supernatants were used to measure the radioactivity. The 100% value corresponds to 13 000 c.p.m.

Discussion

Our results demonstrate that the glucocorticoid-receptor complex from rat liver can be activated in the presence of ATP at low temperature. The activation achieved by incubation of nucleotide with the receptor is comparable with the transformation of the receptor reported by other conventional procedures. Cake & Litwack (1978) have reported a 3-fold increase in the rate of nuclear binding of $[^3\text{H}]$ dexamethasone-receptor complex from rat liver cytosol in the presence of theophylline. ATP-induced activation allows a 10-fold increase in the nuclear binding when compared with values obtained without ATP (Table 1). The analysis of sedimentation properties shows no detectable change in the heat-activated or ATP-activated receptor complex. This is consistent with the findings of Cake & Litwack (1978) on the sedimentation profiles of the glucocorticoid-receptor complex.

The exact role of ATP in receptor function

remains unclear. However, the results presented here indicate that ATP may have a variety of effects on the properties of the glucocorticoid-receptor complex. The results of Table 3 would suggest that ATP binding is a property of an activated receptor, which is consistent with the similar observations reported for progesterone and oestrogen receptors (Miller & Toft, 1978). If this is the case, then the effects seen on the non-activated receptor may be indirect. The presence of ATP increases the steroid-binding capacity (Fig. 3) of unoccupied glucocorticoid receptor, but has no such effect on the hormone-occupied receptor (Fig. 3). This could be brought about by a stabilizing effect of ATP in the absence of steroid, which may interact with unoccupied receptor and expose the steroid-binding site. The glucocorticoid receptor has been shown to be highly unstable in the absence of hormone (Nielsen *et al.*, 1977). It is possible that ATP is antagonizing the effects of cytosol inhibitor, which is reported to destroy the steroid-binding properties of the glucocorticoid receptor in the absence of hormone (Nielsen *et al.*, 1977). Also, the effects of ATP seen on the rate of dissociation of cytosol $[^3\text{H}]$ triamcinolone acetone-receptor complex at 4°C or at 23°C (Fig. 4) suggest that ATP may not only interact with the unoccupied receptor to enhance steroid binding, but also appears to slow down the loss of steroid from the receptor once the complex has formed. Whether these effects are direct or indirect remains to be seen. If, however, ATP binds to both non-activated and activated forms of receptor, but with an altered affinity, then an equilibrium may exist between the two forms of receptor, where ATP binding to a non-activated receptor population may shift the equilibrium in favour of an activated state, the latter showing increased affinity for the nucleotide. Although our results (Table 3) on glucocorticoid interaction with ATP-Sepharose suggest the presence of a nucleotide-binding site(s) on the receptor molecule, one would need direct binding studies with pure receptor preparation to demonstrate such a phenomenon. Another possibility is that the receptor may have more than one nucleotide-binding site for interaction with ATP or other nucleotides, and the effects observed could depend on the functional state (e.g. unoccupied, activated or non-activated form) of the receptor.

ATP may be a source of energy for the soluble cytoplasmic glucocorticoid-receptor complex at physiological conditions and may play a role in the translocation of the receptor complex on to the sites in the nucleus. The energy-dependent processes may involve metabolism of ATP by the suggested involvement of an enzymic process (Moudgil & Toft, 1976). King *et al.* (1971), using a cell-free system from rat uterus, had observed an effect of

ATP on the nuclear transfer of oestrogen receptor. The nuclear binding of oestradiol-receptor complex was measured after the incubation of receptor extract with isolated nuclei. They found that nuclear receptor binding was enhanced if the nuclei were incubated with ATP. Kalimi *et al.* (1973) had reported opposing results, that ATP had no effect on the nuclear uptake of [³H]dexamethasone-receptor complex from rat liver in a cell-free system, but in their experiments ATP was added to an already activated preparation. Our initial results, from similar experiments with [³H]triamcinolone acetate, showed a 2–3-fold increase in the rate of nuclear uptake of receptor (John & Moudgil, 1979).

It remains unclear as to how ATP brings about its effects. It is possible that ATP treatment alters the receptor protein either by binding to it or by indirectly affecting its structure in an irreversible way not requiring the further presence of a nucleotide, since its removal by charcoal treatment allows such preparations to bind to ATP-Sepharose effectively. Had ATP become covalently linked during the activation process, it would have then competitively decreased the retention of the receptor complex on ATP-Sepharose columns (Table 3).

Reports of the inactivation and activation of the steroid-binding capacity of the glucocorticoid-receptor complex suggest that a phosphorylation-dephosphorylation process may be involved in the steroid-binding properties of the receptor (Nielsen *et al.*, 1977; Sando *et al.*, 1979a,b; Trojkovic *et al.*, 1979). Molybdate and other phosphatase inhibitors stabilize the receptor, resulting in higher steroid binding and suggesting the phosphorylated form of glucocorticoid receptor as an active steroid-binding form of the receptor. On the other hand, both salt- and heat-activation of the glucocorticoid receptor is shown to be blocked by molybdate (Leach *et al.*, 1979; Dahmer *et al.*, 1979; Toft *et al.*, 1979). If one assumes that molybdate acts in the above process by inhibiting the actions of phosphatases alone, then phosphorylation of unoccupied receptor and dephosphorylation of the steroid-receptor complex would become a prerequisite for receptor activation. Also, molybdate may actually be acting by binding to the receptor itself rather than by inactivating phosphatases alone, and this interaction may favour steroid binding and block receptor activation. The mode of receptor activation still remains unclear.

Finally, in addition to its advantages in studying the process of receptor transformation, the procedure described here should help to reveal information on the receptor site(s) involved in the process of activation and subsequent nuclear binding of the receptor complex. ATP may simply be binding to a nucleotide-binding site and exposing region(s) of receptor that are necessary for the nuclear uptake of the latter.

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References

- Alberts, B. & Herrick, G. (1971) *Methods Enzymol.* **21**, 198–217
- Atger, M. & Milgrom, E. (1976) *Biochemistry* **15**, 4298–4304
- Baxter, J. D., Rousseau, G. G., Bensen, M. C., Garcea, R. L., Ito, J. I. & Tomkins, G. M. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1892–1896
- Beato, M., Kalimi, M., Konstam, M. & Feigelson, P. (1973) *Biochemistry* **12**, 3372–3379
- Buller, R. E., Toft, D. O., Schrader, W. T. & O'Malley, B. W. (1975) *J. Biol. Chem.* **250**, 801–808
- Burton, K. (1956) *Biochem. J.* **62**, 315–323
- Cake, M. H. & Litwack, G. (1978) *Eur. J. Biochem.* **82**, 97–103
- Cidlowski, J. A. & Munck, A. (1978) *Biochim. Biophys. Acta* **543**, 545–555
- Dahmer, M. K., Leach, K. L., Stratford, C. A., Hammond, N. D. & Pratt, W. B. (1979) *Abstr. Annu. Meet. Endocr. Soc.* 61st abstr. 275
- Fevre, B., Baily, A., Sallas, N. & Milgrom, E. (1979) *Biochim. Biophys. Acta* **585**, 266–272
- Goidl, J. A., Cake, M. H., Dolan, K. P., Parchman, L. G. & Litwack, G. (1977) *Biochemistry* **16**, 2125–2130
- John, J. K. & Moudgil, V. K. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1242–1248
- Kaiser, N. & Edelman, I. S. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 638–642
- Kalimi, M., Beato, M. & Feigelson, P. (1973) *Biochemistry* **12**, 3365–3371
- Kalimi, M., Colmen, P. & Feigelson, P. (1975) *J. Biol. Chem.* **250**, 1080–1086
- King, E. G. (1932) *Biochem. J.* **26**, 292–297
- King, R. J. B. & Mainwaring, W. I. P. (1974) *Steroid-Cell Interactions*, University Park Press, Baltimore
- King, R. J. B., Gordon, J., Marx, J. & Steggle, A. W. (1971) in *Basic Actions of Sex Steroids on Target Organs* (Hubinout, P. O., Leroy, F. & Galand, P., eds.), p. 21–43, S. Karger, Basel
- Leach, K. L., Dahmer, M. K., Hammond, N. D., Sando, J. J. & Pratt, W. B. (1979) *J. Biol. Chem.* **254**, 11884–11890
- Lohmar, P. H. & Toft, D. O. (1975) *Biochem. Biophys. Res. Commun.* **61**, 8–15
- Milgrom, E., Atger, M. & Baulieu, E.-E. (1973) *Biochemistry* **12**, 5198–5205
- Milgrom, E., Atger, M. & Baily, A. (1976) *Eur. J. Biochem.* **70**, 1–7
- Miller, J. B. & Toft, D. O. (1978) *Biochemistry* **17**, 173–177
- Moudgil, V. K. & John, J. K. (1979) *Annu. Meet. Abstr. Endocr. Soc.* 61st abstr. 271
- Moudgil, V. K. & Toft, D. O. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 901–905
- Moudgil, V. K. & Toft, D. O. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3443–3447
- Moudgil, V. K. & Toft, D. O. (1977) *Biochim. Biophys. Acta* **490**, 477–488

- Munck, A. & Foley, R. (1979) *Nature (London)* **278**, 752–754
- Munck, A., Wira, C., Yound, D.A., Moscher, K. M., Hallahan, C. & Bell, P. A. (1972) *J. Steroid Biochem.* **3**, 567–578
- Nielsen, C. J., Sando, J. J. & Pratt, W. B. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1398–1402
- Notides, A. C. & Nielsen, S. (1975) *J. Steroid Biochem.* **6**, 483–486
- Parchman, L. G. & Litwack, G. (1977) *Arch. Biochem. Biophys.* **183**, 374–382
- Sando, J. J., Forest, A. C. L. & Pratt, W. B. (1979a) *J. Biol. Chem.* **254**, 4772–4778
- Sando, J. J., Hammond, N. D., Stratford, C. A. & Pratt, W. B. (1979b) *J. Biol. Chem.* **254**, 4779–4789
- Schrader, W. T., Heuer, S. S. & O'Malley, B. W. (1975) *Biol. Reprod.* **12**, 134–142
- Sherman, M. R., Atienza, S. B. P., Shansky, J. R. & Hoffman, L. M. (1974) *J. Biol. Chem.* **249**, 5351–5363
- Spelsberg, T. C., Knowler, J. T. & Moses, H. L. (1974) *Methods Enzymol.* **31**, 263–279
- Toft, D. O. (1973) in *Receptors for Reproductive Hormones* (O'Malley, B. W. & Means, A. R., eds.), pp. 85–96, Plenum, New York
- Toft, D. O., Moudgil, V. K., Lohmar, P. H. & Miller, J. B. (1977) *Ann. N.Y. Acad. Sci.* **286**, 29–42
- Toft, D. O., Roberts, P. E., Nishigori, H. & Moudgil, V. K. (1979) in *Steroid-Hormone Receptor Systems* (Leavitt, W. W. & Clark, J. H., eds.), pp. 329–341, Plenum, New York
- Trojkovic, D., Blecic, G. & Kanaziv, D. (1979) *Abstr. Int. Congr. Biochem.* **11th** abstr. 11-3-S48
- Yamamoto, K. R. & Alberts, B. M. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2105–2109