Activation of the chick oviduct progesterone receptor by heparin in the presence or absence of hormone

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Activation (transformation) of the chick oviduct progesterone receptor was found to be induced at 0°C by heparin free in solution as well as by chromatography on a column of heparin linked to acrylamide/agarose. The transformed molecule displayed properties of the activated form of [³H]progesterone–receptor complex obtained by heat treatment or by high ionic strength: smaller size ($s_{20,w} = 3.9$ S, Stokes radius = 5.2 nm), lower rate of dissociation ($t_{\frac{1}{2}}$ approx. 50 h at 0°C compared with approx. 20 h for the ‘native’ form) and increased binding to phosphocellulose. In all cases, molybdate was an effective inhibitor of transformation and stabilized a large ‘native’ form ($s_{20,w} = 7.9$ S, Stokes radius = 7.6 nm). Transformation by neither KCl nor heparin depended on the presence of ligand bound to the receptor, and the properties of the receptor molecule produced by treatment of ligand-free receptor with high ionic strength or with heparin were identical with those of the activated progesterone–receptor complex, demonstrating that receptor activation can be obtained experimentally in the absence of hormone. Our data are compatible with a model in which activation implies separation of the 4 S units, which compose the approx. 8 S ‘native’ form.

Steroid hormone–receptor complexes accumulate in the nucleus of target cells under physiological conditions. ‘Transformation’ or ‘activation’ (these terms are both currently used in the literature to describe the same process) (Jensen *et al.*, 1968; Gorski *et al.*, 1968; for reviews, see Baulieu *et al.*, 1975; Milgrom, 1981) of the receptor is probably related to the transfer of hormone–receptor complexes from the cytoplasm to the nucleus, and can be detected in cell-free systems by an increased affinity of receptor for isolated nuclei or certain polyanions (phosphocellulose, DNA–cellulose, ATP–Sepharose) (Higgins *et al.*, 1973; Milgrom *et al.*, 1973; Miller & Toft, 1978). Such transformation is considered to be strictly hormone-dependent under physiological conditions. Hormone–receptor complexes are rapidly activated by temperature (>25°C) or by high ionic strength (>0.3; Milgrom *et al.*, 1973) under cell-free conditions. Higher affinity of

the receptor for polyanions is correlated with an increased affinity of the receptor for its ligand, indicated by a lower rate of dissociation (Weichman & Notides, 1977; Wolfson *et al.*, 1980) and changes in the sedimentation coefficient (Jensen & DeSombre, 1973).

The chick oviduct progesterone receptor can be stabilized in a non-activated form by molybdate ions (Nishigori & Toft, 1980; Wolfson *et al.*, 1980). Molybdate has been shown to prevent the temperature-dependent transformation of hormone–receptor complexes. In this work we report several new aspects of activation for the chick oviduct progesterone receptor. In particular, activation is shown to take place at 0°C by interaction of the receptor with heparin, either free or immobilized on acrylamide/agarose. Moreover, activation by high ionic strength and by heparin is found to occur not only with the hormone–receptor complexes but also with ligand-free receptor. All these processes lead to similar, if not identical, activated receptor form(s) and all are inhibited by molybdate.

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Material and methods

Chemicals

[1,2,6,7-³H]Progesterone (80–110 Ci/mmol) was from CEA (Saclay, France). Radioinert progesterone was a gift from Roussel-Uclaf (Romainville, France). All other chemicals (analytical grade) were from usual commercial sources.

Animals and tissue fractionation

Chickens (5 days old; Warren strain) were primed with 10 daily injections of oestradiol benzoate in sesame oil (1 mg/day). Before being killed, animals received additionally three to five daily injections of the oestrogen (Wolfson *et al.*, 1980). The magnum portion of the oviduct was excised and homogenized in 4 vol. of Tris (10 mM)/EDTA (1.5 mM) buffer containing 12 mM-thioglycerol and 10% glycerol, pH 7.4 (TE buffer), or in the same buffer containing 20 mM-Na₂MoO₄ (TE-M buffer), by using an Ultra-Turrax tissue disintegrator. This and all further operations were carried out at 0–4°C. Phenylmethanesulphonyl fluoride was added to the homogenate to a final concentration of 0.3 mM. The cytosol was obtained by ultracentrifugation at 100 000 g for 60 min.

Labelling of receptor

Receptor-hormone complexes were usually formed by incubation with 20 nM-[³H]progesterone for 1–4 h at 0°C. Under these conditions, 80–90% of all receptor binding sites are saturated. Cortisol (1 μM) was included in the incubation medium to eliminate binding of labelled progesterone to contaminating plasma transcortin and glucocorticosteroid receptors (Mester & Baulieu, 1977). When necessary, unbound hormone was removed by charcoal (0.5%)/dextran (0.05%) adsorption (30 min at 0°C). Non-specific binding was monitored by parallel incubations with a 1000-fold excess of unlabelled progesterone. In all cases, non-specific binding was <10% of the total [³H]progesterone binding.

In these experiments where transformation of ligand-free receptor was studied, sodium molybdate (20 mM) was always added before [³H]progesterone in order to prevent any transformation of the ligand-receptor complexes.

Sucrose-density-gradient ultracentrifugation

Samples (0.2 ml) were layered on to a discontinuous sucrose gradients (5–20%) made in TEK-M buffer (TE buffer + 0.15 M-KCl + 50 mM-Na₂MoO₄), and ultracentrifuged in an SW 60 rotor at 42 000 rev./min for 16–20 h. Glucose oxidase (Sigma; $s_{20,w} = 7.9$ S) and peroxidase (Sigma; $s_{20,w} = 3.6$ S), included in the samples as internal standards, were used to calculate the sedimentation coefficients by the method of Martin & Ames

(1962). Fractions (two drops) were collected manually after piercing the bottom of the tube. Two approaches were used to identify the receptor in the fractions. (1) When the samples analysed contained hormone-free receptor, the fractions were incubated with [³H]progesterone (1 nM) for 3 h at 0°C. Unbound hormone was then removed by charcoal adsorption and centrifugation and radioactivity in the supernatant was determined. (2) When [³H]-progesterone-receptor complexes were fractionated, the free steroid was removed by charcoal before ultracentrifugation and the receptor was identified by direct counting of the collected fractions. Both approaches gave identical results.

Gel filtration

A column of Ultrogel AcA 22 (IBF, Clichy, France) equilibrated in TEK-M buffer was used. Internal diameter of the column was 1.6 cm and bed volume 27.5 ml; 1 ml fractions were collected. The column was calibrated with thyroglobulin [Sigma; Stokes radius (R_s) = 8.5 nm], β-galactosidase (Sigma; R_s = 6.9 nm), ferritin (Boehringer-Mannheim; R_s = 6.1 nm) and catalase (Sigma; R_s = 5.2 nm). Void volume (V_0) was determined with Dextran Blue and the total volume (V_t) with potassium bichromate. R_s of the receptor was determined by plotting log R_s against the distribution coefficient, $K_D = (V_e - V_0)/V_t$, where V_e = volume of elution of protein (Sherman, 1975).

Binding to phosphocellulose

Polypropylene columns (Bio-Rad) were packed with 0.2 ml bed volume of phosphocellulose P 11 (Whatman) equilibrated in TE-M buffer. Samples containing 0.3 M-KCl were diluted 10 times with TE-M buffer. After loading the samples, the columns were washed with 3 ml of TE-M buffer, and then eluted with 2 ml of TE-M buffer containing 1.0 M-KCl. Inclusion of molybdate in all buffers used in chromatography on phosphocellulose abolished activation of the receptor by contact with the resin (Milgrom, 1981) and led to very clear-cut and reproducible results.

Chromatography on immobilized heparin

Cytosol was fractionated on columns of heparin covalently linked to acrylamide/agarose (heparin-Ultrogel; IBF), equilibrated in either TE or TE-M buffer (see also the legend to Fig. 4).

Dissociation kinetics

Portions (1 ml) of [³H]progesterone-receptor (approx. 3.5×10^5 c.p.m./ml) were used in these experiments. Dissociation of the radioactive ligand was initiated by adding a 1000-fold excess of radioinert progesterone in ethanol (10 μl/ml of receptor-containing solution). Incubation was car-

ried out at 0°C. At various time intervals, unbound steroid was removed by charcoal adsorption and centrifugation, and radioactivity of the bound ligand was determined in the supernatant. Non-specific binding was monitored in parallel incubations as indicated above. Under the experimental conditions no degradation of the receptor was detected.

Results

Effects of KCl

Incubation of [³H]progesterone–receptor complexes at 0°C with KCl (>0.3 M) leads to the formation of an approx. 4S species. As reported previously (Milgrom *et al.*, 1973; Wolfson *et al.*, 1981), the salt-transformed complexes bind equally well to phosphocellulose as the heat-activated receptor (Table 1). KCl-induced activation also caused the same decrease (approx. 2.5-fold) in the rate of dissociation as did heat-induced activation (Wolfson *et al.*, 1980). Contrary to previous reports (Nishigori & Toft, 1980), molybdate did effectively inhibit the KCl (0.3 M)-induced activation of receptor (Fig. 1; Table 1). However, the effect of molybdate could be overcome by further increasing the ionic strength: in molybdate (20 mM)-containing buffer, 1 M-KCl led to the conversion of approx. 50% of the receptor population to an approx. 4S form (Fig. 2). The presence of both 8S and 4S forms led to biphasic dissociation kinetics (results not shown).

We have examined the necessity for the presence of progesterone complexed with the receptor during

the ionic strength-dependent activation. The results indicated that ligand-free receptor can be activated by this treatment as well as the hormone–receptor complexes. The sedimentation coefficients determined with the KCl-activated forms without or with ligand were found to be identical, and equal to that of the [³H]progesterone–receptor complex activated by incubation at 25°C (Fig. 1; Table 1). The activated receptor without ligand was considerably less stable than both the non-transformed receptor and the activated hormone–receptor complexes (cf. McBlain *et al.*, 1981); its ligand-binding capacity was completely lost upon chromatography on phosphocellulose.

The KCl-activated, ligand-free receptor was labelled with [³H]progesterone after first adding Na₂MoO₄ (20 mM) in order to block any subsequent activation. The complexes prepared in this way displayed properties of the activated form: increased binding to phosphocellulose and low rate of dissociation of the hormone.

As for progesterone–receptor complexes, activation of the ligand-free receptor by 0.3 M-KCl was blocked by 20 mM-Na₂MoO₄ (Fig. 1).

[³H]Progesterone–receptor complexes previously treated with 0.3 M-KCl were not transformed back to the 8S form by either addition of 20 mM-molybdate for 16 h, or by dialysis for 48 h against low-ionic-strength buffer containing molybdate. Dissociation kinetics of the 4S complexes were also unchanged by these attempts to reverse the activation process (Fig. 3).

Table 1. Sedimentation coefficients, R_s values and binding to phosphocellulose of activated and molybdate-stabilized 'native' [³H]progesterone–receptor complexes

Cytosol was prepared in TE buffer, the receptor–[³H]progesterone complexes were formed at 0°C and activated by various treatments as indicated, or stabilized in the 'native' form by adding Na₂MoO₄ to a concentration of 20 mM. Unbound steroid was removed by charcoal adsorption before determination of the sedimentation coefficient by sucrose-density-gradient centrifugation, of the R_s by gel filtration or of phosphocellulose binding. The data for sedimentation coefficients and phosphocellulose binding are means $\pm$ S.D. of n experiments.

	Activation by:			
	1 h at 25°C	4 h at 0°C, 0.3 M-KCl	Chromatography on heparin–Ultragel*	Molybdate-stabilized complexes
Sedimentation coefficient, $s_{20,w}$ (S)	3.9 $\pm$ 0.2 (n = 3)	3.9 $\pm$ 0.2 (n = 12)	3.8 $\pm$ 0.2 (n = 4)	7.9 $\pm$ 0.1 (n = 9)
Proportion transformed to approx. 4S species (%)	80	100	100	0
R_s (nm)	5.2	5.2	5.2	7.6
Proportion bound to phosphocellulose (%)	47.4 $\pm$ 4.0 (n = 3)	77.6 $\pm$ 4.9 (n = 5)	77.8 $\pm$ 8.8 (n = 2)	5.9 $\pm$ 1.2 (n = 4) 13.7 $\pm$ 0.1† (n = 2)

* Elution with 0.3 M-KCl in TE-M buffer.

† In the presence of 0.3 M-KCl (4 h at 0°C).

Fig. 1. Effects of KCl and molybdate on sedimentation behaviour and on dissociation kinetics of the $[^3\text{H}]$ progesterone-receptor complex

Cytosol was prepared either in TE-M (filled symbols) or in TE (open symbols) buffer. One-half of each preparation was labelled with $[^3\text{H}]$ progesterone (O and ●). A portion of the TE-M-buffered sample labelled with $[^3\text{H}]$ progesterone was set aside; to all remaining samples, KCl was added to a concentration of 0.3 M. After 4 h at 0°C, the concentration of Na_2MoO_4 was adjusted to 20 mM in all samples, and $[^3\text{H}]$ progesterone was added to those samples that had not been labelled before the addition of KCl (□ and ■). After another 1 h at 0°C, portions of all samples were treated with charcoal to remove unbound steroid and centrifuged in sucrose gradients (a). Sedimentation is from right to left. The remaining samples were used to measure rates of dissociation of the $[^3\text{H}]$ progesterone-receptor complexes by displacement with excess unlabelled progesterone at 0°C (b). The internal markers glucose oxidase (GO) and peroxidase (P) are indicated by arrows in (a). Dissociation-kinetics data are represented as percentage of the $[^3\text{H}]$ progesterone bound at time zero; O, dissociation kinetics in the control (not treated with KCl).

Heparin activation

Both progesterone-receptor complexes and hormone-free receptor can be retained on a column of heparin-Ultrogel and eluted with a heparin solution (Wolfson *et al.*, 1981). When $[^3\text{H}]$ progesterone-receptor complexes were chromatographed on such a heparin-Ultrogel column in the absence of molybdate, the eluted hormone-receptor complexes behaved similarly to the heat- and KCl-transformed complexes during chromatography on phosphocellulose. Other parameters of heparin-transformed receptor were also identical with heat- and salt-transformed species: the receptor eluted from the heparin column sedimented at approx. 4S (Fig. 4a), displayed an R_s of 5.2 nm (Table 1) and slow dissociation kinetics (Fig. 4b). Heparin chromatography of hormone-free receptor gave the same results.

Transformation was also obtained by incubation at 0°C of the cytosol with free heparin. As seen in

Fig. 4(a), both native and transformed receptor sedimented slightly faster when in the presence of soluble heparin, probably because of formation of receptor-heparin complexes. The dependence of this transformation on heparin concentration is shown in Fig. 5. The concentration of 0.5 mg of heparin/ml was sufficient, as estimated by transformation of the 8S to the 4S form. The kinetics of the reaction suggested that the reaction is either of first or pseudo-first order (Fig. 6).

Na_2MoO_4 (20 mM) abolished all evidence of transformation, that is, binding to phosphocellulose, 8S → 4S transformation and change in dissociation kinetics (Fig. 4b). Similarly, when 20 mM-molybdate was present during chromatography of the cytosol on the heparin-Ultrogel column, the eluted receptor sedimented at 8S (Fig. 4a). The presence of a mixture of 8S and 4S complexes was reflected in a biphasic pattern of dissociation kinetics (results not shown).

Fig. 2. Effect of increasing ionic strength on sedimentation behaviour of the $[^3\text{H}]$ progesterone-receptor complex in the presence of molybdate

Cytosol was prepared in TE-M buffer and labelled with $[^3\text{H}]$ progesterone; KCl was then added to final concentration of 0 (●), 0.3 (▲), 0.5 (□), 0.75 (○) and 1 M (△). After 4 h at 0°C the samples were treated with charcoal to remove unbound steroid and analysed by centrifugation on sucrose gradients in TEK-M buffer. Sedimentation is from right to left. The position of glucose oxidase as internal marker is indicated by the arrow.

Finally, secondary addition of molybdate to heparin-transformed progesterone-receptor complexes failed to reform an 8 S species. Also, when the cytosol was chromatographed on a heparin-Ultrogel column in the absence of molybdate, and elution was carried out with molybdate-containing buffer, only the 4 S form of the receptor was obtained.

Discussion

In this work, we report for the first time that the polyanion heparin, either free in solution or immobilized on a solid support, can induce steroid-hormone-receptor activation. Our experiments suggest that the 4S forms of the progesterone receptor from chick oviduct cytosol, obtained by treatment of the native receptor with high ionic strength or with heparin, are similar to, if not identical with, the activated receptor formed by heating the hormone-receptor complexes at 25°C as previously described (Miller & Toft, 1978). All these modes of activation yield a form of progesterone receptor that sediments at approx. 4S in sucrose gradients, displays an R_s of approx. 5.2 nm, a lower rate of progesterone dissociation ($t_{1/2}$ approx. 50 h at 0°C) than the native complex ($t_{1/2}$ approx. 20 h) and that binds to phosphocellulose. [It has been previously reported (Schrader *et al.*, 1972) that both the 'A' and 'B' subunits of the chick oviduct progesterone receptor bind to phosphocellulose.]

To form activated receptors, KCl and heparin are more suitable than heating, as they permit main-

Fig. 3. Sedimentation on sucrose gradients and dissociation kinetics of the $[^3\text{H}]$ progesterone-receptor complex transformed by high ionic strength: lack of effect of dialysis against low-salt buffer containing molybdate

Cytosol in TE buffer was labelled with $[^3\text{H}]$ progesterone and treated with 0.3 M-KCl for 2 h at 0°C. Half of the sample was then dialysed overnight against two changes of TE-M buffer. Samples of both dialysed (○) and non-dialysed (●) receptor preparations were analysed by ultracentrifugation at 0°C (a; sedimentation from right to left) and by determining the rate of dissociation at 0°C by displacement with excess unlabelled progesterone (b). The positions of the internal markers glucose oxidase (GO) and peroxidase (P) are indicated in (a).

tenance of low temperature throughout the experiment, thus minimizing receptor degradation. Incubation at 25°C transforms approx. 80% of the receptor-hormone complexes to the approx. 4S form, whereas incubation at 0°C for 4 h with 0.3 M-KCl or with 1 mg of heparin/ml activates approx. 100% of the receptor. The fact that molybdate inhibits all processes leading to transformation suggests that they share at least one common step.

The formation of an approx. 4S species of the ligand-free progesterone receptor indicates that high salt or heparin can act in the absence of progesterone. When these 4S receptor molecules were subsequently complexed with [³H]progesterone (in the presence of molybdate, blocking any further transformation), they were shown to possess the other characteristics of the activated form, namely, slow dissociation kinetics and affinity for phosphocellulose. The yield of transformed receptor obtained in the absence of hormone was approx.

70% of that obtained with the receptor-progesterone complex, suggesting that all receptor molecules can undergo the transformation process in the absence of ligand. (The lower yield of transformed, ligand-free receptor is probably due to its greater lability.)

Since physiological receptor activation within the cell implicates hormone binding (see, e.g., Baulieu *et al.*, 1975), it may be that transformation by temperature cannot occur in the absence of hormone. This is difficult to ascertain because of the instability of receptors under these conditions (McBlain *et al.*, 1981). Transformation by (NH₄)₂SO₄ precipitation in the absence of ligand has been suggested by Jensen & DeSombre (1973) for the oestradiol receptor of the rat uterus. In the chick oviduct, activation by ATP of the progesterone receptor without added ligand was reported by Moudgil *et al.* (1981). However, their data were obtained using tissue from adult hen, probably containing endogenous progesterone. Increased

Fig. 4. Effect of soluble or immobilized heparin on the progesterone receptor

(a) (i) Chromatography on a heparin-Ultrogel column. Cytosol (1 ml) in TE (○) or in TE-M (□) buffer was loaded on to heparin-Ultrogel columns (1 ml) equilibrated in the same buffers. The columns were washed with buffer and eluted with 0.3 M-KCl in TE-M buffer. The eluates were labelled with [³H]progesterone and analysed by centrifugation on sucrose gradients. (The same profiles were obtained when the [³H]progesterone-receptor complexes were formed before chromatography on heparin-Ultrogel.) (ii) Effect of soluble heparin. Cytosol in TE buffer (●) or in TE-M buffer (■) was incubated for 1 h at 0°C with heparin (5 mg/ml). The molybdate concentration was then adjusted to 20 mM, the samples were labelled with [³H]progesterone and centrifuged on sucrose gradients. Sedimentation is from right to left. (b) Effect of heparin on dissociation kinetics of the [³H]progesterone-receptor complex. Cytosol (2 ml) in TE buffer was loaded on to a 1 ml column of heparin-Ultrogel equilibrated in the same buffer. The column was washed with the TE buffer and eluted with a solution of KCl in 0.3 M-KCl in TE-M buffer. The eluate was labelled with [³H]progesterone and the rate of dissociation of the complex was measured at 25°C by displacement with unlabelled progesterone (●). As a control, a sample of the same cytosol to which molybdate (20 mM) was added before heparin chromatography, was run in parallel (△). In (a), the position of the internal markers glucose oxidase (GO) and peroxidase (P) is indicated.

Fig. 5. Receptor transformation by heparin: concentration dependence

Cytosol labelled with [^3H]progesterone was incubated with various concentrations of heparin for 2 h at 0°C . Molybdate was then added to a final concentration of 20 mM, and the incubation mixtures were analysed by sucrose-density-gradient centrifugation. The radioactivities found in the 8S (open columns) and in the 4S peaks (stippled columns) are expressed as percentages of the sum of the two peaks.

binding to nuclei was reported by Buller *et al.* (1975) to result from $(\text{NH}_4)_2\text{SO}_4$ precipitation of ligand-free chick oviduct progesterone receptor. The experimental conditions leading to activation in the absence of hormone are not compatible with normal cell physiology.

Our data suggest a scheme in which the molybdate-stabilized molecule would consist of several 4S binding subunits held together in such a way that their polyanion (DNA, phosphocellulose, but not heparin; cf. Wolfson *et al.*, 1981)-binding site is masked, and the rate of dissociation of progesterone is high. Activation would obligatorily involve separation of the individual 4S subunits, with unmasking of the polyanion-binding sites and tightening of hormone binding. No enzymic process (cf. Nishigori & Toft, 1980) need be implicated, as suggested by the observation that heparin-induced transformation proceeds as a (pseudo) first-order reaction. The 8S complex appears to be held together by relatively weak electrostatic forces: its

Fig. 6. Time-dependence of the progesterone-receptor transformation by heparin

Cytosol labelled with [^3H]progesterone in TE buffer was incubated with heparin (0.5 mg/ml) at 0°C . The 8S $\rightarrow$ 4S transformation was stopped at various time intervals by addition of molybdate to a concentration of 20 mM. The degree of transformation was estimated by sucrose-density-gradient centrifugation. The natural logarithm of the fraction of receptor-bound [^3H]progesterone sedimenting as the 8S form is plotted as a function of time. Non-specific binding in the 4S region has been subtracted.

disruption occurs spontaneously by simply raising the temperature, or can be induced by breaking the electrostatic interactions of the charged regions of the subunits involved, either by KCl or by heparin, a polysulphated polysaccharide. Existence of a low-molecular-weight component stabilizing the 8S complex (as suggested by Jensen & DeSombre, 1973; Bailly *et al.*, 1977; Goidl *et al.*, 1977) is compatible with our data. Molybdate could act either by replacing such a low-molecular-weight component, or, alternatively, by fitting into the 8S structure in such a way as to increase its stability by additional electrostatic interactions. Whether two or several 4S hormone-binding units participate in the 8S structure cannot be deduced from our experiments. Thermodynamic considerations may explain the apparent impossibility of re-associating the 4S transformed subunit into the 8S form on removal of the transforming agent. Although the 8S form is probably a polymeric structure, hormone binding does not display any co-operativity (Y. Chang-Ren, unpublished work).

Besides the theoretical interest suggesting that any process dissociating the 8S structure may release the activated 4S receptor units, the salt and heparin experiments in the absence of hormone have interesting practical consequences. For instance, the salt-activated molecule may be allowed to bind to

digested chromatin in the absence of hormone and subsequently be fractionated by a steroid affinity gel.

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