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EFFECT OF ATP ON THE REGULATION OF THE STEROID BINDING ACTIVITY OF THE OESTRADIOL RECEPTOR

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Summary—We have observed that ATP induces a second type of oestradiol binding site with slightly lower affinity (K_a 3.3 × 10^8 M^{-1}) and lower sedimentation coefficient (4 S) in cytosol from immature lamb uterus and MCF-7 cells. A factor isolated from immature lamb uterine nuclear extract was found to decrease the steroid binding activity of oestradiol receptor that had been purified by heparin Sepharose and oestradiol-Sepharose chromatography. Inhibition of this factor by known phosphatase inhibitors, indicated that this factor may be a phosphatase. Another factor isolated from immature lamb uterine cytosol was found to enhance the effect of ATP on receptor binding in cytosol from immature lamb uterus and MCF-7 cells. The ability of this factor to phosphorylate a partially purified cytosol receptor from immature lamb uterus when incubated with [γ^{32}P]ATP, indicates that this factor is a phosphokinase. The phosphorylated products after labeling with [^{3}H]tamoxifen aziridine were characterized by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Three phosphorylated proteins with molecular weights 150, 97, and 67 kDa bound [^{3}H]tamoxifen aziridine. Ammonium sulphate precipitated cytosol oestradiol receptor from immature lamb uterus was inactivated with receptor inactivating factor and then reactivated with receptor activating factor in the presence of [γ^{32}P]ATP and subsequently affinity labelled with [^{3}H]tamoxifen aziridine. The affinity labelled oestradiol receptor was immunopurified with the monoclonal antibody JS 34/32. Three proteins with molecular weights 67, 50 and 43 kDa specifically bound [^{3}H]tamoxifen aziridine and only 43 kDa receptor fragment was phosphorylated. The relevance of inactivation/reactivation of oestradiol receptor to the dephosphorylation/phosphorylation of receptor is discussed.

INTRODUCTION

Protein phosphorylation–dephosphorylation is an important regulatory process for many biological activities. The covalent modification of steroid hormone receptors through phosphorylation–dephosphorylation is a potentially important reaction.

A hypothesis [1] based on *in vitro* studies, states that the oestradiol receptor from calf uterus is a phosphoprotein. According to this hypothesis, the interaction of hormone with receptor results in receptor transformation and subsequent translocation to the nuclear compartment, where the receptor becomes dephosphorylated by a specific nuclear phosphatase. The dephosphorylated receptor, recycles back to the cytoplasmic compartment, where it becomes rephosphorylated by an endogenous kinase. The kinase has an absolute requirement for Ca^{2+} and calmodulin. This kinase appears to phosphorylate oestradiol receptor on tyrosine [2]. *In vitro* as well as *in vivo* studies have indicated that progesterone [3] and glucocorticoid receptors [4] are phosphorylated on serine residues.

We are reporting the effect of ATP on the binding activity of cytosol oestradiol receptor from human mammary cancer tissue, immature lamb uterus and MCF-7 cells, a hormone sensitive human mammary cancer cell line. The effect of a nuclear inactivating activity (RIAA), with subsequent reactivation of oestradiol receptor with a cytosolic receptor activating activity (RAA) are reported. These studies indicate a relationship between these inactivation-reactivation processes and dephosphorylation-phosphorylation of [^{3}H]tamoxifen aziridine labeled oestradiol receptor in the presence of RIAA, RAA, and [γ^{32}P]ATP.

EXPERIMENTAL

Chemicals

All reagents were of analytical grade. Dithiothreitol, 2-mercaptoethanol, sodium dodecyl sulphate (SDS), phenyl-methyl-sulphonyl fluoride (PMSF)

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and *N*-Tris[Hydroxymethyl]methyl 2-aminoethane sulfonic acid (TES) were from Sigma (St Louis, Mo., U.S.A.). ATP disodium salt was from Fluka (AG, Buch, SG). Dextran grade C, was purchased from Becton, Dickinson & Co., N.Y. 17β-[2,4,6,7,16,17-^{3}H]oestradiol (170 Ci/mmol) and [methyl-^{14}C]albumin (0.02 mCi/mg) were from New England Nuclear (Boston, Mass, U.S.A.). [γ ^{32}P]ATP (3000 Ci/mmol), and [ring-^{3}H]tamoxifen-aziridine (21 Ci/mmol) were from Amersham, U.K. Optifluor, Dimilume-30 scintillation cocktails and Soluene tissue solubilizer were from United Technologies Packard, Ill., U.S.A. Aprotinine, protein A-Sepharose and Protein reference standards were from Pharmacia, Uppsala, Sweden.

The following buffers were used and designated as follows: Tris/HCl 10 mM pH 7.4 containing 1 mM EDTA and 1 mM dithiothreitol (TED-buffer). Phosphate buffer 50 mM pH 7.4 containing 1.5 mM thioglycerol (PT-buffer). Isotonic phosphate/saline buffer 150 mM pH 7.4 (IPS-buffer). Tris–glycine containing SDS pH 8.3 buffer for SDS–polyacrylamide gel electrophoresis (SDS–PAGE). All the procedures were carried out at 0–4°C unless otherwise indicated.

Cells and culture conditions

MCF-7 cells were grown in a humidified atmosphere containing 5% CO_2 in air at 37°C and maintained in monolayer culture in plastic T-175 flasks in Eagle's minimal essential medium (MEM) supplemented with 5% foetal calf serum, L-glutamine (2 mM), penicillin (50 IU/ml), streptomycin (50 μg/ml), and insulin (1.7 μM). The last 48 h, the cells were cultured in 5% dextran-charcoal treated foetal calf serum.

Cytosol and nuclear extract preparation

Cells from nearly confluent T-175 flasks were harvested by incubating with 1 mM EDTA at 37°C for about 45 min in Hanks' balanced salt solution (calcium and magnesium free). The cell suspension was centrifuged at 400 ***g*** for 5 min, washed twice with IPS-buffer pH 7.4 and centrifuged at 400 ***g*** for 5 min. The washed cells were suspended in the desired volume of TED buffer (1×10^7 cells/ml) and homogenized in a Teflon-glass Potter/Elvehjem homogenizer with 20 strokes until more than 90% of the cells were disrupted as seen by phase contrast microscopy. The homogenate was centrifuged at 800 ***g*** for 10 min. The supernatant obtained was further centrifuged at 105,000 ***g*** for 1 h to prepare cytosol. The nuclear pellet from the 800 ***g*** centrifugation was suspended in TED-buffer or 50 mM phosphate buffer pH 7.4 containing 0.5 M NaSCN. Solubilized proteins were then obtained by centrifugation at 180,000 ***g*** for 30 min. Protein measurement in cytosol fractions were performed according to the method of Sedmak *et al.*[5].

Tissues were trimmed of fat and connective tissue, and stored at −70°C. The frozen tissue samples were cut into small pieces, and immediately transferred to the tubes containing homogenization buffer. The human mammary cancer tissue were homogenized in PT-buffer (1:6 w/v) and immature lamb uterus were homogenized in TED buffer (1:6 w/v). The suspensions were homogenized with 3×15 s bursts of a precooled Polytron homogenizer. Cytosol from mammary cancer tissue and immature lamb uterus was prepared as described above. Oestradiol receptor was purified from immature lamb uterus cytosol by chromatography on heparin-Sepharose followed by affinity chromatography on 17β-oestradiol-17-hemisuccinyl-aminohexane Sepharose(oestradiol-Sepharose), according to the method of Puca *et al.*[6]. The receptor inactivating activity (RIAA) and the receptor activating activity (RAA) were purified from immature lamb uterus according to the methods of Auricchio *et al.*[7–8]. Sucrose gradient sedimentation analysis was performed according to the method of Wittliff[9] and ligand binding analysis was performed using the Ligand System, Fortran version 3.2.11, N.I.H.

Inactivation of cytosol receptor

Cytosol was incubated for 2 h at 0°C with 5 nM [^{3}H]oestradiol-17β in the presence and absence of a 100-fold excess cold oestradiol and 10 mM of various phosphatase inhibitors. In some experiments cytosol was incubated with 15 nM [^{3}H]oestradiol-17β overnight at 0°C, and then the oestradiol–receptor complex was precipitated with 25% saturated ammonium sulphate. The precipitated oestradiol–receptor complex was pelleted and reconstituted in TED buffer. Incubation was performed for 30 min at 25°C in the absence (control) and the presence of 50 μg RIAA. The reaction was stopped by cooling the sample to 0°C. The bound hormone was separated from the free hormone by dextran-charcoal adsorption (charcoal 0.25%, dextran 0.0025%, gelatin 0.1%). The specifically bound radioactivity was measured in aliquots of the supernatants, values are expressed as percent of control.

Activation of cytosol receptor

The receptor inactivated as described above was then incubated with 100 μl of RAA in the presence and absence of 10 mM ATP (all the samples contained 10 mM Mg^{2+} and 10 mM molybdate), and was further incubated at 15°C for 10 min. The sample and control were treated with dextran-charcoal suspension and specifically bound radioactivity in each sample was measured. Values are expressed as percent relative to control.

Affinity labelling of receptor and SDS–PAGE

Partially purified immature lamb uterus cytosol oestradiol receptor was inactivated with RIAA and was used as substrate for RAA in the presence of 0.15 mM [γ ^{32}P]ATP (sp. act. 1 Ci/mmol) and 20 nM [^{3}H]tamoxifen aziridine [10], in the presence or

absence of a 200-fold excess cold oestradiol. Incubation was continued for 1 h at 30°C. The separation of bound and free ligands was with a dextran-charcoal pellet. The supernatants were brought to 1% SDS, 130 mM mercaptoethanol, 20% glycerol, and were incubated at 60°C for 15 min. Aliquots were electrophoresed on 7.5% separating gel according to Laemmli[11].

Incubation of [^{3}H]tamoxifen aziridine labelled oestradiol receptor with the monoclonal antibody JS 34/32 and adsorption to protein A-Sepharose

Unlabelled immature lamb uterus cytosol in TED-buffer or [^{3}H]tamoxifen aziridine labelled cytosol in TEGMP-buffer pH 7.4 [10 mM TES, 50 mM NaCl, 10% (w/v) glycerol, 1.5 mM EDTA, 10 mM molybdate, 1.5 mM thioglycerol, 1 mM PMSF, 77 μg/ml aprotinine and 20 mM sodium fluoride] at 0°C, were added ammonium sulphate to 25% saturation. The precipitates containing most of the cytosol receptor were pelleted and reconstituted with TED-buffer or TEGMP-buffer pH 7.4 respectively. The reconstituted pellet of the [^{3}H]tamoxifen aziridine labelled cytosol in TEGMP-buffer pH 7.4 was kept at 0°C throughout the experiment.

The reconstituted pellet of unlabelled cytosol oestradiol receptor was inactivated with RIAA and was used as substrate for RAA in the presence of 0.15 mM [γ^{32}P]ATP (sp. act. 1 Ci/mmol). Samples were subsequently added 20 nM [^{3}H]tamoxifen aziridine, in the presence or absence of a 200-fold excess cold oestradiol. Incubation was performed at 0°C for 18 h. Monoclonal antibody JS 34/32 [12] or non-immune rat IgG (1 mg protein/ml) was added at a final concentration of 25 μg/ml (2.5% of total volume) and the mixture was incubated for 16 h at 0–2°C.

Incubates were added to a 100 μl bed volume of protein A-Sepharose pre-equilibrated in TEG-buffer pH 7.6 [10 mM TES, 50 mM NaCl, 10% (w/v) glycerol, 4 mM EDTA], and mixed by slow end to end rotation at 0–4°C for 4 h. Protein A-Sepharose was then pelleted by centrifugation, the supernatant was removed, and the pellet was washed 3 times by suspension in 1 ml of TEGMD-buffer pH 7.6 [10 mM TES, 50 mM NaCl, 10% (w/v) glycerol, 4 mM EDTA, 10 mM molybdate, 2 mM dithiothreitol (DTT)]. The pellet was washed sequentially with 3 × 1 ml volumes of the following buffers: (1) TEG-buffer with 0.4 M NaCl and 10 mM sodium molybdate pH 7.6, (2) TEG-buffer with 0.4 M NaCl, 0.2% Triton X-100 and 10 mM sodium molybdate pH 7.6, (3) 10 mM Tris/HCl-buffer containing 10% (w/v) glycerol, 10 mM molybdate, 2 mM DTT pH 6.8. These sequential washing eliminates proteins not directly bound by the antibody [13]. The washed pellet was suspended in sample buffer containing 4% SDS and 10% mercaptoethanol boiled for 4 min. Aliquots were electrophoresed on 7.5% separating gel according to Laemmli[11].

Gel electrophoresis

The SDS–PAGE was performed using the BIO-RAD disc electrophoresis apparatus and at constant current of 5 mA/gel, until the dye front reached 1 cm from the gel end. The gels were frozen immediately using dry ice and stored at −70°C. The gels were sliced into 2 mm slices, each slice was solubilized in Soluene at 65°C for 3 h. Scintillation cocktail (Dimilum-30) was added and the radioactivity was measured in each slice. Standard protein markers of high and low molecular weight were run on separate gels. Molecular weights of (mol. wt) protein standards were: (1) ferritin half subunit, 220 kDa, (2) phosphorylase b, 94 kDa, (3) albumin, 67 kDa, (4) ovalbumin, 43 kDa, (5) dye front.

Specific radioactive binding activity

Specifically bound radioactivity was calculated as the difference between the total binding and the non-specific binding.

Statistical analysis

Wilcoxon's-test for two means was used to determine the significance of the difference between control mean and each individual mean.

RESULTS

Aliquots of cytosol from human breast tumour, immature lamb uterus or MCF-7 cells prepared in TED-buffer containing 10 mM molybdate were incubated with 5 nM [^{3}H]oestradiol-17β in the presence and absence (control) of 10 mM ATP for 10 min at 15°C and then overnight at 0–4°C. Non specific binding was assessed in samples containing a 100-fold excess of non-radioactive oestradiol. All the samples contained 10 mM Mg^{2+} and molybdate. Unbound radioactivity was removed with a dextran-charcoal suspension. The specific binding of control (in the absence of ATP) was taken as 100 percent. Results of such experiments are presented in Fig. 1. It is apparent that physiological levels of ATP significantly increased specific binding activity of the oestradiol receptor. This effect of ATP on oestradiol receptor binding was further confirmed by sucrose gradient analysis of immature lamb uterus and MCF-7 cell cytosol (Fig. 2). In the presence of 10 mM ATP, the binding sites in the 4 S region were increased, while those in 8 S region remained unchanged. It is evident that ATP/Mg^{2+} activates a type of low molecular weight binding sites (4 S), without influencing binding in the 8 S region. Ligand binding analysis (Fig. 3) on cytosol from MCF-7 cells, indicated that in the absence of ATP cytosol contains only one type of receptor binding sites with high affinity and low capacity for [^{3}H]oestradiol-17β (K_a $2.15 \times 10^9\ M^{-1}$). In the presence of ATP/Mg^{2+} there are two types of binding sites, one high affinity type (K_a $3.2 \times 10^9\ M^{-1}$) and one with lower affinity (K_a

$3.3 \times 10^8\ M^{-1}$). The total concentration of binding sites was increased between 50 and 130×10^{-12} M. This increase of low affinity binding sites of cytosol oestradiol receptor, in the presence of ATP demonstrated by ligand binding analysis, confirmed the corresponding increase of low molecular weight binding sites (4 S) of the cytosol oestradiol receptor by sucrose gradient analysis. The Scatchard plot (inset) also showed a decrease in concentration of binding sites for the high affinity type in the presence of ATP. This decrease in concentration of binding sites was not significant and was not reproduced in all experiments.

RIAA (50 μg) was found to decrease the steroid binding activity of the purified receptor ($P = 0.0024$) and was characterized by its inhibition by known phosphatase inhibitors (Fig. 4). Molybdate, glucose-1-phosphate, tungstate, pyrophosphat at 10 mM concentration each inhibited the inactivation of oestradiol receptor. In contrast 20 mM sodium fluoride was ineffective. Using p-nitrophenyl phosphate as substrate, it was found that 50 μg (50 μl) of RIAA is equivalent to one milliunit of phosphatase activity. Table 1, shows the dose–response of the RIAA. In this experiment cytosol from immature lamb uterus was incubated for 2 h at 0°C with 5 nM [^{3}H]oestradiol-17β with and without a 100-fold excess cold oestradiol. Incubation was performed for 30 min at 25°C in the absence (control) and presence of 25, 50 and 100 μg RIAA. The reaction was stopped by cooling the samples at 0°C. It is evident from Table 1

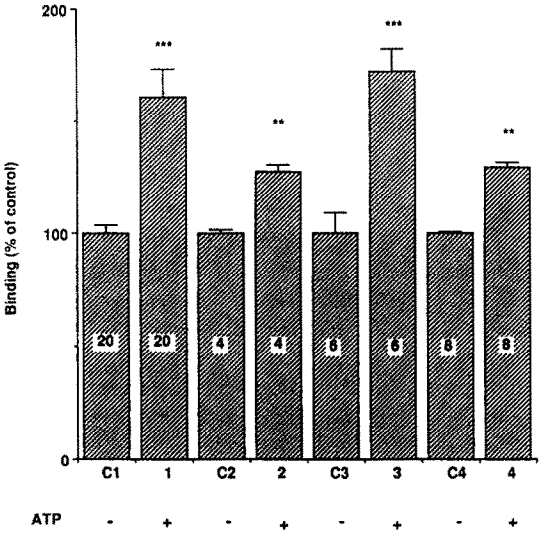

Fig. 1. The effect of ATP on oestradiol receptor. Number in columns are number of experiments. C = control, 1 = human mammary cancer tissue (C1 = 114 fmol/mg protein), 2 = immature lamb uterine cytosol (C2 = 104 fmol/mg protein), 3 = MCF-7 cell cytosol (C3 = 108 fmol/l $\times 10^7$ cells), 4 = MCF-7 cell nuclear extract (C4 = 30.9 fmol/l $\times 10^7$ cells). Control is taken as 100%. Values are given as percent relative to control. Bars are mean ± SEM. *$0.05 > P > 0.01$, **$0.001 > P > 0.0001$, ***$0.0001 > P > 0.00001$. All receptor measurements were single point saturation assay.

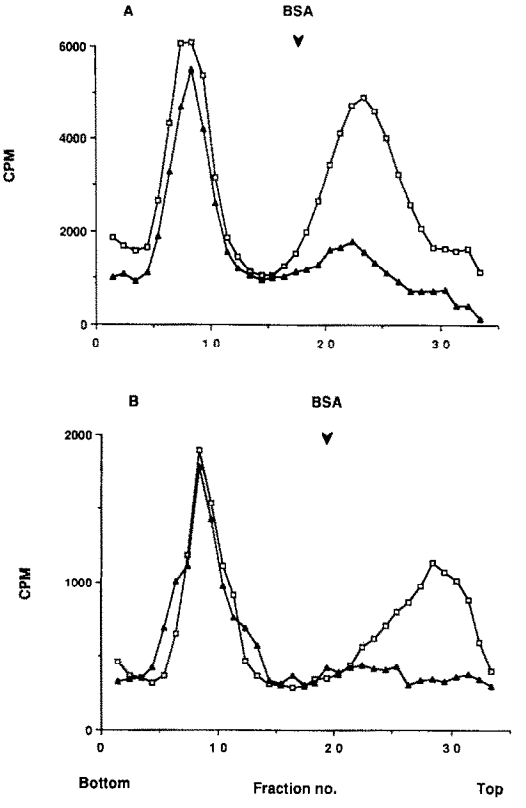

Fig. 2. Sedimentation pattern of cytosol oestradiol receptor in 20–30% sucrose gradients. Immature lamb uterus (A), MCF-7 cells (B). Aliquots of cytosol (15 mg/ml for A and 4.4 mg/ml for B) prepared in TED-buffer containing 10 mM molybdate were incubated with 5 nM [^{3}H]oestradiol-17β in the presence and absence of 10 mM ATP/Mg^{2+} for 10 min at 15°C and then overnight at 0–4°C. Unbound radioactivity was removed with a dextran-charcoal pellet. Aliquots (200 μl), were layered on top of the gradients containing 10 mM molybdate. Gradients were centrifuged at 369,000 g for 3 h using the vertical rotor Vti 65.2 of a Beckman ultracentrifuge. Fractions were collected from the bottom of the tubes (9 drops/fraction) and radioactivity in each fraction was determined. [^{14}C]BSA was used as marker. Closed triangles: binding activity without ATP. Open squares: binding activity with ATP.

that RIAA effects a dose related inhibition of the binding activity of the oestradiol receptor. RIAA significantly reduced the binding of oestradiol to ammonium sulphate precipitated receptor, and RAA restored binding in the presence of ATP and Mg^{2+} (Fig. 5). ATP alone in the absence of RAA following treatment with RIAA did not significantly affect binding activity. Similar inactivation/reactivation experiments has been performed on cytosol (A) and nuclear extract (B) of MCF-7 cells (Fig. 6). RIAA had no significant effect on receptor binding. However, RAA in the presence of ATP and Mg^{2+} significantly increased binding activity. Effects were seen of both RAA and ATP alone.

[^{3}H]Tamoxifen aziridine affinity labelling of partially purified immature lamb uterus cytosol oestradiol receptor demonstrated the presence of 4 peaks of

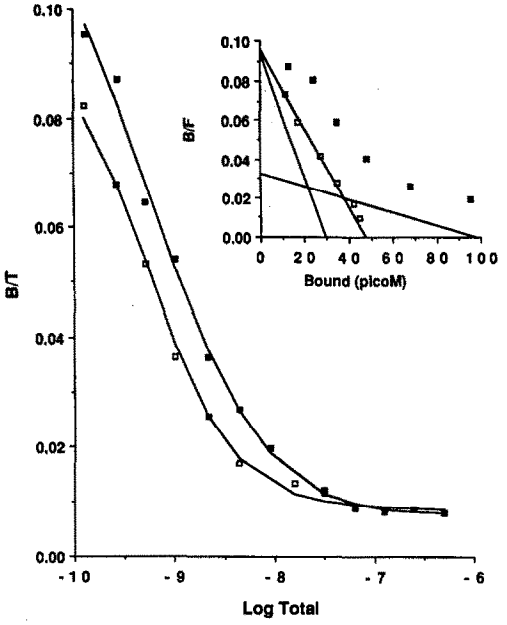

Fig. 3. Ligand binding analysis of [^{3}H]oestradiol-17β binding in cytosol from MCF-7 cells. Cytosols were prepared in 50 mM phosphate buffer pH 7.4, containing 1.5 mM thioglycerol and 10 mM molybdate. Aliquots of cytosol were incubated with increasing concentrations of radioactive oestradiol (0.15–5.0 nM) in the presence and absence of 10 mM ATP/Mg^{2+} for 10 min at 15°C and then overnight at 0–4°C. Binding parameters were calculated using the Ligand System, Fortran version 2.3.11, N.I.H. Open Squares: binding activity without ATP. Closed squares: binding activity with ATP. Inset: Scatchard plot constructed from calculated values of association constant and concentration of binding sites. Symbols show the actual binding data.

Table 1. Dose–response of the RIAA

	ER (fmol/mg protein)	Inhibition (%)
Control	289	
RIAA 25 μg	237	18
RIAA 50 μg	170.5	41
RIAA 100 μg	103	64

Cytosol from immature lamb uterus was incubated for 2 h at 0°C with 5 nM [^{3}H]oestradiol-17β in the presence and absence of a 100-fold excess cold oestradiol. Incubation was performed for 30 min at 25°C in the absence (control) and presence of 25, 50 and 100 μg RIAA. The reaction was stopped by cooling the samples to 0°C. The bound hormone was separated from the free hormone by dextran-charcoal adsorption (charcoal 0.25%, dextran 0.0025%, gelatin 0.1%). The specifically bound radioactivity was measured in aliquots of the supernatants, values are expressed as inhibition percent of control and also the concentration of the inactivated oestradiol receptor as fmol/mg protein.

binding activity with approximate molecular weights of 150, 97, 67 and 50 kDa under the denaturing conditions of SDS–PAGE (Fig. 7A, control).

The purified receptor was inactivated with RIAA and then subjected to reactivation with [γ^{32}P]-ATP in the absence (Fig. 7B) and presence of RAA (Fig. 7C). Subsequently the receptor was covalently labelled with [^{3}H]tamoxifen aziridine, and the phosphorylated products were identified by SDS–PAGE (Fig. 7C). In the presence of RAA the three proteins of 150, 97, and 67 kDa were phosphorylated, with a concomitant increase in the [^{3}H]tamoxifen aziridine. These results indicate that the RAA is a phosphokinase, and that the increased binding activity was due to the phosphorylation of the receptor.

In order to determine whether these peaks of [^{3}H]tamoxifen aziridine binding activity are receptors

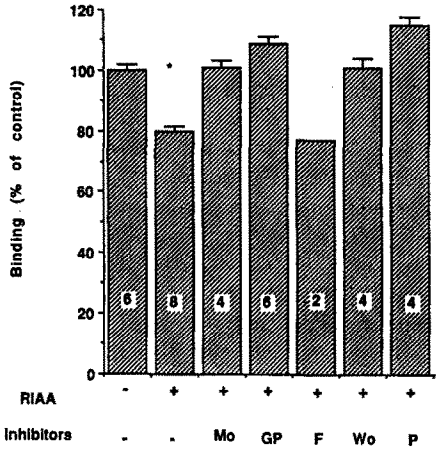

Fig. 4. Inhibition of receptor inactivating activity (RIAA). Aliquots of purified immature lamb uterus oestradiol receptor were incubated in the absence (control) and presence of RIAA plus phosphatase inhibitors. The inhibitor concentrations were 10 mM except for sodium fluoride where 20 mM was used. Inhibitors used were: sodium molybdate (Mo), glucose-1-phosphate (GP), sodium fluoride (F), sodium tungstate (Wo), Pyrophosphate (P). Number in columns are number of experiments. Control is taken as 100%. Values are given as percent relative to control. Bars are mean ± SEM. *$P = 0.0024$.

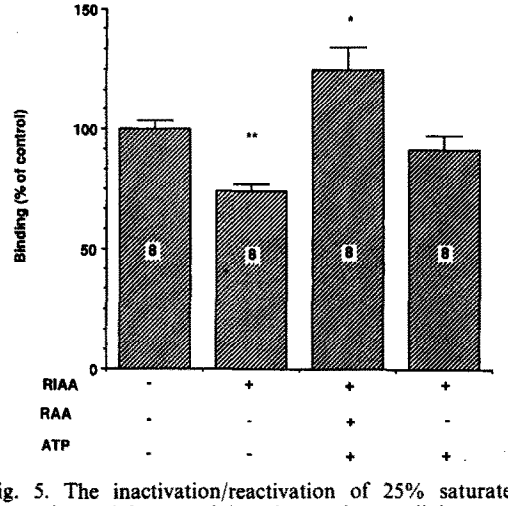

Fig. 5. The inactivation/reactivation of 25% saturated ammonium sulphate precipitated cytosol oestradiol receptor from immature lamb uterus as described in experimental procedures. Number in columns are number of experiments. Control (1751.5 fmol/mg protein) is taken as 100%. Values are given in percent relative to control. Bars are mean ± SEM. *$0.05 > P > 0.01$, **$0.001 > P > 0.0001$.

or some other non-specific tamoxifen aziridine binding proteins, immunoprecipitation of oestradiol receptor with the monoclonal antibody JS 34/32 was performed. The monoclonal antibody JS 34/32 [12] was raised against a purified preparation of oestradiol receptor from calf uterus. This antibody recognizes a conformational epitope on the oestradiol receptor (Moncharmont B., personal communication).

Immature lamb cytosol in TED-buffer at 0°C, was added ammonium sulphate to 25% saturation. The precipitate was solubilized in TED-buffer and incubated with RIAA at 25°C for 30 min followed by incubation in the absence (Fig. 8B) and presence of RAA and [γ^{32}P]ATP as described under Methods (Fig. 8C). Control (Fig. 8A) is an equivalent amount of untreated ammonium sulphate precipitate cytosol solubilized in TED-buffer. Finally the samples were treated with 20 nM [^{3}H]tamoxifen aziridine ([^{3}H]TA) in the presence or absence of a 200-fold excess cold oestradiol. Incubation was performed at 0°C for 18 h. The affinity labelled oestradiol receptor was immunoprecipitated with monoclonal antibody JS 34/32. Non-immune rat IgG was used as control antibody.

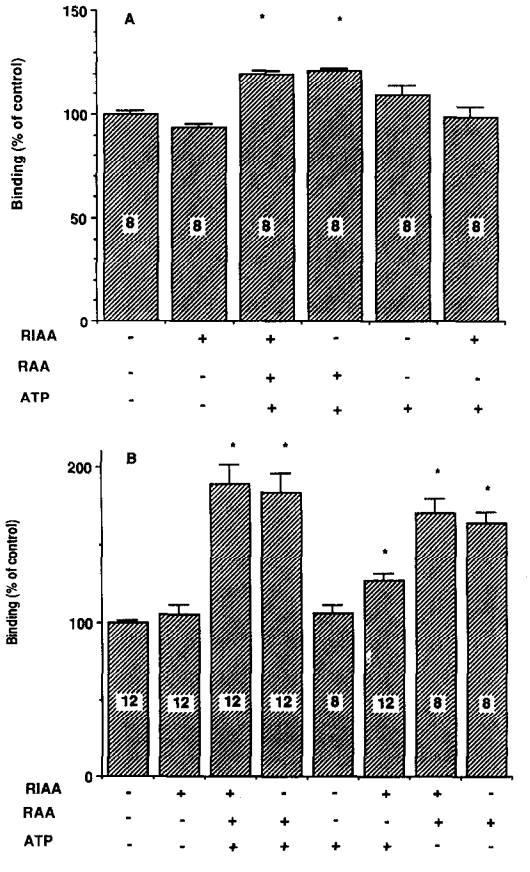

Fig. 6. The inactivation/reactivation of MCF-7 cells oestradiol receptor. Cytosol (A) and nuclear extract (B). Number in columns are number of experiments. Control (A: 151.1 fmol/l × 10^7 cells, B: 36.2 fmol/l × 10^7 cells) is taken as 100%. Values are given as percent relative to control. Bars are mean ± SEM. *$0.05 > P > 0.01$.

The control (Fig. 8A) demonstrated that the JS 34/32 monoclonal antibody precipitated three [^{3}H]tamoxifen aziridine binding proteins with approximate molecular weights of 67, 50 and 43 kDa. These three [^{3}H]tamoxifen aziridine binding proteins were not precipitated by non-immune rat IgG antibody. Under the conditions used the 67 kDa receptor was extensively proteolyzed. In the presence of RIAA (Fig. 8B) the [^{3}H]tamoxifen aziridine binding activity of 43 kDa was reduced. The [^{3}H]tamoxifen aziridine binding activity 67 and 50 kDa proteins remained unchanged. In the presence of RAA and [γ^{32}P]ATP (Fig. 8C), only 43 kDa protein was phosphorylated, with a concomitant increase in the [^{3}H]tamoxifen aziridine. The 67 and 50 kDa proteins were not phosphorylated. In these experiments cytosol was prepared in TED-buffer without molybdate and any proteases inhibitors. It was apparent that the receptor preparation was subjected to considerable proteolysis. Therefore, cytosol was prepared in buffer containing molybdate and proteases inhibitors, incubated with [^{3}H]tamoxifen aziridine overnight at 0°C, added ammonium sulphate to 25% saturation. The precipitate was solubilized in the same buffer containing molybdate and proteases inhibitors. The affinity labelled oestradiol receptor was immunoprecipitated with the monoclonal antibody JS 34/32.

Under these conditions of receptor stabilization and inhibition of proteolysis, the immunoprecipitation of oestradiol receptor with the monoclonal antibody JS 34/32 but not with control antibody demonstrated (Fig. 9) the presence of undegraded [^{3}H]tamoxifen aziridine binding activity of 67 and 50 kDa. The [^{3}H]tamoxifen aziridine binding activity of 43 kDa was present but in reduced quantity.

DISCUSSION

The studies showed that ATP increased the cytosol oestradiol binding activity of human mammary cancer tissue, MCF-7 cells and immature lamb uterus. Incubation with ATP generated a new population of binding sites with a lower sedimentation rate, approximately 4 S, without any significant influence on binding in the 8 S region. Scatchard analysis showed that the new binding sites had lower affinity than the normal oestradiol receptor, K_a $3.3 \times 10^8\ M^{-1}$ and $3.2 \times 10^9\ M^{-1}$ respectively. The Scatchard plot also showed a minor decrease in concentration of binding sites for the high affinity type in the presence of ATP. This decrease in concentration of high affinity binding sites could not account for the large increase in concentration of binding sites of the lower affinity type and was not seen in all experiments. It should be pointed out that there is a fairly large statistical uncertainty in the calculation of binding parameters in a model with two binding components. Recent reports [14–15] indicated that there are two types of oestradiol receptor with different affinity in the chick oviduct. A low affinity receptor exists in a

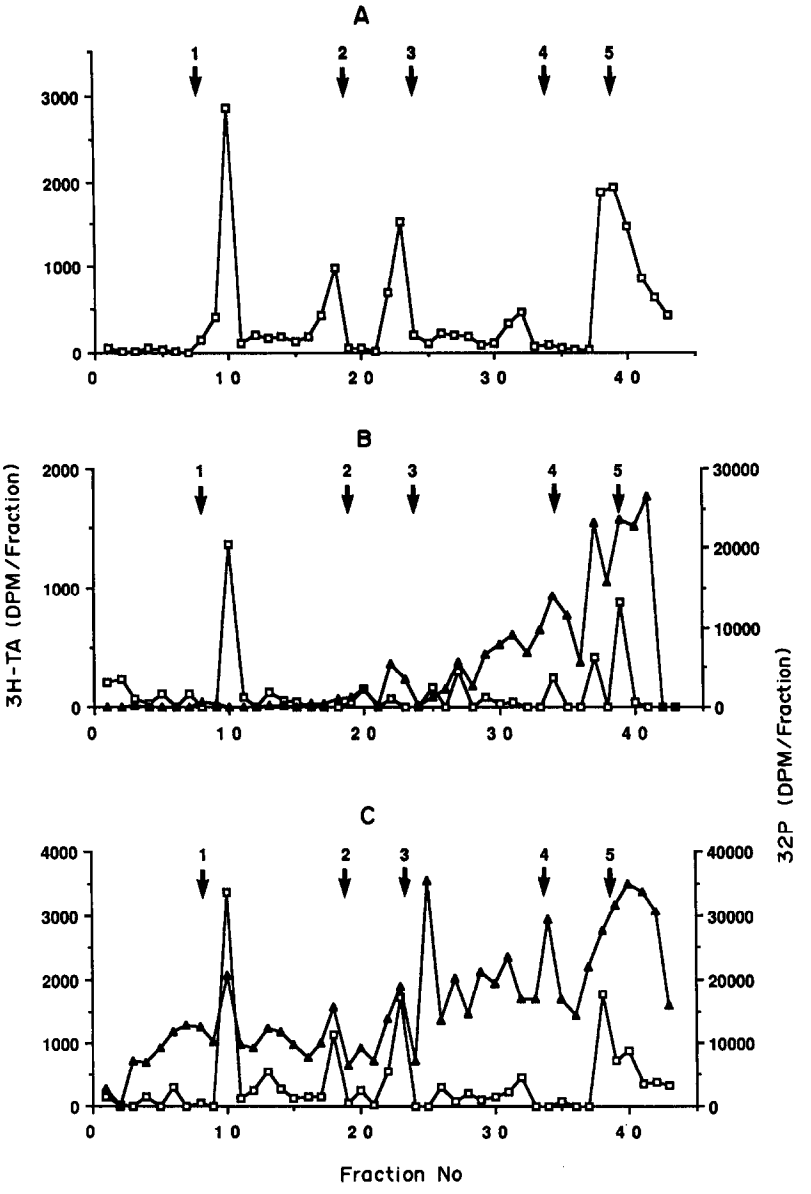

Fig. 7. Purified receptor was incubated with RIAA at 25°C for 30 min followed by incubation with Mg^{2+}/molybdate/[γ^{32}P]-ATP in the absence (B) and presence (C) of RAA at 15°C for 10 min. Finally the receptor was treated with [^{3}H]tamoxifen aziridine ([^{3}H]TA) for 1 h at 30°C with and without 4×10^{-6} M oestradiol. Control (A) is an equivalent amount of untreated receptor. The figure shows the oestradiol competable binding. Open squares [^{3}H]TA, closed triangles ^{32}P. The arrows indicate the positions of proteins of known molecular weight. Molecular weights of (mol. wt) protein standards were: (1) ferritin half subunit, 220 kDa, (2) phosphorylase b, 94 kDa, (3) albumin, 67 kDa, (4) ovalbumin, 43 kDa, (5) dye front.

non-oestradiol binding form which can be converted to the binding form *in vitro* by ATP or ADP. This activation required the presence of Mg^{2+}. The conversion of the non-binding form to the binding form could be similar to that of the activation of low affinity components in the presence of ATP and Mg^{2+} in immature lamb uterus and MCF-7 cell cytosol.

The inhibition of RIAA by various phosphatase inhibitors indicates that RIAA is a phosphatase. Auricchio *et al.*[16] reported that RIAA can not inactivate the oestradiol receptor in the presence of antioestrogen. We have incubated the purified receptor with RIAA first, then with RAA and [γ^{32}P]-ATP and finally used an exchange assay in the presence of [^{3}H]tamoxifen aziridine. In this way we were able to show the effect of RIAA on inactivation of purified receptor. The effect of molybdate on receptor inactivation is difficult to interpret. The mechanism seems to be complex, and not solely due to inhibition of phosphatase [17, 18]. RIAA significantly reduced the binding of oestradiol to the cytosol and ammonium sulphate precipitate receptor from immature lamb

uterus, while RIAA appears to have no effect on MCF-7 cell oestradiol receptor. This could be due to species specificity. We have found a dose–response relationship between the amount of RIAA used and reduction in cytosol receptor binding. However, receptor binding activity could not be reduced by more than about 50–60%. This is in agreement with results published by Auricchio *et al.*, and could be due to the

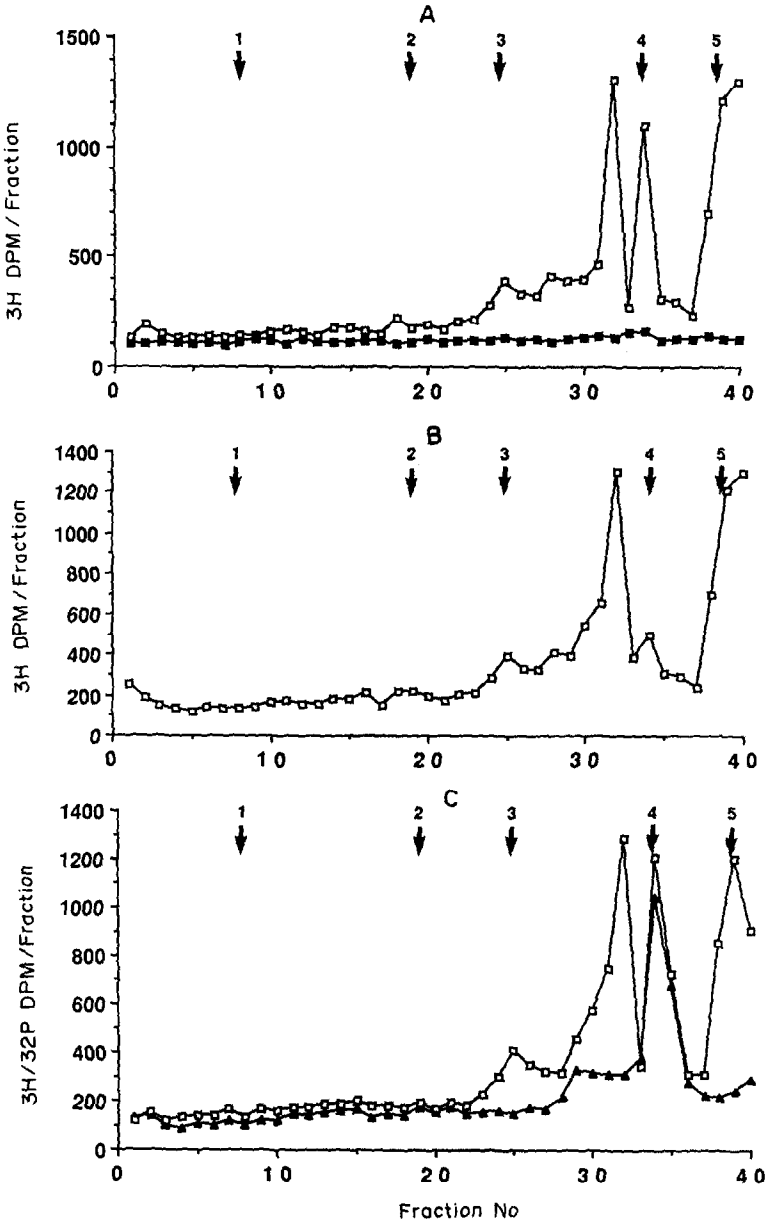

Fig. 8. Immature lamb uterus cytosol ammonium sulphate precipitated in TED-buffer at 0°C. The solubilized precipitate was incubated with RIAA at 25°C for 30 min followed by incubation with Mg^{2+}/molybdate in the absence (B) and presence of RAA and [γ^{32}P]ATP at 15°C for 10 min (C). Control (A) is an equivalent amount of untreated ammonium sulphate precipitated cytosol solubilized in TED-buffer. Finally the solubilized precipitate was treated with 20 nM [^{3}H]tamoxifen aziridine ([^{3}H]TA) in the presence and absence of a 200-fold excess cold oestradiol. Incubation was performed at 0°C for 18 h. Monoclonal antibody JS 34/32 or non-immune rat IgG (was added to samples at a final concentration of 25 μg/ml (2.5% of total volume) and the mixture was incubated for 16 h at 0–2°C. Incubation with protein A-Sepharose and washing of immunoaffinity labelled receptor coupled to protein A-Sepharose and SDS–PAGE, were performed as described in experimental procedures. The figure is representative of three experiments. It shows the oestradiol competable binding. Open squares [^{3}H]TA, closed triangles ^{32}P, closed squares [^{3}H]TA of sample incubated with non-immune rat antibody. The arrows indicate the positions of proteins of known molecular weight. Molecular weights of (mol. wt) protein standards were: (1) ferritin half subunit, 220 kDa, (2) phosphorylase b, 94 kDa, (3) albumin, 67 kDa, (4) ovalbumin, 43 kDa, (5) dye front.

presence of two different receptor forms (i.e. high and low affinity forms). Possibly, one form of the receptor is phosphorylated and can be dephosphorylated by RIAA, while the other form is not phosphorylated, and as a result RIAA has no effect on that form of the receptor. RAA restored binding in the presence of ATP/Mg^{2+}, and appears to be a phosphokinase.

In MCF-7 cells, effects were seen of both RAA and ATP alone, probably due to the presence of endogenous RAA and ATP. The effect of RIAA and RAA was independent of receptor occupancy by oestradiol (data not shown).

Auricchio *et al.*[19, 20] reported that the oestradiol receptor from calf and rat uterus is phosphorylated on tyrosine residues. However, progesterone [3] and glucocorticoid [4] receptor are phosphorylated on the amino acid serine. Treatment of MCF-7 cells with phorbol ester resulted in a reduction of oestradiol receptor binding activity after a rapid increase in phosphorylation of the receptor on serine and threonine [21]. In order to determine the relevance of these processes of inactivation/reactivation to that of the dephosphorylation/phosphorylation of oestradiol receptor under the denaturing conditions of SDS–PAGE, one has to use [^{3}H]tamoxifen aziridine, a covalent ligand. A non-covalently bound ligand would dissociate from the oestradiol receptor under denaturing conditions.

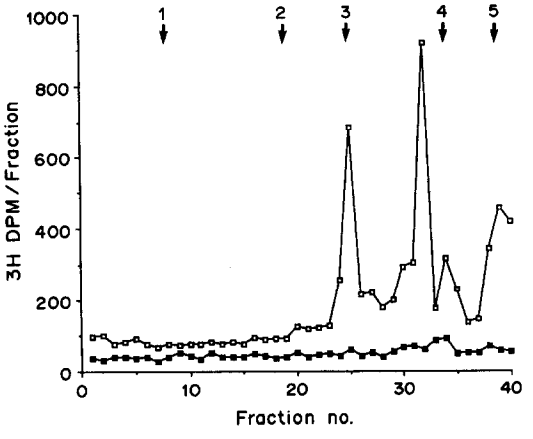

Fig. 9. Immature lamb uterus cytosol in TEGMP-buffer pH 7.4 [10 mM TES, 50 mM NaCl, 10% (w/v) glycerol, 1.5 mM EDTA, 10 mM molybdate, 1.5 mM thioglycerol, 1 mM PMSF, 77 μg/ml aprotinine and 20 mM sodium fluoride] was incubated with 20 nM [^{3}H]tamoxifen aziridine in the presence and absence of a 200-fold excess cold oestradiol, overnight at 0°C, and then ammonium sulphate precipitated. The precipitate solubilized in TEGMP-buffer at 0°C. Monoclonal antibody JS 34/32 or non-immune rat IgG (was added to samples at a final concentration of 25 μg/ml (2.5% of total volume) and the mixture was incubated for 16 h at 0–2°C. Incubation with protein A-Sepharose and washing of immunoaffinity labelled receptor coupled to protein A-Sepharose and SDS–PAGE, were performed as described in experimental procedures. The figure is representative of two experiments. It shows the oestradiol competable binding. Open squares [^{3}H]TA, closed squares [^{3}H]TA of sample incubated with non-immune rat antibody. The arrows indicate the positions of proteins known molecular weight. Molecular weights of (mol. wt) protein standards were: (1) Ferritin half subunit, 220 kDa, (2) Phosphorylase b, 94 kDa, (3) Albumin, 67 kDa, (4) Ovalbumin, 43 kDa, (5) dye front.

On sucrose gradients the purified receptor moved as a 5S "transformed" receptor dimer and Scatchard analysis of the purified receptor indicated that there was only one class of high affinity binding sites (K_a $1.1 \times 10^{10}\,M^{-1}$) for [^{3}H]oestradiol-17β (data not shown). The covalent labelling of the purified receptor with [^{3}H]tamoxifen aziridine (Fig. 7A control) showed 3 prominent peaks of binding activity with approximate molecular weights of 150, 97 and 67 kDa under the denaturing conditions of SDS–PAGE. A small peak of binding activity corresponding to the molecular weight 50 kDa was also present.

The purified receptor was inactivated by RIAA and then subjected to reactivation with RAA in the presence of [γ^{32}P]ATP and subsequently covalently labelled with [^{3}H]tamoxifen aziridine showed that the three proteins of 150, 97 and 67 kDa were, phosphorylated, with concomitant increase in [^{3}H]tamoxifen aziridine binding.

The high ratio of phosphorus to tritium probably has three main explanations:

(1) The receptor preparation was purified by affinity chromatography and eluted from the affinity column with oestradiol. Accordingly, the receptor is "occupied" and it has been found virtually impossible to get a quantitative exchange of oestradiol with tamoxifen aziridine.

(2) The receptor preparation is obviously not a homogeneous protein.

(3) It has recently been reported [22] that the murine glucocorticoid receptor contains two to three phosphorylated sites. Similar observations [23] were made for mouse L cell glucocorticoid receptor which contains four phosphorylated residues.

Immunoprecipitation of the affinity labelled oestradiol receptor from immature lamb uterus cytosol with the monoclonal antibody JS 34/32 demonstrated the presence of the [^{3}H]tamoxifen aziridine binding activity of 67, 50 and 43 kDa. Only the [^{3}H]tamoxifen aziridine binding activity of 43 kDa was phosphorylated. [^{3}H]tamoxifen aziridine binding proteins were not precipitated with non-immune IgG and the binding was displaced by excess cold oestradiol. The [^{3}H]tamoxifen aziridine binding proteins of 150 and 97 kDa were absent. These two proteins were copurified with oestradiol receptor under the conditions used for the isolation of the receptor [6], and appear to be non-specifically labelled. The method of Puca *et al.* [6] for purification of oestradiol receptor allows purification of the native form of receptor. We chose this method to partially purify the receptor because it did not use molybdate to stabilize the receptor. It was known that, RIAA was not able to inhibit the purified molybdate stabilized receptor. Immunoprecipitation analysis demonstrated the lack of phosphorylation of the 67 kDa oestradiol

receptor. This result however, does not disprove that the 67 kDa receptor protein is phosphorylated. The smaller fragment of 43 kDa which binds [^{3}H]tamoxifen aziridine is phosphorylated. The proteolysis of the receptor has the following reasons:

(1) The cytosol was prepared in the absence of molybdate, ligands and protease inhibitors (Fig. 8).

(2) Incubation of the unoccupied receptor for 30 min at 25°C in the absence of any stabilizing agents favours the proteolysis of the receptor.

Preparation of cytosol in buffer containing molybdate and proteases inhibitors (Fig. 9) demonstrated the presence of undegraded 67 and 50 kDa receptor proteins. The 43 kDa protein was also present but in reduced quantity, which indicated that the proteolysis of the receptor had not been completely inhibited. Thus it appears that in the absence of any stabilizing agents or proteolysis inhibitors the proteolysis of the receptor had taken place during the cytosol preparation. It is possible that proteolysis of the receptor facilitates the phosphorylation of the 43 kDa fragment.

Dephosphorylation/phosphorylation reactions are among the most important post-translational control mechanisms for biological activity, and clarification of this process may shed light on the mechanism of hormone–receptor interactions. The data presented, provide evidence for the involvement of such mechanisms in the inactivation/reactivation of oestradiol receptor from immature lamb uterus cytosol and probably in the MCF-7 cells *in vitro*. It is important to determine whether oestradiol receptor is a phosphoprotein *in vivo*. By covalent labelling in cell culture it is possible to study the phosphorylation of receptor, by immunoaffinity purification and subsequent characterization of the binding under the SDS–PAGE conditions. Such studies are at present in progress at this laboratory.

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REFERENCES

1. Auricchio F., Migliaccio A., Castoria G., Rotondi A. and Lastoria S.: Direct evidence of *in vitro* phosphorylation-dephosphorylation of the estradiol-17β receptor. Role of Ca^{2+}-calmodulin in the activation of hormone binding sites. *J. Steroid Biochem.* **20** (1984) 31–35.
2. Migliaccio A., Rotondi A. and Auricchio F.: Calmodulin-stimulated phosphorylation of 17β-estradiol receptor on tyrosine. *Proc. Natn. Acad. Sci. U.S.A.* **81** (1984) 5921–5925.
3. Dougherty J. J., Puri R. K. and Toft D. O.: Phosphorylation *in vivo* of chicken oviduct progesterone receptor. *J. Biol. Chem.* **257** (1982) 14226–14230.
4. Housley P. R. and Pratt W. B.: Direct demonstration of glucocorticoid receptor phosphorylation by intact L-cells. *J. Biol. Chem.* **258** (1983) 4630–4635.
5. Sedmak J. J. and Grossberg S. E.: A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. *Analyt. Biochem.* **79** (1977) 544–552.
6. Puca G. A., Medici N., Molinari A. M., Moncharmont B., Nola E. and Sica V.: Estrogen receptor of calf uterus: an easy and fast purification procedure. *J. Steroid Biochem.* **12** (1980) 105–113.
7. Auricchio F., Migliaccio A. and Rotondi A.: Inactivation of oestrogen receptor *in vitro* by nuclear dephosphorylation. *Biochem. J.* **194** (1981) 569–574.
8. Auricchio F., Migliaccio A., Castoria G., Lastoria S. and Schiavone E.: ATP-dependent enzyme activating hormone binding of estradiol receptor. *Biochem. Biophys. Res. Commun.* **101** (1981) 1171–1178.
9. Wittliff J. L.: *Application data, Spinco DS-509.* Beckman Instruments (1977).
10. Katzenellenbogen J. A., Carlson K. E., Heiman D. F. Robertson D. W., Wei L. L. and Katzenellenbogen B. S.: Efficient and highly selective covalent labeling of estrogen receptor with [^{3}H]Tamoxifen aziridine. *J. Biol. Chem.* **258** (1983) 3487–3495.
11. Laemmli U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** (1970) 680–685.
12. Moncharmont B., Su Jui-Lan and Parikh I.: Monoclonal antibodies against estrogen receptor: Interaction with different molecular forms and functions of the receptor. *Biochemistry* **21** (1982) 6916–6921.
13. Sanchez E. R., Redmond T., Scherrer L. C., Bresnick E. H., Welsh M. J. and Pratt W. B.: Evidence that the 90-kilodalton heat shock protein is associated with tubulin-containing complexes in L cell cytosol and in intact PtK cells. *Molec. Endocr.* **2**, (1988) 756–760.
14. McNaught R. W., Raymoure W. J. and Smith R. G.: Receptor interconversion model of hormone action. I. ATP-mediated conversion of estrogen receptors from a high to lower affinity state and its relationship to antiestrogen action. *J. Biol. Chem.* **261** (1986) 17011–17017.
15. Raymoure W. J., McNaught R. W., Greene G. L. and Smith R. G.: Receptor interconversion model of hormoneaction. II. Nucleotide-mediated conversion of estrogen receptors from nonsteroid binding to the lower affinity binding state. *J. Biol. Chem.* **261** (1986) 17018–17025.
16. Auricchio F., Migliaccio A. and Castoria G.: Dephosphorylation of oestradiol nuclear receptor in *in vitro*. *Biochem. J.* **198** (1981) 699–702.
17. Dahmer M. K., Housley P. R. and Pratt W. B.: Effect of molybdate and endogenous inhibitors on steroid-receptor inactivation, Transformation, and translocation. *A. Rev. Physiol.* **46** (1984) 67–81.
18. Pratt W. B.: Transformation of glucocorticoid and progesterone receptors to the DNA-binding state. *J. Cell. Biochem.* **35** (1987) 35–51.
19. Auricchio F., Migliaccio A., Castoria G., Rotondi A., Domenico M. D. and Pagano M.: Activation-inactivation of hormone binding sites of the oestradiol-17β receptor is a multiregulated process. *J. Steroid Biochem.* **24** (1986) 39–43.
20. Auricchio F., Migliaccio A., Castoria G., Rotondi A., Domenico M. D., Pagano M. and Nola E.: Phosphorylation on tyrosine of oestradiol-17β receptor in uterus and interaction of oestradiol-17β and glucocorticoid receptors with antiphosphotyrosine antibodies. *J. Steroid Biochem.* **27** (1987) 245–253.
21. Dickson R. B. and Lippman M. E.: Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. *Endocr. Rev.* **8** (1987) 29–43.

22. Mendel D. B., Bodwell J. E. and Munck A.: Activation of cytosolic glucocorticoid-receptor complexes in intact WEHI-7 cells does not dephosphorylate the steroid-binding protein. *J. Biol. Chem.* **262** (1987) 5644–5648.

23. Dalman F. C., Sanchez E. R., Lin A. L.-Y., Perini F. and Pratt W. B.: Localization of phosphorylation sites with respect to the functional domains of the mouse L cell glucocorticoid receptor. *J. Biol. Chem.* **263** (1988) 12259–12267.