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Biochemistry

Calmodulin-stimulated phosphorylation of 17β-estradiol receptor on tyrosine

(Ca^{2+}-calmodulin/phosphotyrosine/receptor kinase/steroid receptors)

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ABSTRACT **The calf uterine 17β-estradiol receptor is a phosphoprotein. Phosphorylation–dephosphorylation of the receptor is controlled by a cytosol receptor kinase that activates the hormone binding and by a nuclear phosphatase that inactivates this binding. This report concerns the nature of the 17β-estradiol receptor kinase. Highly purified calf uterus 17β-estradiol receptor preinactivated by the nuclear phosphatase was used as substrate of the purified receptor kinase. Ca^{2+} and calmodulin stimulate both the kinase-dependent activation of the hormone binding and ^{32}P incorporation from [γ-^{32}P]-ATP into the receptor. Maximal stimulation of hormone binding activation requires 1 μM Ca^{2+} and 0.6 μM calmodulin. Fifteen micromolar trifluoperazine is the lowest concentration that will prevent completely Ca^{2+}-calmodulin stimulation of the kinase. The receptor is phosphorylated by the receptor kinase exclusively on tyrosine. Phosphorylation of proteins on tyrosine is a rare event implicated in hormone-induced cell growth and cell transformation.**

Reversible covalent modifications regulate the activities of several enzymic and nonenzymic proteins. Since the finding that glycogen phosphorylase exists in a dephosphorylated and phosphorylated form, several proteins have been shown to undergo the same interconversion, which is controlled by protein kinase and phosphoprotein phosphatase (1, 2). Receptors of immunoglobulin E (3), acetylcholine (4, 5), epidermal growth factor (6), and insulin (7) are phosphoproteins, and evidence is accumulating that steroid receptors are phosphorylated. Phosphorylation appears to be a prerequisite for the hormone binding activity of steroid receptors: cortisol binding in thymocytes has been correlated to the level of ATP (8); ATP enhances the hormone binding of crude androgen receptor (9); hormone-free glucocorticoid receptor is inactivated by an exogenous alkaline phosphatase and by a process inhibited by molybdate and reactivated by a process requiring ATP (10, 11). In addition, phosphorylation–dephosphorylation appears to play a role in converting the receptor to the form that is retained by nuclei or by ATP or DNA covalently linked to Sepharose or to similar polymers (12–14).

Recently chicken progesterone receptor has been phosphorylated *in vivo* (15) and by an exogenous cAMP-dependent kinase *in vitro* (16). The glucocorticoid receptor of intact fibroblasts also has been phosphorylated (17). The phosphorylation of these two steroid receptors is on serine; the role in receptor function of this phosphorylation is unknown.

Direct evidence is now available that the estradiol receptor is a phosphoprotein and that phosphorylation is required for hormone binding (18). Phosphorylation–dephosphorylation of this receptor is controlled by a cytosol kinase (19) and by nuclear phosphatase, respectively (20–22). After the phosphorylated estradiol receptor has migrated into the nuclear compartment in complex with the hormone, it is apparently inactivated by the nuclear phosphatase and rapidly released into the cytoplasm (23). The two enzymes that regulate the hormone binding have been partially purified and characterized (19–22).

The nature of the protein kinase and phosphatase, the factors that regulate these enzymes, and the amino acid residues that are phosphorylated are not known. For instance, could the kinase be activated by cyclic nucleotides and/or calmodulin, and are serine, threonine and/or tyrosine residues phosphorylated? In preliminary studies with crude preparations of estrogen receptors, we found that Ca^{2+} can stimulate estradiol binding in the presence of the kinase (19). In the current studies we have used highly purified estrogen receptors and find that calmodulin, in a Ca^{2+}-dependent manner, can activate this kinase. We have further analyzed the nature of the phosphorylation and report that tyrosine is the only amino acid of the estradiol receptor phosphorylated by the kinase.

MATERIALS AND METHODS

Materials. All reagents were of analytical grade. Cellulose thin-layer plates (250 μm) were from Machery-Nagel (Düren, F.R.G.). 17β-[2,4,6,7-^{3}H]Estradiol (85 Ci/mmol; 1 Ci = 37 GBq) and [γ-^{32}P]ATP (3000 Ci/mmol) were from the Radiochemical Centre. Trifluoperazine dihydrochloride was generously donated by Smith Kline & French (Welwin Garden City, Herts, U.K.).

Buffers. The following buffers were used: 50 mM Tris·HCl (pH 7.4) containing 2 mM dithiothreitol and 1 mM EDTA without (TED buffer) or with 0.25 M sucrose (TED/sucrose buffer); 10 mM Tris·HCl (pH 7.4) containing 1 mM dithiothreitol and 5 mM $MgCl_2$ (TDM buffer). In most of the experiments described in this paper the EDTA in the TED buffer was substituted by 0.2 mM EGTA (TGD buffer).

Purification of Receptor Phosphatase and Receptor Kinase. Calf uterus was homogenized in TGD/sucrose buffer, and the phosphatase was purified from nuclear extract by CM-cellulose chromatography as reported (21). The supernatant from the original homogenization was centrifuged to prepare cytosol that was the source of the receptor kinase. The latter was purified according to a previously described procedure based on ammonium sulfate fractionation, heparin-Sepharose, and DEAE-cellulose chromatography (19). This procedure results in at least 300-fold, and possibly >2000-fold, purification of the enzyme over crude cytosol (19). The arbitrary units of both enzymes were calculated as reported (19, 21).

Purification of Cytosol 17β-Estradiol Receptor. The receptor was purified from calf uterus by affinity chromatography on heparin-Sepharose followed by affinity chromatography on 17β-estradiol-17-hemisuccinyl-hexane-agarose as reported (24). The final purification step of the original procedure,

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Sephadex G-200 chromatography, was omitted. Extensive washing of the estradiol-agarose column before elution, yields pure receptor, as indicated by gel electrophoresis showing a single protein band coincident with a single peak of 17β-[^{3}H]estradiol bound to the receptor (18).

Polyacrylamide Gel Electrophoresis. Slab gel electrophoresis under nondenaturating and denaturating conditions was performed as reported (18). The final polyacrylamide concentration of gel was 5% (wt/vol).

After electrophoresis, some gel lanes were stained for proteins by the silver stain method (25). Others were cut into 3-mm slices with a Bio-Rad gel slicer apparatus. In some experiments each slice was solubilized by 3 hr of incubation with 1 ml of Soluene (Packard), then added to 3 ml of Beckman MP scintillation liquid, and assayed for radioactivity. In other experiments the ^{32}P-phosphorylated receptor was extracted from the gel.

Extraction of Receptor from Gel Lanes. Slices containing the receptor were rehydrated in 1% $NaDodSO_4$ by boiling for 30 min, and then they were homogenized and extracted with 1 ml of 1% $NaDodSO_4$. Bovine serum albumin was added (0.5 mg/ml) as carrier, and proteins were precipitated with 5 vol of acetone at −17°C (26); the pellets were dried and submitted to acid hydrolysis.

Phospho Amino Acid Analysis. The acid hydrolysis of samples containing ^{32}P-phosphorylated receptor was performed under vacuum in 6 M HCl at 110°C for 3 hr. The samples were then lyophilized and solubilized with 100 μl of H_2O containing 250 μg of each of the following three phospho amino acids: phosphotyrosine, synthesized according to a previous report (27), phosphothreonine, and phosphoserine. Twenty-five-microliter aliquots were submitted to thin-layer electrophoresis at pH 1.9 in formic acid/acetic acid/H_2O, 44:156:1800, 1000 V for 2.5–3 hr. When indicated, this electrophoresis was followed by electrophoresis in the second dimension at pH 1.9 for 30 min. Bidimensional electrophoresis was also performed at pH 1.9, 1000 V for 90 min, followed by a run at pH 3.5 in acetic acid/pyridine/H_2O, 50:5:945, 1000 V for 45 min. The plates were stained with ninhydrin and then exposed for autoradiography with Kodak XAR film at −80°C with a Lanex intensifying screen in a Kodak X-Omat cassette for 90 min.

Inactivation of the 17β-Estradiol Receptor by Incubation with the Nuclear Phosphatase. Aliquots of the purified receptor preparations (7.7–19 pmol of the 17β-estradiol binding sites) in TGD buffer containing 1 μM 17β-[^{3}H]estradiol (6 Ci/mmol) were incubated with 21–79 arbitrary units of purified nuclear phosphatase (21) in a final volume of 1 ml for 20 min at 25°C; 30–70% of the hormone binding sites were inactivated during incubation. These receptor preparations were used as substrate of the receptor kinase to monitor the hormone binding activation and ^{32}P phosphorylation of the receptor.

Hormone Binding Activity of Purified Receptor. Bound estradiol was separated from free hormone by dextran-coated charcoal treatment as described (21). Supernatant (0.2 ml) was added to 3 ml of scintillation liquid and the radioactivity was measured in a Beckman LS-7000 spectrometer with 45% efficiency.

RESULTS

Ca^{2+} and Calmodulin Stimulate the Receptor Kinase Activation of Estradiol Binding Receptor. 17β-[^{3}H]Estradiol receptor was extensively purified (24). Polyacrylamide gel electrophoresis under nondenaturing conditions of the purified receptor produces a single band of protein coincident with a peak of 17β-[^{3}H]estradiol binding (18). The hormone binding of this receptor was partially inactivated by the purified nuclear phosphatase in TGD buffer. ATP and purified receptor kinase were added to the buffer and the activation was monitored during incubation under several conditions.

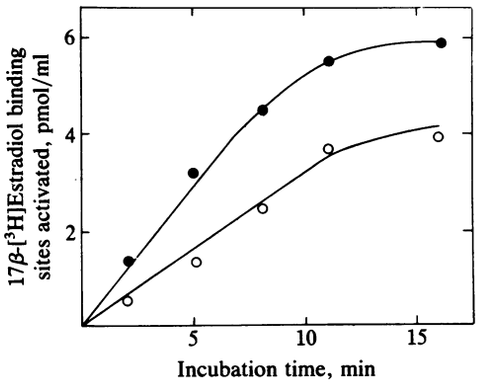

FIG. 1. Effect of Ca^{2+} and calmodulin on time course of hormone binding activation of 17β-estradiol receptor by the kinase. Purified estradiol receptor binding 10 pmol of hormone was partially inactivated by incubation with the nuclear phosphatase; 4.5 pmol of hormone binding sites were still active at the end of the incubation. The receptor was then used as a substrate of the kinase. The binding activation of the receptor was monitored at 15°C in 50 mM Tris·HCl (pH 7.4) containing 2 mM dithiothreitol and 0.2 mM EGTA (TGD buffer) at the indicated times in the presence of 5 mM $MgCl_2$, 10 mM Na_2MoO_4, 0.15 mM ATP, and 41 arbitrary units of purified kinase with (●) or without 1 μM free Ca^{2+} obtained by adding $CaCl_2$ and 0.6 μM calmodulin (○) in a final volume of 1.5 ml. Free Ca^{2+} concentration was calculated by using an apparent binding constant for Ca/EGTA of 7.61×10^6 M^{-1} (28) and verified with a Ca^{2+}-specific electrode (Radiometer). Hormone binding was assayed by treating the receptor sample with dextran-coated charcoal.

The time course of hormone binding activation by the kinase in the absence and in the presence of Ca^{2+} and calmodulin is shown in Fig. 1. The activation is linear for about 8 min and it is stimulated by Ca^{2+} and calmodulin. Added separately, Ca^{2+} and calmodulin have no stimulatory effect on this activation (not shown).

A dose–response curve for calmodulin-stimulated activation of hormone binding is shown in Fig. 2. The half-maximal rate of activation was reached at ≈60 nM calmodulin. The effect of various concentrations of Ca^{2+} on the activation is shown in Fig. 3. Half-maximal stimulation is observed with a

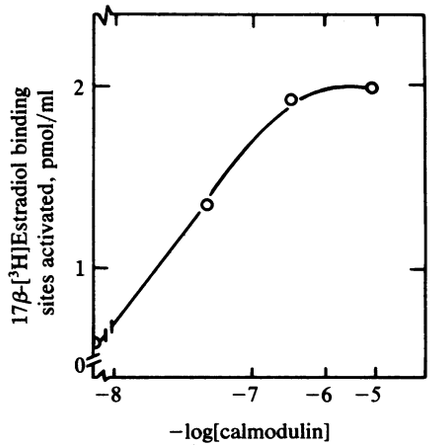

FIG. 2. Dependence of kinase activation of hormone binding of 17β-estradiol receptor on calmodulin. Pure estradiol receptor binding 7.7 pmol of hormone was inactivated by incubation with the nuclear phosphatase and then used as a substrate of the kinase. The binding activation of the receptor was monitored in TGD buffer for 10 min at 15°C, in the presence of 5 mM $MgCl_2$, 10 mM Na_2MoO_4, 0.15 mM ATP, 16 arbitrary units of purified kinase, 1 μM free Ca^{2+} obtained by adding $CaCl_2$, and the indicated amounts of calmodulin in a final volume of 1.5 ml.

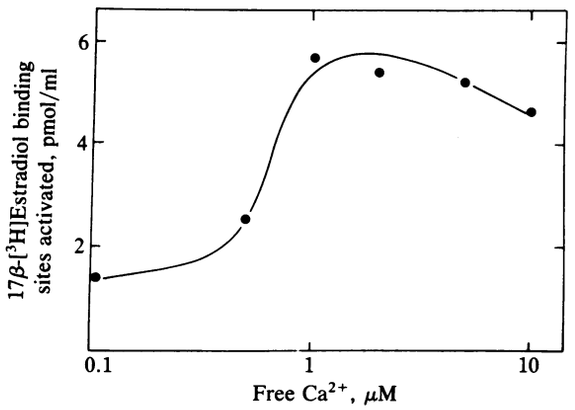

FIG. 3. Dependence of kinase activation of hormone binding of 17β-estradiol receptor on calcium. Pure 17β-estradiol receptor binding 10 pmol of hormone was inactivated by incubation with the nuclear phosphatase and then used as a substrate of the kinase. The binding activation of the receptor was monitored in TGD buffer for 10 min at 15°C in the presence of 5 mM $MgCl_2$, 10 mM Na_2MoO_4, 0.15 mM ATP, 41 arbitrary units of purified kinase, 0.6 μM calmodulin, and the indicated amounts of free Ca^{2+} obtained by adding $CaCl_2$.

free Ca^{2+} concentration of about 0.80 μM. When the activation was measured in the presence of various concentrations of trifluoperazine, a drug known to inhibit calmodulin-sensitive enzymes (29), half-maximal inhibition of Ca^{2+}- and calmodulin-stimulated activation was observed at a concentration of about 7 μM (Fig. 4). A high concentration of trifluoperazine in the absence and in the presence of calmodulin was equally effective in partially inhibiting the activation (Fig. 4). This fact suggests that endogenous calmodulin contamination of the kinase, the phosphatase, or the receptor is not responsible for the activation observed in the absence of exogenous calmodulin. Various concentrations of cAMP and cGMP (from 1 μM to 1 mM) have no effect on the activation of hormone binding by the purified kinase (not shown).

Ca^{2+} and Calmodulin Stimulate Phosphorylation of Estradiol Receptor by the Receptor Kinase. The pure 17β-estradiol receptor, preinactivated by the nuclear phosphatase, incorporates ^{32}P when activated by the kinase in the presence of [γ-^{32}P]ATP (18). The experiments reported in the previous section show that Ca^{2+} and calmodulin stimulate the activation of hormone binding by the kinase. The question now arises as to whether Ca^{2+} and calmodulin stimulate phosphorylation of receptor to the same extent. A pure receptor preparation was inactivated by the nuclear phosphatase, then divided in two portions, and activated by the cytosol kinase by using [γ-^{32}P]ATP with and without Ca^{2+} and calmodulin. After 8 min of incubation calmodulin stimulated the activation of hormone binding 3.28-fold (see legend to Fig. 5). The two samples of activated receptors were submitted to polyacrylamide gel electrophoresis under nondenaturating conditions. A single ^{32}P peak coincident with the 17β-[^{3}H]estradiol bound to the receptor was observed in both cases (Fig. 5), thereby confirming that the receptor is phosphorylated during activation by kinase (18). Data reported in the legend to Fig. 5 show that calmodulin stimulates ^{32}P incorporation into receptor 3.21-fold. Phosphorylation of receptor did not continue after 15 min of incubation with the kinase either with or without calmodulin when activation of 17β-[^{3}H]estradiol binding has reached the plateau (not shown). The excellent correlation between stimulation by

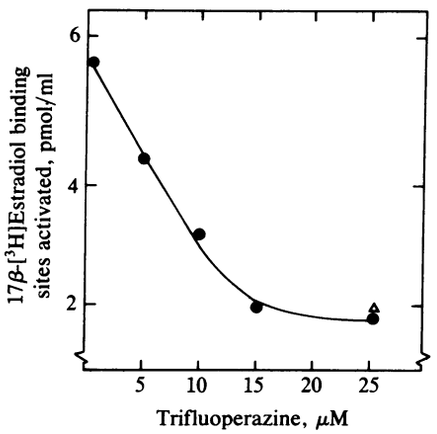

FIG. 4. Effect of trifluoperazine on the receptor kinase activation of 17β-estradiol in the presence of Ca^{2+} and calmodulin. Pure 17β-estradiol receptor binding 15 pmol of hormone was inactivated by incubation with the nuclear phosphatase and used as a substrate of the kinase. The binding activation of the receptor was performed in TGD buffer as described in the legend to Fig. 2 with the difference that 0.6 μM calmodulin, 28 arbitrary units of kinase, and the indicated amounts of trifluoperazine were used (●). In one sample the binding activation was measured in the absence of exogenous calmodulin (△).

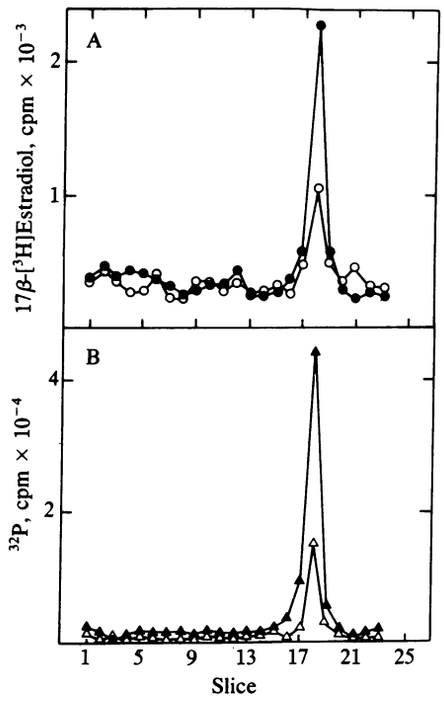

FIG. 5. Phosphorylation of 17β-estradiol receptor in the absence and presence of Ca^{2+} and calmodulin. Pure 17β-estradiol receptor binding 13.6 pmol of hormone was inactivated by incubation with the nuclear phosphatase and used as a substrate of the kinase. The binding activation of the receptor was monitored in TGD buffer under the following conditions: the incubation temperature was 15°C; 5 mM $MgCl_2$, 10 mM Na_2MoO_4, 0.15 mM [γ-^{32}P]ATP (6 Ci/mmol), and 21 arbitrary units of purified kinase were added, with or without 1 μM free Ca^{2+} obtained by adding $CaCl_2$ and 0.6 μM calmodulin in a final volume of 1.5 ml. After 8 min of incubation the activation was 2.17 pmol in the absence and 7.12 pmol in the presence of Ca^{2+} and calmodulin. Seventy-five microliter aliquots of each of the two samples of reactivated receptors were then submitted to slab gel electrophoresis at 4°C after exhaustive dialysis against TGD buffer. After electrophoresis the gel lanes were sliced and each slice was solubilized by Soluene and assayed for ^{3}H (*A*) and ^{32}P (*B*). The incorporation of ^{32}P into receptor was 15,628 cpm in the absence and 50,162 cpm in the presence of Ca^{2+} and calmodulin. Open symbols, sample incubated without Ca^{2+} and calmodulin; closed symbols, sample incubated with Ca^{2+} and calmodulin.

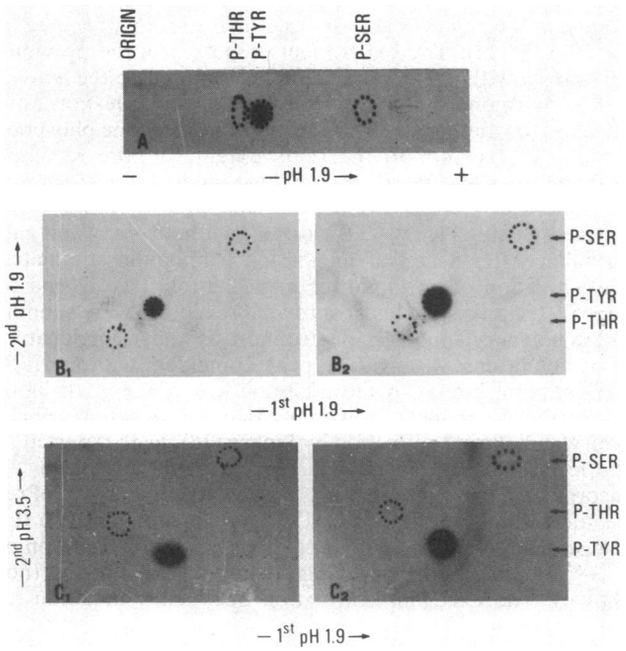

FIG. 6. Phospho amino acid analysis of the phosphorylated 17β-estradiol receptor. Pure 17β-estradiol receptor binding 8.71 pmol of hormone was inactivated by incubation with the nuclear phosphatase and used as a substrate of the kinase. The binding of the receptor was activated during 10 min of incubation in TGD buffer (see legend to Fig. 1) under the following conditions: the incubation temperature was 15°C; 5 mM $MgCl_2$, 10 mM Na_2MoO_4, 0.15 mM [γ-^{32}P]ATP (6 Ci/mmol), and 27 arbitrary units of purified kinase were added, with or without 1 μM free Ca^{2+} obtained by adding $CaCl_2$ and 0.6 μM calmodulin in a final volume of 1.5 ml. The activated samples were dialyzed extensively against TGD buffer; then 150-μl aliquots were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis using three different gel lanes for each aliquot. The receptor was then extracted from gel and finally subjected to acid hydrolysis, lyophilized, and solubilized with 100 μl of H_2O. Twenty-five-microliter samples were applied on cellulose thin-layer plates. Electrophoresis was run at pH 1.9 in one direction (*A*), at pH 1.9 in two directions (*B*), and at pH 1.9 in the first direction and at pH 3.5 in the second direction (*C*). B_1 and C_1 show the electrophoresis of amino acids from receptor activated in the absence and *A*, B_2, and C_2 in the presence of Ca^{2+} and calmodulin. Samples of phosphoserine (P-SER), phosphothreonine (P-THR), and phosphotyrosine (P-TYR) were added to the radioactive samples analyzed. The dotted lines represent the ninhydrin-stained standards superimposed on autoradiography.

calmodulin of both activation of hormone binding and phosphorylation of receptor lends support to the view that phosphorylation is required for 17β-estradiol binding to the receptor.

The Kinase Phosphorylates Tyrosine Residues of Receptor. Two samples of the receptor phosphorylated with [γ-^{32}P]-ATP by the kinase in the absence and in the presence of Ca^{2+} and calmodulin, respectively, were submitted to $NaDodSO_4$ gel electrophoresis according to a previously reported procedure (18). The phosphorylated receptors were then eluted from the gel and submitted to acid hydrolysis followed by one-dimensional electrophoresis at pH 1.9. As previously reported, this electrophoresis separates phosphotyrosine clearly from phosphothreonine and effectively from phosphoserine (30, 31). Phosphotyrosine is the only phospho amino acid detectable in both samples (Fig. 6*A* shows the amino acids from receptor activated in the presence of calmodulin). This result is confirmed by bidimensional electrophoresis at pH 1.9, which gives a better separation of phosphotyrosine from phosphothreonine (Fig. 6*B*) and it is corroborated by a classical bidimensional electrophoresis at pH 1.9 and pH 3.5 (32) shown in Fig. 6*C*. About 75% of the radioactivity applied on the plates was recovered in correspondence of phosphotyrosine.

The analysis of phospho amino acids was also performed on receptor phosphorylated with [γ-^{32}P]ATP and not submitted to gel electrophoresis. The results were identical to those shown in Fig. 6*C*. Hence, it is unlikely that other proteins not included in the gel are phosphorylated by the kinase.

DISCUSSION

All eukaryotic cells studied thus far contain a calcium binding protein, known as "calmodulin" (33). This protein is an intracellular calcium receptor. It binds Ca^{2+} with high affinity and specificity, and many of the effects of calcium on intracellular metabolism are mediated by its binding to this structurally conserved, small protein. Calmodulin affects cyclic nucleotide metabolism via phosphodiesterase and adenylate cyclase, glycogen metabolism via a glycogen synthase kinase and phosphorylase kinase, transport of calcium out of cells via Ca^{2+},Mg^{2+}-ATPase, and other important intracellular processes, including protein phosphorylation (34–36).

Our recent observation that the calf uterine estradiol receptor can be phosphorylated–dephosphorylated *in vitro* by two endogenous enzymes that regulate its hormone binding activity and that, in crude receptor-containing preparations, the receptor phosphorylating enzyme is stimulated by Ca^{2+} (19), led us to investigate whether this stimulation is mediated by calmodulin. A purified system has been used, which presumably does not contain endogenous calmodulin. In this system Ca^{2+} stimulated hormone binding activation of the receptor by the kinase only in the presence of exogenous calmodulin. An antidepressant drug, trifluoperazine, which in the presence of Ca^{2+} binds to calmodulin making it biologically inactive, prevents Ca^{2+} and calmodulin interference with the hormone binding activation of the receptor. Ca^{2+} stimulation of kinase begins at a concentration of about 1 μM. This suggests that *in vivo* Ca^{2+} stimulates the phosphorylation and the hormone binding activity of the estradiol receptor only when its intracellular concentration increases. In fact, in mammalian cells the steady-state concentration of Ca^{2+} in cytosol ranges from 0.01 to 0.1 μM. Cell stimulation may cause a transient increase of Ca^{2+} to 1 μM or higher. At this concentration Ca^{2+} binds to calmodulin and the complex stimulates several enzymic activities (34), including, presumably, the receptor kinase. The finding that estradiol increases uterine uptake of Ca^{2+} (37, 38) suggests the intriguing possibility that estradiol stimulates its binding to its own receptor via increased Ca^{2+} uptake and consequent stimulation of receptor phosphorylation.

The fact that low concentrations of calmodulin activate the kinase suggests that this protein regulates the kinase activity *in vivo*. The interaction calmodulin–kinase could be exploited to further purify the kinase by binding the enzyme to a calmodulin-agarose column as has been reported for the calmodulin-stimulated glycogen synthase kinase (39).

In the presence of ATP, cGMP added to cytosol of human endometrium or cells of endometrial cancer lines increases specific estrogen binding sites, whereas addition of cAMP causes a decrease of these sites (40, 41). These findings suggest that the effects of the cyclic nucleotides are related to phosphorylation of the receptor, although under our conditions cyclic nucleotides do not appear to affect the activation of binding sites by the purified receptor kinase. Nevertheless, it cannot be ruled out that cyclic nucleotides affect the receptor kinase activity indirectly—for instance, via a hypothetical protein kinase phosphorylating the receptor kinase.

It has been postulated that a tyrosyl residue at the binding site of 17β-estradiol receptor is essential for hormonal binding (42). In addition, it appears that tyrosine phosphorylation plays a key role in the control of cellular growth. In fact,

tumor virus-encoded transformation proteins such as the avian sarcoma virus-transforming protein, pp 60 src, are phosphorylated on tyrosine (32). Epidermal growth factor receptor and insulin receptor are also phosphorylated on tyrosine residues (26, 43). The data here reported show that also in the case of estradiol receptor the phosphorylated residue is tyrosine and that this phosphorylation is necessary for hormone binding of the receptor. In addition, they show that tyrosine phosphorylation can be regulated by calmodulin. Whereas it has been reported that calmodulin-stimulated kinases phosphorylate proteins on phosphoserine and phosphothreonine (44, 45), calmodulin has now been demonstrated to regulate phosphorylation on tyrosine. Therefore, the 17β-estradiol receptor kinase is apparently a member of a new class of protein kinases—namely, a calmodulin-stimulated tyrosine kinase.

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1. Krebs, E. G. & Beavo, J. A. (1979) *Annu. Rev. Biochem.* **48,** 923–959.
2. Cohen, P. (1982) *Nature (London)* **296,** 613–620.
3. Fewtrell, C., Goetze, A. & Metger, H. (1982) *Biochemistry* **21,** 2004–2010.
4. Gordon, A. S., Davis, C. G., Milfay, D. & Diamond, I. (1977) *Nature (London)* **267,** 539–540.
5. Teichberg, V. I., Sobel, A. & Changeux, J. P. (1977) *Nature (London)* **267,** 540–542.
6. Cohen, S., Carpenter, G. & King, L. (1980) *J. Biol. Chem.* **255,** 4834–4842.
7. Kasuga, M., Karlsson, F. A. & Khan, C. R. (1982) *Science* **215,** 185–187.
8. Munck, A. & Brinck-Johnson, T. (1968) *J. Biol. Chem.* **243,** 5556–5565.
9. Liao, S., Rossini, G. P., Hiipakka, R. A. & Chen, C. (1980) in *Perspectives in Steroid Receptor Research*, ed. Bresciani, F. (Raven, New York), pp. 99–112.
10. Sando, J. J., Hammond, N. D., Stratford, C. A. & Pratt, W. B. (1979) *J. Biol. Chem.* **254,** 4779–4789.
11. Sando, J. J., La Forest, A. C. & Pratt, W. B. (1979) *J. Biol. Chem.* **254,** 4772–4778.
12. Miller, J. B. & Toft, D. O. (1978) *Biochemistry* **17,** 173–177.
13. Moudgil, U. K. & Eessalu, T. E. (1981) *FEBS Lett.* **122,** 189–192.
14. Kalimi, M., Colmen, P. & Feigelson, P. (1975) *J. Biol. Chem.* **250,** 1080–1088.
15. Dougherty, J. J., Puri, R. K. & Toft, D. (1982) *J. Biol. Chem.* **257,** 14226–14230.
16. Weigel, N. L., Tash, J. S., Means, A. R., Schrader, W. T. & O'Malley, B. W. (1981) *Biochem. Biophys. Res. Commun.* **102,** 513–519.
17. Housley, P. R. & Pratt, W. B. (1983) *J. Biol. Chem.* **258,** 4630–4635.
18. Migliaccio, A., Lastoria, S., Moncharmont, B., Rotondi, A. & Auricchio, F. (1982) *Biochem. Biophys. Res. Commun.* **109,** 1002–1010.
19. Auricchio, F., Migliaccio, A., Castoria, G., Lastoria, S. & Schiavone, E. (1981) *Biochem. Biophys. Res. Commun.* **101,** 1171–1178.
20. Auricchio, F. & Migliaccio, A. (1980) *FEBS Lett.* **117,** 224–226.
21. Auricchio, F., Migliaccio, A. & Rotondi, A. (1981) *Biochem. J.* **194,** 569–574.
22. Auricchio, F., Migliaccio, A. & Castoria, G. (1981) *Biochem. J.* **198,** 699–702.
23. Auricchio, F., Migliaccio, A., Castoria, G., Lastoria, S. & Rotondi, A. (1982) *Biochem. Biophys. Res. Commun.* **106,** 149–157.
24. Puca, G. A., Medici, N., Molinari, A. M., Moncharmont, B., Nola, E. & Sica, V. (1980) *J. Steroid Biochem.* **12,** 105–113.
25. Merril, C. R., Coloman, D., Sedman, S. A. & Ebert, M. H. (1981) *Science* **211,** 1437–1438.
26. Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W. & Osathanoudh, R. (1982) *J. Biol. Chem.* **257,** 15162–15166.
27. Mitchell, H. K. & Lunan, K. D. (1964) *Arch. Biochem. Biophys.* **106,** 219–222.
28. Portzell, H., Caldwell, P. C. & Renng, J. C. (1964) *Biochim. Biophys. Acta* **79,** 581–591.
29. Weiss, B. & Levin, R. M. (1979) *Adv. Cyclic Nucleotide Res.* **9,** 285–303.
30. Echart, W., Hutchison, M. A. & Hunter, T. (1979) *Cell* **18,** 925–933.
31. Ramakrishna, S., Pucci, D. L. & Benjamin, W. (1981) *J. Biol. Chem.* **256,** 10213–10216.
32. Hunter, T. & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1311–1315.
33. Means, A. R. & Dedman, J. R. (1980) *Nature (London)* **285,** 73–77.
34. Cheung, W. Y. (1980) *Science* **207,** 19–27.
35. Means, A. R. & Chafouleas, J. G. (1982) *Annu. Rev. Physiol.* **44,** 667–682.
36. Cohen, P. (1982) *Nature (London)* **296,** 613–620.
37. Pietras, R. J. & Szego, C. M. (1975) *Nature (London)* **253,** 357–359.
38. Batra, S. & Sjogren, C. (1983) *Life Sci.* **32,** 315–319.
39. Ahmad, Z., De Paoli-Roach, A. A. & Poach, P. J. (1982) *J. Biol. Chem.* **257,** 8348–8355.
40. Fleming, H., Blumenthal, R. & Gurpide, E. (1982) *Endocrinology* **111,** 1671–1677.
41. Fleming, H., Blumenthal, R. & Gurpide, E. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2486–2490.
42. Puca, G. A. & Bresciani, F. (1970) *Nature (London)* **225,** 1251–1252.
43. Ushiro, H. & Cohen, S. (1980) *J. Biol. Chem.* **255,** 8363–8365.
44. Campbell, K. & McLennan, D. H. (1982) *J. Biol. Chem.* **257,** 1238–1246.
45. Goldenring, J. R., Gonzales, B., McGuire, J. S. & De Lorenzo, R. J. (1983) *J. Biol. Chem.* **258,** 12632–12640.