Calmodulin Enhances the Stability of the Estrogen Receptor*

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The estrogen receptor mediates breast cell proliferation and is the principal target for chemotherapy of breast carcinoma. Previous studies have demonstrated that the estrogen receptor binds to calmodulin-Sepharose in vitro. However, the association of endogenous calmodulin with endogenous estrogen receptors in intact cells has not been reported, and the function of the interaction is obscure. Here we demonstrate by co-immunoprecipitation from MCF-7 human breast epithelial cells that endogenous estrogen receptors bind to endogenous calmodulin. Estradiol treatment of the cells had no significant effect on the interaction. However, incubation of the cells with tamoxifen enhanced by 5–10-fold the association of calmodulin with the estrogen receptor and increased the total cellular content of estrogen receptors by 1.5–2-fold. In contrast, the structurally distinct calmodulin antagonists trifluoperazine and CGS9343B attenuated the interaction between calmodulin and the estrogen receptor and dramatically reduced the number of estrogen receptors in the cell. Neither of these agents altered the amount of estrogen receptor mRNA, suggesting that calmodulin stabilizes the protein. This hypothesis is supported by the observation that, in the presence of Ca\(^{2+}\), calmodulin protected estrogen receptors from in vitro proteolysis by trypsin. Furthermore, overexpression of wild type calmodulin, but not a mutant calmodulin incapable of binding Ca\(^{2+}\), increased the concentration of estrogen receptors in MCF-7 cells, whereas transient expression of a calmodulin inhibitor peptide reduced the estrogen receptor concentration. These data demonstrate that calmodulin binds to the estrogen receptor in intact cells in a Ca\(^{2+}\)-dependent, but estradiol-independent, manner, thereby modulating the stability and the steady state level of estrogen receptors.

Alterations in intracellular free Ca\(^{2+}\) concentrations are frequently translated into cellular events via the Ca\(^{2+}\) sensor protein, calmodulin. Calmodulin, an acidic effector protein that regulates multiple intracellular processes (1), has an essential role in cell proliferation and cell cycle progression (2–4). Inactivation of calmodulin blocks the cell cycle (4, 5), whereas overexpression of calmodulin in a mouse mammary cell line increases the rate of cell division (4). In addition, calmodulin is directly involved in DNA synthesis (2). Interestingly, calmodulin concentrations are increased in several malignancies, including those of the liver (6), lung (7), keratinocytes (8), and breast (9–11). Whether the increased calmodulin contributes to neoplastic transformation or is a consequence of the altered cellular homeostasis that occurs during malignancy has not been ascertained.

It has been reported that calmodulin associates in vitro in a Ca\(^{2+}\)-dependent manner with the estrogen receptor isolated from rat uterus cytosol (12, 13), and that this interaction stimulates binding of the estrogen receptor to the estrogen response element (13). The estrogen receptor, which regulates the expression of specific genes and participates in breast cell proliferation (14), is the principal target for chemotherapy of breast carcinoma (15). Several lines of evidence implicate a role for Ca\(^{2+}\) and calmodulin in mediating the actions of estrogen as follows. (i) Estrogen induces Ca\(^{2+}\) fluxes in breast carcinoma cells (16); (ii) Ca\(^{2+}\)-channel antagonists inhibit the proliferation of breast carcinoma cells (17); (iii) tamoxifen, the primary chemotherapeutic agent used to treat breast cancer, deregulates intracellular Ca\(^{2+}\) homeostasis (18); (iv) sustained increased intranuclear Ca\(^{2+}\) concentrations induce DNA fragmentation and apoptosis of breast cancer cells in a calmodulin-dependent manner (19); (v) calmodulin stimulates tyrosine phosphorylation and activation of the estrogen receptor (20); and (vi) calmodulin is required for the formation of the estrogen receptor-estrogen response element complex and for activation of an estrogen responsive promoter (21). Together these findings suggest that Ca\(^{2+}\)/calmodulin could be a participant in the mitogenic effects of estrogen. Therefore, we set out to examine the physiological significance of the interaction of calmodulin with the estrogen receptor.

EXPERIMENTAL PROCEDURES

Materials—The antibody to calmodulin was described previously (22). The anti-estrogen receptor antibody was obtained from Santa Cruz. Calmodulin-Sepharose was purchased from Amersham Pharmacia Biotech. Affi-Gel was from Bio-Rad. Tissue culture reagents were obtained from Life Technologies, Inc. Fetal bovine serum was from BioWhittaker. Restriction enzymes were purchased from New England Biolabs. Purified human estrogen receptor was from PanVera, Ca\(^{2+}\)-free pig brain calmodulin was obtained from Ocean Biologies, and sequencing grade L-1-tosylamide-2-phenylethyl chloromethyl ketone -treated trypsin was obtained from Promega. All other reagents were of standard analytical grade.

Cell Culture and Lysis—MCF-7 human breast epithelial cells were grown to 80% confluence in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. To examine the effect of estradiol, the medium was replaced with phenol red-free Dulbecco’s modified Eagle’s medium, and the fetal bovine serum was treated with dextran-coated charcoal (23). Where indicated, cells were treated with estradiol, tamoxifen, or the calmodulin antagonists trifluoperazine (TFP) or CGS9343B. The
concentrations and incubation times are indicated in the figure legends. To lyse the cells, the medium was removed, cells were washed three times with phosphate-buffered saline (145 mM NaCl, 12 mM Na₂HPO₄, and 4 mM NaH₂PO₄, pH 7.2) and 1 ml of lysis buffer (50 mM Tris-base, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and pepstatin containing 1 mM CaCl₂ or 1 mM EGTA) was added. The lysates were collected and quick-frozen in methanol/solid CO₂.

**Construction of Plasmids and Transfection**—The mammalian expression vector, POP3, was purchased from Stratagene. An oligonucleotide linker flanked by NcoI and A/P-II-containing XbaI, MnaI, and BpiI cleavage sites was fashioned and inserted into the NcoI and A/P-II sites of POP3. This introduced XbaI, MnaI and BpiI sites in the polylinker region to form POP3-Xb. A purumycin resistance gene was removed from pBabe-puro (24) with BanHI and Clal. The fragment was treated with S1 nuclease to form blunt ends. POP3 was digested with AarII and treated with calf intestinal phosphatase to prevent self-ligation. The purumycin gene was ligated into the AarII site to form POP3-Xb-puro.

The vector containing the cDNA for mammalian calmodulin was obtained from Dr. A. Persechini (University of Rochester, Rochester, NY) and was designated pMZ-xCaM1 (25). To excise the calmodulin cDNA for insertion into POP3-Xb-puro, the PstI site of pMZ-xCaM1 was changed to an A/P-II site by insertion of an oligonucleotide linker containing PstI and AarII sites flanked by PstI. The vector was digested with NcoI, then treated with Klenow enzyme and deoxynucleotides to form a blunt end, and finally, digested with AarIII. To accommodate the fragment, POP3-Xb-puro was digested with AarIII and XbaI. The fragment was ligated with the vector to form POP3-Xb-puro-mCaM.

The vector containing the cDNA for mutant calmodulin that does not bind Ca²⁺ (CaMΔCa) was a gift from Dr. K. Beckingham (Rice University, Houston, TX) and was designated pRBS2 (26). To excise the calmodulin cDNA for insertion into POP3-Xb-puro, pRBS2 was digested with BpiI, then treated with S1 nuclease to form a blunt end, and finally, digested with XbaI. To accommodate the fragment, POP3-Xb-puro was digested with BpiI, treated with S1 nuclease to form a blunt end, and then digested with XbaI. The fragment was ligated with the vector to form POP3-Xb-puro-CaMΔCa.

A synthetic gene encoding the calmodulin binding sequence of myosin light chain kinase inserted in the eukaryotic expression vector pSVL (27) was kindly provided by Dr. John Dedman (University of Cincinnati, Cincinnati, OH). Transient transfection of this construct into mammalian cells neutralizes calmodulin function (27).

Vectors were transiently transfected into MCF-7 cells by the calcium phosphate precipitation method. After 6 h in the medium with 10% fetal bovine serum. Unless indicated otherwise, cells were lysed 48 h post-transfection.

**Calmodulin-Sepharose Chromatography**—Cell lysates, normalized for total protein, were incubated for 2 h at 4 °C with 2 ml of calmodulin-Sepharose in the presence of 1 mM CaCl₂ or 1 mM EGTA. Samples were washed five times in lysis buffer containing 1 mM CaCl₂ or 1 mM EGTA, resolved by SDS-PAGE, and immunoblotting was performed as described below.

**Immunoprecipitation and Immunoblotting**—Equal amounts of protein lysate were immunoprecipitated with anti-calmodulin monoclonal antibody linked to Affi-Gel as described previously (28). Samples were washed five times in lysis buffer, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane. Immunoblots were probed with anti-estrogen receptor or anti-calmodulin antibody. Complexes were visualized with a horseradish peroxidase-conjugated secondary antibody and detected by enhanced chemiluminescence.

For immunoprecipitation of pure proteins, 1 μg of estrogen receptor was incubated with 1 μM tamoxifen or an equal volume of vehicle at 4 °C. After 30 min, 5 μg of calmodulin was added in lysis buffer (final volume of 300 μl) containing 1 mM CaCl₂ or 1 mM EGTA, and incubation was continued for 30 min. Samples were immunoprecipitated with anti-calmodulin antibody and processed by Western blotting as described above.

**In Vitro Proteolysis of the Estrogen Receptor**—Pure estrogen receptor (8 μg) was incubated at 4 °C with 10.5 μg calmodulin (5-fold molar excess) or 10.5 μg of myoglobin in 125 μl of 50 mM Tris, pH 7.6, containing 1 mM CaCl₂ or 1 mM EGTA. After 20 min, 1,1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (1:100 trypsin:estrogen receptor, w/w) was added, and samples were incubated at 25 °C. At the time points indicated in the figure legend, 50-μl aliquots were removed from the reaction mixture and immediately added to boiling SDS. Samples were resolved by 12% SDS-PAGE, and proteins were detected with Coomassie Blue R-250. The extent of digestion, expressed relative to undigested protein, was quantified by laser-scanning densitometry from Coomassie Blue-stained gels.

**Northern RNA analysis—Total RNA was isolated from MCF-7 cells with RNAzol B (Teltest, Inc.). Two micrograms of RNA was mixed with denaturing buffer (60% deionized formamide, 7% formaldehyde, 1 × borate buffer, 6% glycerol, 0.02% bromphenol blue, and 0.02% xylene cyanol), resolved on a 1% agarose, 3% formaldehyde gel in borate buffer (5 mM boric acid, 5 mM sodium borate, 10 mM sodium sulfate, and 1 mM EDTA, pH 8.0) and transferred onto a nitrocellulose membrane, and cross-linked with UV light. The estrogen receptor cDNA (kindly provided by Dr. P. Yen, National Institutes of Health) was removed from pcDNA3 with EcoRI and purified by low melt agarose. Approximately 20 ng of estrogen receptor cDNA was labeled with [32P]dCTP using a random primer labeling kit obtained from Life Technologies, Inc. Northern blots were probed with radiolabeled cDNA, and estrogen receptor mRNA was identified by autoradiography.

**RESULTS**

**Binding of Estrogen Receptors to Calmodulin**—The interaction of calmodulin with the estrogen receptor was initially characterized in MCF-7 breast epithelial cells by calmodulin-Sepharose chromatography. This approach demonstrated that calmodulin binds to the estrogen receptor (Fig. 1B), corroborating previous observations with cytosol from rat uterus (12, 13). Binding of the estrogen receptor to calmodulin-Sepharose was abrogated when Ca²⁺ was chelated with EGTA (data not shown). To evaluate the effect of estradiol and tamoxifen on the association of calmodulin with the estrogen receptor, we first examined the level of estrogen receptors in cells incubated with each agent (Fig. 1A). Estradiol alone decreased the concentration of estrogen receptors by 50%, whereas tamoxifen increased the amount of estrogen receptor in the cell lysate by 1.5–2-fold, inclusion of estradiol during cell culture prevented the augmentation by tamoxifen (Fig. 1A). Analysis by calmodulin-Sepharose chromatography revealed that estradiol treatment did not significantly alter the binding of estrogen receptors to calmodulin (Fig. 1B). In contrast, 1 μM tamoxifen enhanced binding of the estrogen receptor to calmodulin by 5–10-fold (Fig. 1B). The enhanced binding induced by tamoxifen is substantially greater than the 1.5–2-fold increase in estrogen receptor content produced by tamoxifen. When cells were treated with both tamoxifen and estradiol, the increased binding of estrogen receptors to calmodulin produced by tamoxifen was attenuated (Fig. 1B). Under the conditions tested, neither estradiol (data not shown) nor tamoxifen (Fig. 2) influenced the amount of calmodulin in the cells nor the amount of calmodulin immunoprecipitated.

**Co-immunoprecipitation of Estrogen Receptors with Calmodulin and the Effect of Tamoxifen on the Interaction**—Although the in vitro interaction of estrogen receptors with calmodulin-Sepharose has been described (12, 13), the binding of estrogen receptors to endogenous calmodulin in intact cells has not been previously reported. Binding of endogenous calmodulin to the estrogen receptor was revealed by co-immunoprecipitation using anti-calmodulin monoclonal antibody (Fig. 1C). Analogous to the data obtained with calmodulin-Sepharose, co-immunoprecipitation demonstrated that estradiol did not significantly alter the interaction between the estrogen receptor and calmodulin. Although fewer estrogen receptors co-immunoprecipitated with calmodulin in cells stimulated with estradiol than in control cells (Fig. 1C, compare lanes 1 and 2), the magnitude of
indicated. Data are representative of two independent experimental determinations. A, MCF-7 cells, treated for 16 h with either vehicle, 10 nM estradiol \( (E_2) \), 1 \( \mu \)M tamoxifen \( (Tam) \), or both agents, were washed three times as described under "Experimental Procedures." A, cells were lysed, and equal amounts of protein were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with anti-estrogen receptor antibody. The positions of migration of the estrogen receptor \( (ER) \) is indicated. B, equal amounts of protein from MCF-7 cell lysates were incubated with calmodulin \( (CaM) \)-Sepharose in the presence of 1 mM CaCl\(_2\). After washing the beads, proteins were resolved by SDS-PAGE and processed as described above. C, equal amounts of protein were immunoprecipitated \( (IP) \) with anti-calmodulin antibody \( (\alpha CaM) \), described under "Experimental Procedures." Samples were processed as described under B above. The position of migration of the estrogen receptor \( (ER) \) is indicated. Data are representative of at least two independent experimental determinations. D, pure estrogen receptor was incubated with 1 \( \mu \)M calmodulin or vehicle. After 30 min, 5 \( \mu \)g of calmodulin was added in the presence of 1 mM Ca\(^{2+}\) or 1 mM EGTA. Samples were immunoprecipitated with anti-calmodulin antibody and processed as described under B above.

the decrease was similar to that produced by estradiol in cell lysates (Fig. 1A). These data indicate that estradiol does not significantly modulate the binding of the estrogen receptor to calmodulin. By contrast, tamoxifen substantially enhanced in a dose-dependent manner the interaction between calmodulin and the estrogen receptor (Fig. 2A). The maximum increase was observed at 0.25–0.5 \( \mu \)M tamoxifen. Although tamoxifen also increased the total amount of estrogen receptor in the cells, the magnitude of the increase was substantially less than the magnitude of the increase in the amount of estrogen receptor that co-immunoprecipitated with calmodulin (compare Figs. 2, A and B). Moreover, the maximum increase in total estrogen receptors was detected at 0.5–1 \( \mu \)M tamoxifen, a concentration slightly higher than that at which maximum binding was observed. Neither the total amount of calmodulin in the cell nor the amount of calmodulin immunoprecipitated was significantly altered by treatment (Fig. 2).

To verify that the tamoxifen-induced augmentation of the interaction between calmodulin and the estrogen receptor is direct, \textit{in vitro} analysis with pure proteins was performed. Pure estrogen receptors were preincubated with tamoxifen or vehicle, followed by incubation with calmodulin. The amount of estrogen receptor that co-immunoprecipitated with calmodulin was increased approximately 2-fold by tamoxifen (Fig. 1D). Chelation of Ca\(^{2+}\) with EGTA revealed that the binding of estrogen receptors to calmodulin is Ca\(^{2+}\)-dependent (Fig. 1D).

**Effect of Calmodulin Antagonists on the Interaction between Estrogen Receptors and Calmodulin—**To examine the function of the interaction of calmodulin with the estrogen receptor, cells were incubated with two structurally distinct, cell-permeable calmodulin antagonists, TFP (29) or CGS9343B (30). Exposure to 10 \( \mu \)M TFP or 40 \( \mu \)M CGS9343B for 16 h reduced the total number of estrogen receptors in the cells to 50% of control and virtually undetectable levels, respectively (Fig. 3A). These concentrations of the antagonists had no effect on cell viability.
Calmodulin Stabilizes the Estrogen Receptor

Effect of calmodulin antagonists on the interaction between calmodulin and the estrogen receptor. MCF-7 cells were treated for 30 min with vehicle, 25 μM TFP or 40 μM CGS9343B. Cells were lysed, and equal amounts of protein were immunoprecipitated (IP) with anti-calmodulin monoclonal antibody (αCaM) (panel A) or applied directly to SDS gels (panel B) as described under “Experimental Procedures.” Proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with anti-estrogen receptor or anti-calmodulin antibody. The positions of migration of the estrogen receptor (ER) and calmodulin (CaM) are indicated. Data are representative of three independent experimental determinations. C, the relative amount of estrogen receptor that co-immunoprecipitated with calmodulin in A was quantified. The results represent means ± S.E., relative to control (vehicle), of three independent experimental determinations. *, significantly different from no treatment (p < 0.001).

(data not shown). CGS9343B induced a dose-dependent decrease in the number of estrogen receptors; this reduction was not modulated by estradiol (Fig. 3B). The effect of the calmodulin antagonists was specific for estrogen receptors as they did not alter the amount of cellular IQGAP1 (data not shown), a major calmodulin-binding protein in breast epithelial cells (28).

To examine the effects of the antagonists on the interaction between calmodulin and the estrogen receptor, cells were incubated with each calmodulin antagonist for a short time period. Treatment of MCF-7 cells with TFP or CGS9343B for 30 min altered neither the total amount of estrogen receptor nor of calmodulin in the cell lysate (Fig. 4B). By contrast, the amount of estrogen receptor bound to calmodulin was decreased by both TFP and CGS9343B (Fig. 4A). Compared with vehicle, the relative amount of estrogen receptor that co-immunoprecipitated with calmodulin was 0.42 ± 0.04 and 0.38 ± 0.04 (mean ± S.E., n = 3; p < 0.001) for TFP and CGS9343B, respectively (Fig. 4C).

Effect of Calmodulin Antagonists on Estrogen Receptor Gene Expression—Together, our data demonstrate that stimulation and inhibition of the interaction of calmodulin with the estrogen receptor increases and reduces the total cellular content of estrogen receptors, respectively. These results indicate that calmodulin influences either the expression or stability of the estrogen receptor. To discriminate between these two possibilities, Northern blot analysis was compared among MCF-7 cells treated for 16 h with vehicle, tamoxifen, or CGS9343B (Fig. 5). Neither compound significantly altered the amount of estrogen receptor mRNA, indicating that calmodulin does not modulate estrogen receptor gene expression. Thus, calmodulin binding most likely stabilizes the estrogen receptor against proteolysis. This hypothesis was evaluated both in vitro with purified proteins and in intact cells.

Effect of Calmodulin on Proteolysis of the Estrogen Receptor in Vitro—In vitro proteolysis revealed that the estrogen receptor was very sensitive to digestion by 1-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin. Incubation at 37 °C (1:7 trypsin:estrogen receptor, w/w) resulted in complete disappearance of the full-length receptor within 1 min (data not shown). Analysis at 25 °C with a very low ratio of trypsin to estrogen receptor (1:100, w/w) was required to detect limited proteolysis (Fig. 6). Because Ca\(^{2+}\) regulates the binding of calmodulin to the estrogen receptor (see Fig. 1D), the effect of calmodulin on proteolysis of the estrogen receptor was performed both in the presence and absence of Ca\(^{2+}\). In the presence of Ca\(^{2+}\), calmodulin reduced the susceptibility of the estrogen receptor to proteolysis by trypsin. The rates of disappearance of intact estrogen receptor and the appearance of degradation products were substantially reduced by Ca\(^{2+}\)/calmodulin (Fig. 6A). Myoglobin, which has a molecular mass close to that of calmodulin, was used as a control protein. Chelation of Ca\(^{2+}\), which substantially decreases the activity of trypsin (31), markedly slowed the rate of digestion of the estrogen receptor (compare the samples with myoglobin in Figs. 6, A and B). In the absence of Ca\(^{2+}\), the protective effect of calmodulin on proteolysis of the estrogen receptor by trypsin was substantially attenuated (Fig. 6B).

Overexpression of Calmodulin or Calmodulin-binding Peptide—To further evaluate the ability of calmodulin to protect the estrogen receptor in intact cells, MCF-7 cells were transiently transfected with a mammalian expression vector directing the expression of wild type calmodulin or a mutant calmodulin that is incapable of binding Ca\(^{2+}\), CaMΔCa (26). Immunoblotting with anti-calmodulin antibodies revealed that the concentration of calmodulin (wild type and mutant) in transfected cells was 1.5–2-fold higher than that in cells transfected with vector alone (Fig. 7A, lower panel). As noted earlier,
the binding of calmodulin to the estrogen receptor is dependent upon Ca\(^{2+}\); CaM\(_{ΔCa}\) should therefore not alter the stability of the estrogen receptor. Probing immunoblots of cell lysates with anti-estrogen receptor antibody demonstrated a 1.69 \(\pm\) 0.27-fold (mean \(\pm\) S.E., \(n = 3\)) increase in the amount of estrogen receptor in cells overexpressing wild type calmodulin (Fig. 7A). By contrast, overexpression of CaM\(_{ΔCa}\) had no effect on the amount of estrogen receptor (Fig. 7A).

Targeted expression of an inhibitor peptide derived from myosin light chain kinase can neutralize the function of calmodulin in the nucleus of transiently transfected mammalian cells (27). Transient transfection of this peptide into MCF-7 cells decreased by \(\sim\)50\% the total amount of estrogen receptor in the cells after 48 h (Fig. 7B). Thus, selectively increasing or decreasing the relative amount of functional calmodulin resulted in a concomitant increase and decrease, respectively, of estrogen receptor levels in the cells.

**DISCUSSION**

Our data clearly document an interaction between endogenous calmodulin and endogenous estrogen receptors in human breast epithelial cells. The binding is regulated by Ca\(^{2+}\), which is required for the interaction. Incubation of MCF-7 cells for 16 h with estradiol or the antiestrogen tamoxifen decreased and increased, respectively, the total amount of estrogen receptor in the cells (see Figs. 1 and 2 and Refs. 32 and 33). Moreover, concentrations of tamoxifen as low as 0.25 \(\mu\)M substantially increased the binding of the estrogen receptor to calmodulin. Tamoxifen acts as a calmodulin antagonist in vitro (34–36) and has been reported to inhibit the binding of the estrogen receptor to calmodulin (12). Our data differ from the last report. Several factors may account for the discrepant results. The studies of Bouhoute and Leclercq (12) were carried out on rat uterine cytosol by adding tamoxifen after cell lysis, at the time of mixing with calmodulin-Sepharose. In our analyses, intact MCF-7 human breast epithelial cells were incubated with tamoxifen, the tamoxifen was removed before the cells were lysed, and calmodulin was immunoprecipitated. This strategy revealed that pretreatment of MCF-7 cells with therapeutic tamoxifen concentrations increased binding of estrogen receptors to calmodulin. Our experimental technique represents the physiological conditions associated with the use of tamoxifen as a chemotherapeutic agent in patients. Moreover, our approach examined interactions between physiologically relevant concentrations of calmodulin and estrogen receptors. Tissue differences are also likely to be relevant. Tamoxifen is
an estrogen receptor antagonist in breast, whereas in the uterus it is an estrogen receptor agonist (37). Thus, it is possible that cofactors for the estrogen receptor in the different cell types may alter the interaction of tamoxifen-bound estrogen receptors with calmodulin.

Interestingly, as was observed with purified proteins (21), estradiol did not alter the binding of estrogen receptors to calmodulin. Thus, estradiol and tamoxifen, both of which bind to the estrogen receptor, produce different effects on the interaction between calmodulin and the estrogen receptor. Although the calmodulin binding domain of the estrogen receptor has not been identified, these apparently discrepant data can be interpreted in the context of recently solved crystal structures (38). The conformation of the human estrogen receptor ligand binding domain bound to tamoxifen is distinct from that bound to estradiol (38). Similar findings were obtained using peptides, leading to the suggestion that different estrogen receptor-ligand complexes could contact different proteins within the cell (39). It seems reasonable to infer from our data that the conformation of tamoxifen-bound estrogen receptor has increased affinity for calmodulin, whereas estrogen does not change the conformation of the calmodulin binding domain of the receptor. Another possible, but less likely, explanation is that tamoxifen alters the conformation of calmodulin, thereby increasing binding to the estrogen receptor.

We also documented that the binding of calmodulin directly affects the stability and therefore the steady state level of estrogen receptors. This finding was demonstrated both in vitro with purified estrogen receptors and in intact cells. Two complementary strategies were adopted to establish this finding in intact cells. In the first, we observed that two cell-permeable calmodulin antagonists, namely CGS9343B and TFP, attenuated calmodulin binding to the estrogen receptor and dramatically reduced the cellular content of estrogen receptors. In particular, CGS9343B lowered in a dose-dependent manner the amount of estrogen receptors in MCF-7 cells to virtually undetectable levels at 40 μM CGS9343B. Calmodulin antagonists are frequently used to evaluate the role of calmodulin in cellular functions (40, 41), but at high concentrations have been reported to alter the functions of other proteins. To minimize nonspecific interactions, we used relatively low concentrations of the antagonists, namely 10 μM TFP and 40 μM CGS9343B. Analysis performed with purified proteins in vitro revealed that TFP had an IC50 of 12.5 μM for inhibition of calmodulin-stimulated cAMP phosphodiesterase, whereas CGS9343B was specific for calmodulin at 1000 μM (30), a concentration 25-fold higher than the highest concentration used in this study. Moreover, the two structurally distinct compounds produced similar effects on estrogen receptor concentrations, further decreasing the likelihood of a nonspecific effect.

To validate the results obtained with the calmodulin antagonists, we employed a second approach. The amount of available intracellular calmodulin was increased and decreased by transient expression of the cDNA for calmodulin or the cDNA of a calmodulin-binding peptide, respectively. Increasing intracellular calmodulin concentrations led to estrogen receptor accumulation in MCF-7 cells, whereas the inhibitor peptide mimicked the effect of the calmodulin antagonists and decreased estrogen receptor content. Consistent with the binding and in vitro proteolysis data, analysis with CaM ATP revealed that Ca2+ binding to calmodulin was required for the protective effect on the estrogen receptor. Interestingly, a direct positive correlation has been reported between the concentrations of calmodulin and estrogen receptors in human breast tumors (42).

The mechanism by which calmodulin protects the estrogen receptor from proteolysis is not known. The interaction of calmodulin with targets frequently results in a conformational change in the target protein (1). Thus, the direct binding to calmodulin probably induces a tertiary conformation of the estrogen receptor that restricts access to its normal proteolytic cleavage sites. This model is supported by the in vitro trypsin digestion data presented in Fig. 6. Additional mechanisms may be operative in intact cells. For example, calmodulin could modulate the interaction between the estrogen receptor and other proteins. In addition to binding DNA hormone response elements, estrogen receptors bind to adaptor proteins that modulate their function (43, 44). These include the steroid receptor coactivator (SRC) family, receptor interacting proteins (RIP), and members of the heat shock protein (hsp) family, including hsp90 (43, 45). Although previously unclear, the role of hsp90 in the regulation of the estrogen receptor appears to be a molecular chaperone effect that may be important for the correct folding of the receptor (46). Interestingly, hsp90 also binds calmodulin (47, 48), and this interaction prevents hsp90 from binding to F-actin (47). Moreover, recent evidence documents that calmodulin facilitates the effects of hsp90 on the dissociation of endothelial nitric-oxide synthase from caveolin-1 (49). It is therefore possible that synergistic interactions between calmodulin and hsp90 could stabilize the estrogen receptor.

The estrogen receptor is involved in breast cell proliferation, and exogenous estrogens have been implicated in the development of breast carcinoma (50). Importantly, the estrogen receptor is the primary target for chemotherapy of breast tumors (15). Therefore, a clear understanding of the function and the regulation of the estrogen receptor is a prerequisite for optimal pharmacotherapy of breast carcinoma. Reports from several groups link calmodulin and the estrogen receptor. For example, binding to calmodulin enhances the affinity of the estrogen receptor for the estrogen response element (13), and calmodulin has a fundamental role in estradiol-regulated gene expression in breast carcinoma cells (21). The concentration of calmodulin in cells is tightly regulated. Overexpression of calmodulin cDNA increases calmodulin mRNA 20–50-fold, whereas calmodulin protein concentrations are only 2–4-fold higher (4, 51). Nevertheless, higher concentrations of calmodulin are present in rapidly growing cells, and increased intracellular concentration of calmodulin have been reported in several malignancies (6–10), including breast carcinoma (9–11). These data, coupled with the findings presented here that calmodulin enhances the stability of the estrogen receptor, suggest that calmodulin may be a component of estrogen-induced cell proliferation. Calmodulin antagonists, which inhibit growth of human breast carcinoma cell lines (52) and augment antiestrogen therapy (53, 54), could be potentially useful in the treatment of breast carcinoma in patients.

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