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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The purpose of this proposal is to evaluate the participation of Ca^{2+} and calmodulin in estrogen receptor (ER) function and tamoxifen resistance. The focus is directed towards the interaction between calmodulin and ER in ER signaling, as well as the possible involvement of Ca^{2+} and calmodulin in tamoxifen resistance. Major findings to date are: (i) calmodulin binds directly to ER and stabilizes the receptor; (ii) calmodulin protects ER from degradation in the proteasome pathway; (iii) calmodulin is necessary for estrogen-stimulated transcriptional activation by ER; (iv) the effect of calmodulin in ER transcriptional activation is independent of its effect on ER stability; and (v) inhibition of calmodulin function in the nucleus, but not at the plasma membrane, eliminates estrogen-induced transcriptional activation by ER. These data reveal that calmodulin is a component of both ER stability and ER transcriptional activity. This information could have potential therapeutic implications in patients with breast cancer.

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INTRODUCTION

The presence of estrogen receptors (ER) in breast tumors has important implications for the choice of therapy and prognosis. A detailed understanding of the mechanism of action of tamoxifen, the most widely used chemotherapeutic agent for breast cancer, is necessary to develop improved chemotherapeutic agents. Calmodulin binds to both ER and tamoxifen. The hypothesis to be evaluated in this proposal is that the Ca^{2+}-mediated binding of calmodulin to ER has an important role in estrogen function and that Ca^{2+} and calmodulin modulate tamoxifen function and resistance. The role of calmodulin in ER function and subcellular location will be examined. In addition, the effect on tamoxifen resistance of modulating intracellular Ca^{2+} and calmodulin concentrations will be assessed. An enhanced understanding of the interaction between calmodulin and ER could ultimately lead to the development of small molecules that specifically modulate the binding of calmodulin to ER.

BODY

Research accomplishments are described according to the Tasks listed in the approved Statement of Work.

Task 1. Determine whether calmodulin modulates ER function

i. Examine the effect of calmodulin on ER stability

We observed that the cell-permeable calmodulin antagonists CGS9343B and trifluoperazine reduced the level of endogenous ER in T47D and ZR-75 cells. In addition, the calmodulin antagonist CGS9343B decreased the amount of transfected ER in ER-negative COS cells. These observations suggest that the stabilizing effect of calmodulin on ER is independent of cell type. The latter data were published in *The Journal of Biological Chemistry*: Li L, Li Z, Sacks DB. Calmodulin regulates the transcriptional activity of estrogen receptors. Selective inhibition of calmodulin function in subcellular compartments. *J Biol Chem* 2003; 278:1195-1200 (reprint appended).

ii. Develop breast epithelial cell lines which have increased or decreased calmodulin levels

MCF-7 cell lines that inducibly increase calmodulin concentrations in the presence of tetracycline were developed. Unfortunately, we have had less success in developing breast epithelial cell lines that inducibly reduce calmodulin expression. Nevertheless, we are continuing with the planned antisense approach. It is generally accepted that it may be necessary to screen up to 20 oligomers before identifying one that functions adequately. In addition, we shall attempt to reduce endogenous calmodulin with the

recently described technique of RNA interference. This approach is complicated by the three genes that encode calmodulin, but we may succeed.

iii. Determine whether ubiquitination and/or heat shock protein are components of calmodulin-regulated ER stability

Initial findings reveal that the proteasome inhibitors lactacystin and MG132 prevent calmodulin antagonists from reducing ER levels. By contrast, the protease inhibitors calpeptin and calpain inhibitor II were ineffective at blocking the reduction in ER produced by calmodulin antagonists Moreover, the calmodulin antagonist CGS9343B appears to increase ubiquitination of ER. The heat shock protein (hsp) 90 inhibitor geldanamycin enhanced the reduction in the amount of ER produced by calmodulin antagonism. Moreover, preliminary analysis suggests that CGS9343B may reduce the binding of ER to hsp90. These findings suggest that synergistic interactions between calmodulin and hsp may stabilize ER. These observations will be investigated further.

iv. Examine the effect of calmodulin on the subcellular location of ER by confocal microscopy

Studies have been initiated to address this task. We have obtained high quality confocal images of endogenous calmodulin and ER in MCF-7 cells. Initial analysis reveals that estrogen increases the amount of ER and calmodulin in the nucleus. By contrast, CGS9343B uniformly reduces ER in all subcellular regions and attenuates estrogen-induced nuclear translocation of ER. Examination of ZR-75 cells and the effects of altering intracellular Ca^{2+} concentrations will be conducted in the second year as planned.

v. Analyze the effect of calmodulin on the function of ER

Because of the very exciting initial findings, considerable effort was directed toward this task. The cell-permeable calmodulin antagonist CGS9343B inhibited estrogen-induced transcriptional activity of ER in MCF-7 cells. The inhibition was dose-dependent, with estrogen stimulation essentially abolished at 40 μM CGS9343B. Virtually identical findings were observed in ER-positive T47D breast epithelial cells and in COS cells transiently transfected with ER. More detailed investigation revealed that the inhibition was independent of the time of estrogen stimulation and, most importantly, independent of the reduction in ER produced by CGS9343B. A complementary strategy to inhibit calmodulin was adopted using transient transfection of an inhibitor peptide derived from the calmodulin-binding domain of myosin light-chain kinase. This peptide (termed CaMBP, for calmodulin-binding peptide) was selectively targeted to the nucleus or plasma membrane. The peptides do not significantly reduce endogenous calmodulin or ER levels. Inhibition of calmodulin function in the nucleus eliminated estrogen-induced transcriptional activation by ER without altering basal transcription. By contrast, neutralizing membrane calmodulin function only slightly attenuated estrogen-stimulated transcriptional activation by ER. These data were published this year in *The Journal of*

Biological Chemistry: Li L, Li Z, Sacks DB. Calmodulin regulates the transcriptional activity of estrogen receptors. Selective inhibition of calmodulin function in subcellular compartments. *J Biol Chem* 2003; 278:1195-1200 (reprint appended).

vi. Analyze the interaction between calmodulin and ER in living cells by fluorescence microscopy

These studies are scheduled to commence in the second year of funding as originally proposed.

Task 2. To evaluate the role of Ca^{2+} and calmodulin in tamoxifen resistance

This task is scheduled to commence in the second year of funding as originally proposed.

KEY RESEARCH ACCOMPLISHMENTS

- created MCF-7 cells that inducibly overexpress calmodulin under control of a tetracycline promotor
- demonstrated that calmodulin antagonists reduce endogenous ER in several ER-positive cell lines and in ER transiently transfected into ER-negative cells
- calmodulin binding reduces the ubiquitination of ER
- calmodulin protects ER from degradation in the proteasome pathway
- calmodulin is required for estrogen-stimulated transcriptional activation by ER
- the effect of calmodulin on ER transcriptional activation is independent of its effect on ER stability
- developed a novel strategy to selectively inhibit calmodulin function in discrete subcellular domains
- inhibition of calmodulin function in the nucleus eliminated estrogen-induced transcriptional activation by ER

REPORTABLE OUTCOMES

- 1 manuscript published (appended): Li L, Li Z, Sacks DB. Calmodulin regulates the transcriptional activity of estrogen receptors. Selective inhibition of calmodulin function in subcellular compartments. *J Biol Chem* 2003; 278:1195-1200

- 1 abstract published (appended): Li L, Li Z, Sacks D. Calmodulin regulates the transcriptional activity of estrogen receptors. *Proceedings of the American Association for Cancer Research* 2003; 44:3194

- 1 presentation to be given in April 2003 in Toronto, Canada at the annual meeting of the American Association for Cancer Research

CONCLUSIONS

The work performed to date has yielded some insights into the role of calmodulin in ER function. We observed that cell-permeable calmodulin antagonists reduce endogenous ER in several ER-positive breast epithelial cell lines. In addition, calmodulin antagonists reduced the stability of ER transfected into ER-negative cell lines, suggesting that calmodulin stabilization of ER is independent of cell type. Initial findings support the hypothesis that calmodulin reduces the ubiquitination and degradation of ER by the proteasome pathway. Moreover, inhibition of calmodulin function prevented estrogen-induced transcriptional activation by ER. The interaction of calmodulin with ER in the nucleus appears necessary for estrogen-stimulated transcriptional activation.

Collectively these findings implicate calmodulin in several aspects of ER function, including ER stability and ER transcriptional activity. This information could potentially lead to the development of small molecules that significantly modulate the binding of calmodulin to ER, with possible therapeutic implications in breast cancer.

REFERENCES

1. Li L, Li Z, Sacks DB. Calmodulin regulates the transcriptional activity of estrogen receptors. Selective inhibition of calmodulin function in subcellular compartments. *J Biol Chem* 2003; 278:1195-1200

2. Li L, Li Z, Sacks D. Calmodulin regulates the transcriptional activity of estrogen receptors. *Proceedings of the American Association for Cancer Research* 2003; 44:3194.

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Calmodulin Regulates the Transcriptional Activity of Estrogen Receptors

SELECTIVE INHIBITION OF CALMODULIN FUNCTION IN SUBCELLULAR COMPARTMENTS*

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The steroid hormone estrogen elicits biological effects in cells by binding to and activating the estrogen receptor (ER). Estrogen binding induces a conformational change in the receptor, inducing nuclear translocation and transcriptional activation of ER. The ubiquitous Ca^{2+}-binding protein calmodulin has been shown to interact directly with ER and enhance its stability. To further elucidate the functional sequelae of the association between calmodulin and ER, we examined the effect on ER transcriptional activation of specifically inhibiting calmodulin. The cell-permeable calmodulin antagonist CGS9343B prevented estrogen-induced transcriptional activation by ER, without altering basal transcription. The inhibition was dose-dependent and independent of the time of estrogen stimulation. To validate these findings, calmodulin function was also neutralized by targeted expression of a specific inhibitor peptide. By inserting localization signals, the inhibitor peptide was selectively targeted to different subcellular domains. Inactivation of calmodulin function in the nucleus virtually eliminated estrogen-stimulated ER transcriptional activation. By contrast, when membrane calmodulin was specifically neutralized, estrogen-stimulated transcriptional activation by ER was only slightly attenuated. Importantly, the inhibitor peptides did not significantly reduce the amount of ER in the cells. Together, these data demonstrate that calmodulin is a fundamental component of ER transcriptional activation.

The classic steroid hormone estrogen promotes the proliferation of both normal and malignant breast epithelial cells and shortens the cell cycle. Estrogen mediates its biological effects in cells through the estrogen receptor (ER),[1] a member of the nuclear receptor family of ligand-dependent transcription factors (reviewed in Refs. 1 and 2). Analogous to other steroid hormone receptors, ER is an intracellular transcription factor composed of six domains. Estrogen binding to the C-terminal hormone-binding domain induces conformational changes in ER, thereby promoting its dimerization and nuclear localization. The DNA-binding domain of the activated ER binds to DNA sequences, termed estrogen response elements, found in the regulatory regions of target genes. Several factors, including coactivators, corepressors, and integrator proteins, are important in ER-mediated transcription (reviewed in Refs. 3 and 4). It is becoming apparent that transcriptional regulation requires the recruitment by ER of multiple, distinct proteins that cooperate to achieve the required response (3). These factors can alter the magnitude of cellular responses to estrogen. There are yet additional factors that modulate ER function. For example, ER interacts with members of the heat-shock protein family (1), and dissociation of heat-shock protein seems to be necessary for ER to activate transcription. One of the major roles of ligand binding is to change the nature of protein-protein interactions between steroid receptors and other proteins (2). Conversely, other proteins can alter the state of ER independent of ligand binding. For example, phosphorylation of ER by several protein kinases, including a calmodulin-stimulated kinase, modulates ER transcriptional activation (5).

Calmodulin, a ubiquitous modulator of Ca^{2+} signaling (6), regulates the function of multiple, diverse proteins (7, 8). A substantial body of evidence supports a role for Ca^{2+} and calmodulin in estrogen action (Ref. 9, and references therein). For example, calmodulin binds to ER in a Ca^{2+}-dependent manner (9, 10) and is required for formation of the ER-estrogen response element complex (11). In addition, calmodulin stimulates 17-β-estradiol (E_2) binding to ER, inducing tyrosine phosphorylation and activation of the ER (12). Recent evidence from our laboratory indicates that endogenous ER binds to endogenous calmodulin, thereby stabilizing ER (9). Together with the report that calmodulin antagonists inhibit the growth of human breast carcinoma cell lines (13), these finding suggest that Ca^{2+}/calmodulin may participate in ER signaling pathways. Therefore, we set out to examine whether calmodulin modulates the transcriptional activation of ER.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture reagents were purchased from Invitrogen and fetal bovine serum (FBS) was obtained from Biowhittaker. Charcoal-treated FBS was from Cocalico Biologicals, Inc. MCF-7 and T47D breast epithelial cells as well as COS-7 green monkey kidney cells were obtained from the American Type Culture Collection. pcDNA3-CaMBP4-Flag (calmodulin-binding peptide with C-terminal-tagged Flag) was kindly provided by Drs. Marcia Kaetzel, Thomas Freeman, and John Dedman (University of Cincinnati). ERE3-TK-Luc reporter was a generous gift from Dr. Myles Brown (Dana-Farber Cancer Institute). CGS9343B was generously donated by Drs. E. Moret and B. Schmid (Novartis, Basel, Switzerland). Permanox plastic eight-well

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[1] The abbreviations used are: ER, estrogen receptor; E_2, 17-β-estradiol; FBS, fetal bovine serum; Luc, luciferase; PVDF, polyvinylidene difluoride; CaMBP, calmodulin-binding peptide; CaMBP/m, EYFP-CaMBP-Flag membrane expression construct; CaMBP/n, EYFP-CaMBP-Flag nuclear expression construct; EYFP, yellow-green fluorescent variant of the enhanced green fluorescent protein; TK, thymidine kinase.

chamber culture slides were from Nalge Nunc International. FuGENE 6 was purchased from Roche Molecular Biochemicals. Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore Corporation. pEYFP-Mem vector was purchased from Clontech. pRL-TK plasmid was from Promega.

Antibodies—Anti-ERα (Ab-15) antibodies were manufactured by Neomarkers. Anti-ERβ and anti-Flag antibodies were from Upstate Biotechnology. Anti-calmodulin monoclonal antibodies have been characterized previously (14). Anti-green fluorescent protein antibodies and anti-Flag M2 agarose affinity beads were purchased from Clontech and Sigma, respectively. Tetramethyl rhodamine isothiocyanate-labeled goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. Anti-mouse Ig, horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences.

Plasmid Construction—A synthetic gene that encodes the myosin light chain kinase calmodulin-binding sequence was used (15). The Flag-tagged construct, which comprises four tandem calmodulin-binding peptide (CaMBP) repeats, is termed CaMBP4-Flag. The pcDNA3.1-CaMBP4-Flag was used as template. A 356-bp fragment that contains the four CaMBP repeats and a C-terminal-tagged Flag fusion protein was amplified by PCR and *Bsr*G1 sites were designed at both ends for insertion into pEYFP-Mem (Clontech) to produce a membrane-targeted construct. The oligonucleotides used in PCR were 5′-CGCTGTACATC-GAGTCTAGCGCCACCATG-3′ and 3′-CGCTGTACAGGATCCTTATC-ACTTGTCATC-5′. pEYFP-Mem encodes a fusion protein that consists of the N-terminal 20 amino acids of neuromodulin and a yellow-green fluorescent variant of the enhanced green fluorescent protein (EYFP). The neuromodulin fragment contains a signal for post-translational palmitoylation that targets EYFP to membranes. To label CaMBP4-Flag with EYFP, the pEYFP-Mem plasmid was cut with *Bsr*G1, and the CaMBP4-Flag was inserted and ligated with T4 DNA ligase. The construct was named CaMBP/m. Because the CaMBP localizes in the nucleus (15), CaMBP4-Flag was inserted into pEYFP lacking any localization sequences to develop the nuclear targeted construct, termed CaMBP/n. The sequence of all constructs was confirmed by restriction mapping and DNA sequencing. All plasmids were purified using the Qiagen DNA Purification Kit (Qiagen) following the instructions provided by the manufacturer.

Cell Culture and Transfection—MCF-7 and COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS. T47D cells were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS. MCF-7 and T47D cells were plated in 100-mm dishes (for Western blots) or 96-well plates (for measurement of transcription); COS-7 cells were plated in 12-well dishes. DNA was transiently introduced into cells 24 h after plating using FuGENE 6 according to the manufacturer's instructions. When transfecting cells in 100-mm dishes, 4 μg of CaMBP/m, CaMBP/n, or EYFP-Mem vector was used. When measuring transcriptional activity in MCF-7 and T47D cells, transfections were performed in triplicate with 200 ng of total DNA per well, comprising 10 ng of pRL-TK (which encodes *Renilla reniformis* luciferase, used as an internal control for transfection efficiency), 40 ng of ERE3-TK-Luc reporter, and 150 ng of CaMBP/m, CaMBP/n, or EYFP-Mem vector. For COS-7 cells, 0.65 μg of total DNA, containing 0.1 μg of pcDNA3-ERα, 0.5 μg of ERE3-TK-Luc reporter, and 50 ng of pRL-TK, was used. Six hours after transfection, the medium was replaced with phenol red-free culture medium containing 10% charcoal-treated FBS. Twenty-four hours later, E_2 or an equal amount of vehicle was added to the cells in the absence or presence of CGS9343B. Cells were incubated for the times indicated in the figure legends, lysed, and processed as described below.

Luciferase Reporter Assay—Equal numbers of cells were lysed in 50 μl (for 96-well plates) or 200 μl (for 12-well plates) Passive Lysis Buffer (Promega), and luciferase activity was measured using the dual luciferase reporter assay (Promega), essentially as described previously (16). Briefly, light emission from firefly luciferase activity was measured using a 300–650-nm photomultiplier tube in a Turner Design 20/20 DLReady luminometer for 12 s. Stop & Glo reagent was added to quench the firefly luciferase, and *Renilla* (control) luciferase activity in the same sample tube was then measured for an additional 12 s. Firefly luciferase activities were normalized for transfection efficiency to the *Renilla* luciferase internal control. Where indicated, cells were incubated with CGS9343B or an equal volume of ethanol (vehicle). The concentrations and incubation times are indicated in the figure legends. The concentrations of CGS9343B used did not reduce the viability of the cell lines used in this study (data not shown). In addition, E_2 did not enhance the signal in cells transfected with the TK-Luc plasmid lacking estrogen response element (data not shown).

Immunoprecipitation and Immunoblotting—Cells were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 0.1% Triton X-100, 0.1% protease inhibitor mixture (Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma)) and equal amounts of protein lysate were resolved directly by SDS-PAGE or immunoprecipitated with anti-Flag M2 agarose affinity beads. Samples were washed five times in buffer A, resolved by SDS-PAGE, and transferred to PVDF membrane. Immunoblots were probed with anti-ERα, anti-ERβ, anti-calmodulin (14), or anti-green fluorescent protein antibodies. Complexes were visualized with the appropriate horseradish peroxidase-conjugated secondary antibody and developed by enhanced chemiluminescence.

Immunofluorescence Staining—MCF-7 cells, grown on Permanox plastic slides, were transiently transfected with 0.8 μg CaMBP/m, CaMBP/n or EYFP-Mem empty vector using FuGENE 6. After 24 h, slides were processed for immunocytochemisty essentially as described previously (17). Slides were incubated for 1 h with mouse anti-Flag or anti-calmodulin monoclonal antibody, washed four times with phosphate buffered saline (145 mM NaCl, 12 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.2), and then incubated with tetramethyl rhodamine isothiocyanate-labeled goat anti-mouse IgG for 1 h and mounted with Aqua Polymount (Polysciences, Inc.).

Digital micrographs were acquired using a Zeiss Axiovert S100 microscope with the MRC-1024 Confocal Imaging System (Bio-Rad), and were imported into a Dell Power Edge 2200 computer for processing using the Lasersharp 3.0 program (BioRad). Confocal data were converted to TIFF files. Data were obtained from multiple cells in at least three different fields from multiple wells, each from at least two independent experimental determinations.

Miscellaneous—Densitometry of enhanced chemiluminescence signals was performed using the Scion Image software for PC (Scion Corporation). Protein concentrations were determined with the detergent-compatible protein assay (Bio-Rad). Statistical significance was assessed by Student's *t* test using InStat software (GraphPad Software, Inc.). Cell viability was assessed by monitoring exclusion of trypan blue.

RESULTS AND DISCUSSION

Calmodulin Antagonist Reduces ERα Protein Level in MCF-7 Cells—We demonstrated previously that incubation of MCF-7 cells with calmodulin antagonists for 16 h reduced the amount of ERα (9). ERβ was not examined. Therefore, we incubated MCF-7 cells with the cell-permeable calmodulin antagonist CGS9343B (18–20) for different time intervals. Examination of equal amounts of protein lysate by Western blotting revealed that ERα was decreased in a time-dependent manner (Fig. 1*A*). Our prior analysis showed that the reduction in ERα seemed to be caused primarily by calmodulin stabilization of the ERα protein (9). This finding is supported by reverse transcription-PCR, which demonstrated that transcription of the ERα gene is not reduced by CGS9343B (data not shown). In contrast to the reduction in ERα protein levels, CGS9343B had no effect on the amount of ERβ in the cells (Fig. 1*B*). These data are consistent with the recent observation that ERβ does not bind to calmodulin (21). Therefore, all further analyses were restricted to ERα. Note that CGS9343B did not reduce the cell viability in any cell lines examined in this study at the concentrations used in this work (data not shown).

The Calmodulin Antagonist CGS9343B Inhibits the Transcriptional Activity of ER—In addition to its stabilizing effect on ER (9), calmodulin is required for formation of the ER-ERE complex (11). The latter data suggest that calmodulin may modulate transcriptional activation by ER. To examine this hypothesis, MCF-7 cells were transiently transfected with an ER-responsive reporter plasmid and incubated with or without CGS9343B. E_2-stimulated ER transcription in MCF-7 cells by 4–5-fold (Fig. 2). Exposure of cells to 40 μM CGS9343B for 16 h completely eliminated E_2-induced transcription, without altering basal transcription (Fig. 2*A*). More detailed analysis revealed that the inhibition produced by CGS9343B was dose-dependent, with E_2 stimulation essentially abolished at 40 μM CGS9343B (Fig. 2*B*). The abrogation of E_2-stimulated transcription by CGS9343B could be caused by reduction in ER, disruption of the association of calmodulin with ER (9), or another mechanism. To evaluate the first possibility, E_2-stim-

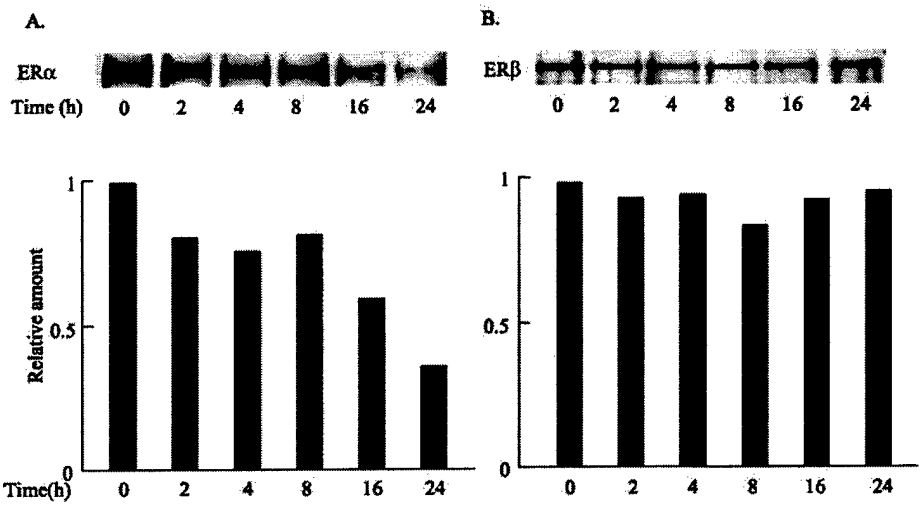

FIG. 1. **Effect of the calmodulin antagonist CGS9343B on ER content.** MCF-7 cells were incubated with 40 μM CGS9343B for the indicated time periods. After lysis, equal amounts of protein were resolved by SDS-PAGE, transferred to PVDF, and membranes were probed for ERα (*A*) or ERβ (*B*). The relative amounts of ERα and ERβ were quantified by densitometry. The results, presented in the *graphs*, are expressed relative to 0 h. A representative experiment is shown.

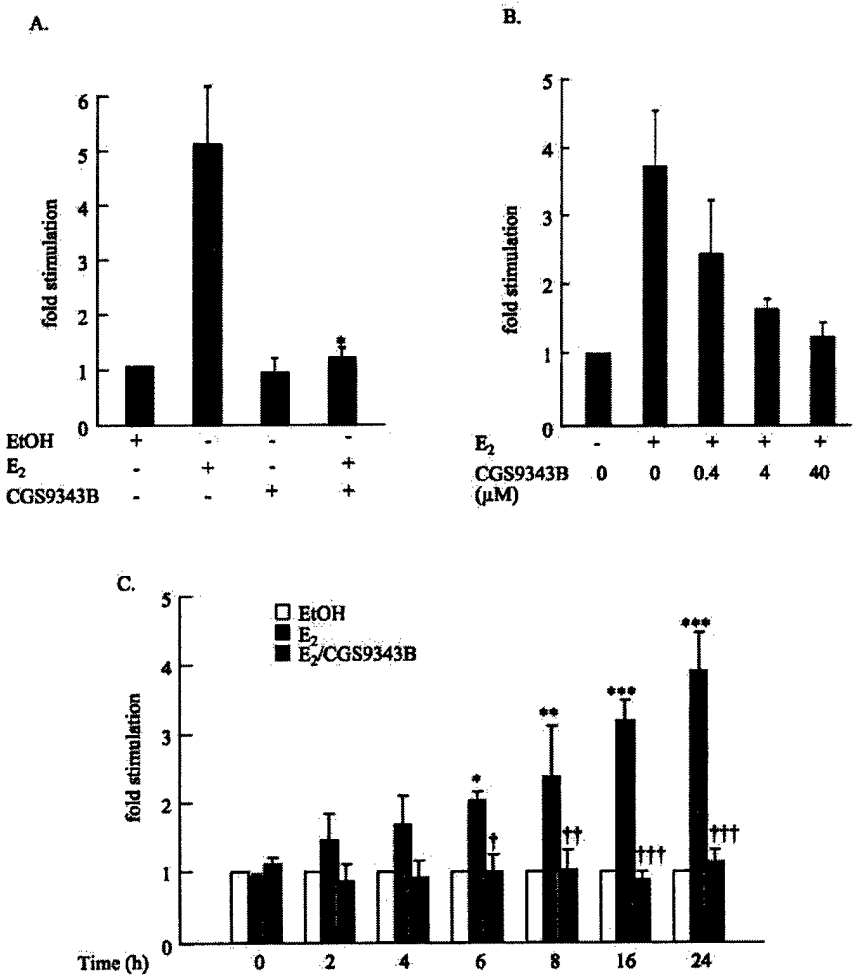

FIG. 2. **E_2-induced transcriptional activity in MCF-7 cells was inhibited by the calmodulin antagonist CGS9343B.** MCF-7 cells were transiently co-transfected with ERE3-TK-Luc and pRL-TK as described under "Experimental Procedures." pRL-TK was used to normalize for transfection efficiency. After lysis, luciferase activity was determined by luminometry. In all cases, lysates were prepared from equivalent numbers of cells. *A*, cells were treated with vehicle (EtOH) or 10 nM E_2 for 16 h in the absence or presence of 40 μM CGS9343B. Results are expressed relative to cells treated with vehicle alone, which was set as 1. *, significantly different from E_2-stimulated ER transcription ($p < 0.05$). *B*, cells were treated as described in *A*, except that the concentration of CGS9343B was varied. *C*, cells were treated with vehicle (EtOH) (*clear bars*) or 10 nM E_2 in the absence (*gray bars*) or presence (*black bars*) of 40 μM CGS9343B for the indicated times. Results are expressed relative to cells treated with vehicle alone, which was set as 1. Significantly different from vehicle: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Significantly different from E_2-stimulated ER transcription: †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$. For all, data are the means of at least three separate experiments, each performed in triplicate. Means ± S.E. are shown.

ulated transcription was examined at different time intervals. E_2-enhanced transcriptional activity in a time-dependent manner (Fig. 2*C*). Neither E_2 nor CGS9343B significantly altered transcription at 0 h. As seen with 16 h of incubation, CGS9343B completely prevented enhancement of transcription by E_2 at all time points (Fig. 2*C*). Note that incubation with CGS9343B for 8 h reduced ERα by only 19% (Fig. 1), far less than its effect on transcription. Together, these results suggest that the absence of E_2-stimulated ER transcription is not caused merely by a reduction in ER; an interaction of calmodulin with ER seems necessary for transcriptional activation.

To confirm the biological relevance of our observations, analy-

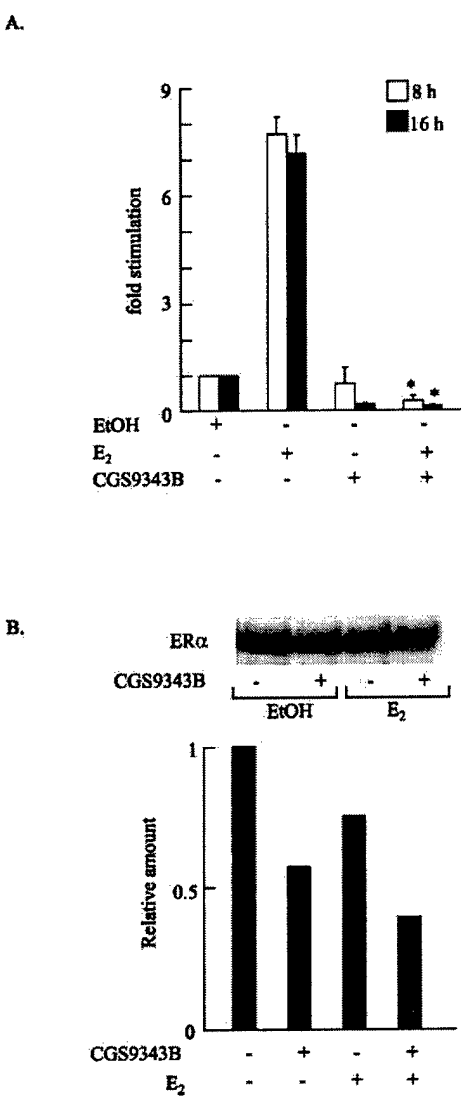

FIG. 3. **CGS9343B inhibited E_2-induced transcriptional activity in T47D cells.** *A*, T47D cells were transiently cotransfected with ERE3-TK-Luc and pRL-TK and lysates were prepared as described in the legend to Fig. 2. Cells were treated with vehicle or 10 nM E_2 for 8 h (*white bars*) or 16 h (*black bars*) in the absence or presence of 40 μM CGS9343B. Results are expressed relative to cells treated with vehicle alone, which was set as 1. *, significantly different from E_2-stimulated ER transcription ($p < 0.001$). Data are from four separate experiments, each performed in triplicate. Means ± S.E. are shown. *B*, T47D cells were treated with 40 μM CGS9343B in the absence or presence of 10 nM E_2 for 16 h. Equal amounts of lysate were resolved by SDS-PAGE, transferred to PVDF, and the blot was probed for ERα. The relative amounts of ERα were quantified by densitometry. The results, presented in the graphs, are expressed relative to vehicle alone. A representative experiment of three separate determinations is shown.

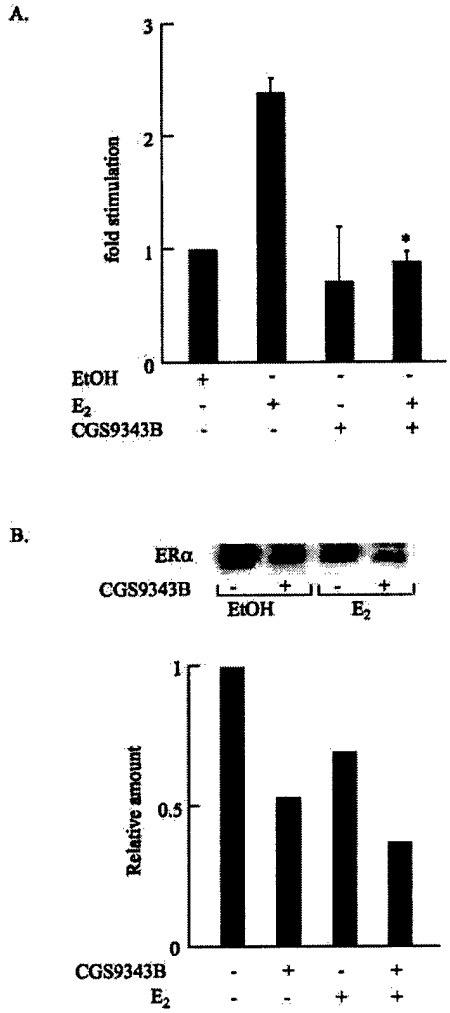

FIG. 4. **CGS9343B inhibited E_2-induced transcriptional activity of transfected ERα.** *A*, COS-7 cells were transiently cotransfected with pcDNA3-ERα, ERE3-TK-Luc, and pRL-TK as described under "Experimental Procedures." 24 h later, cells were treated with vehicle or 10 nM E_2 for 16 h in the absence or presence of 40 μM CGS9343B and subsequently assayed for luciferase activity. Results are expressed relative to cells treated with vehicle alone, which was set as 1. *, significantly different from E_2-stimulated ER transcription ($p < 0.05$). Data are the means of three separate experiments, each performed in duplicate. Means ± S.E. are shown. *B*, aliquots of the lysates used in the transcription assay were cleared by centrifugation and equal amounts of protein were analyzed by Western blotting. The relative amounts of ERα were quantified by densitometry. The results, presented in the graphs, are expressed relative to vehicle alone. A representative experiment of three separate determinations is shown.

sis was performed in T47D cells, another ERα-positive cell line. Analogous to the observations in MCF-7 cells, incubation of T47D cells with 40 μM CGS9343B completely prevented enhancement of transcription by E_2 (Fig. 3*A*). Incubation with CGS9343B for both 8 and 16 h produced essentially identical results. The magnitude of the inhibition of transcription produced by CGS9343B was substantially greater than the extent of the reduction of ERα protein in T47D cells, which was 44–48% (Fig. 3*B*). Note that although E_2 reduced ERα protein, the magnitude of the reduction produced by CGS9343B was independent of E_2. These findings mimic our prior observations in MCF-7 cells (9).

To attempt to eliminate the possibility that the inhibition of transcriptional activity of ER by CGS9343B may have been caused by a decrease in receptor abundance, transfected ERα was also examined. ERα was cotransfected into COS-7 cells with the luciferase reporter gene. Consistent with its effects on endogenous ER, CGS9343B completely inhibited E_2-stimulated transcription of transfected ER (Fig. 4*A*). Although COS-7 cells do not have endogenous ERα or ERβ and might not contain all the components necessary for ER degradation, CGS9343B reduced transfected ERα in COS-7 cells by approximately the same extent as the reduction observed with endogenous ER (Fig. 4*B*).

Development of CaMBPs to Specifically Inhibit Calmodulin in Selected Subcellular Domains—CGS9343B is reported to be a specific antagonist for calmodulin at concentrations up to 1 mM (18), a concentration 25-fold higher than the highest concentration used in this work. Nevertheless, caution should

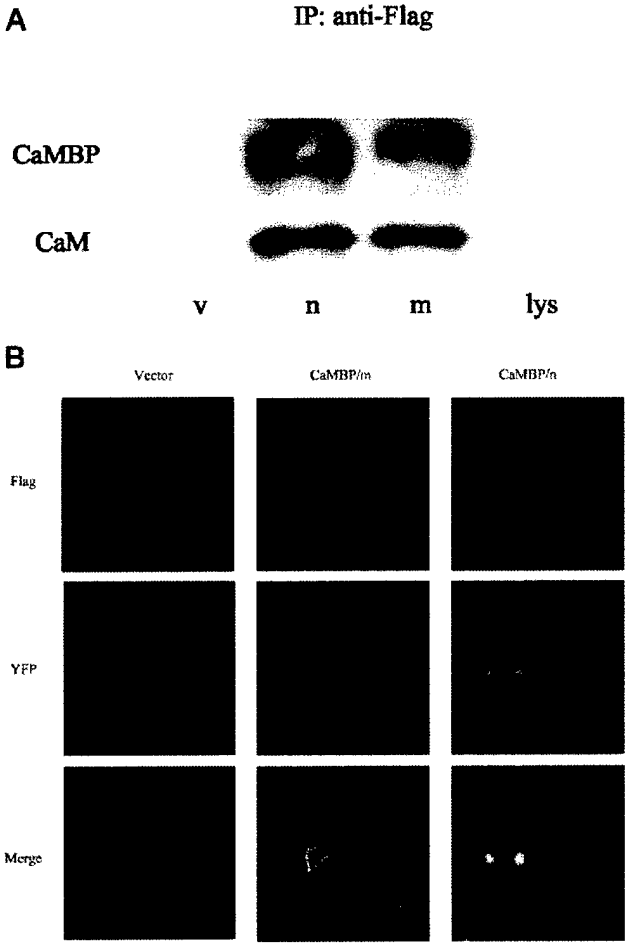

FIG. 5. **Characterization of specific CaMBPs.** *A*, MCF-7 cells were transiently transfected with EYFP-Mem vector alone (*v*), CaMBP/n (*n*), or CaMBP/m (*m*). Mock transfected cell lysate was used as control (*lys*). 48 h after transfection, cells were lysed in buffer containing 1 mM $CaCl_2$ and equal amounts of lysate were immunoprecipitated (*IP*) with anti-Flag affinity gel. Eluted proteins were resolved by SDS-PAGE and transferred to PVDF. The blot was probed for yellow fluorescent protein to identify the CaMBPs (*top*) and calmodulin (*CaM*) (*bottom*). *B*, MCF-7 cells, transfected as described in *A* above, were processed for immunocytochemistry as detailed under "Experimental Procedures." Cells were probed with anti-Flag antibody (*Flag*) and visualized with tetramethyl rhodamine isothiocyanate-labeled secondary antibody, which fluoresces *red* (*top*). Yellow fluorescent protein (*YFP*) is shown in the *center* (*green*). Merged images are presented in the bottom. *Yellow* indicates colocalization. Data are representative of at least three experimental determinations.

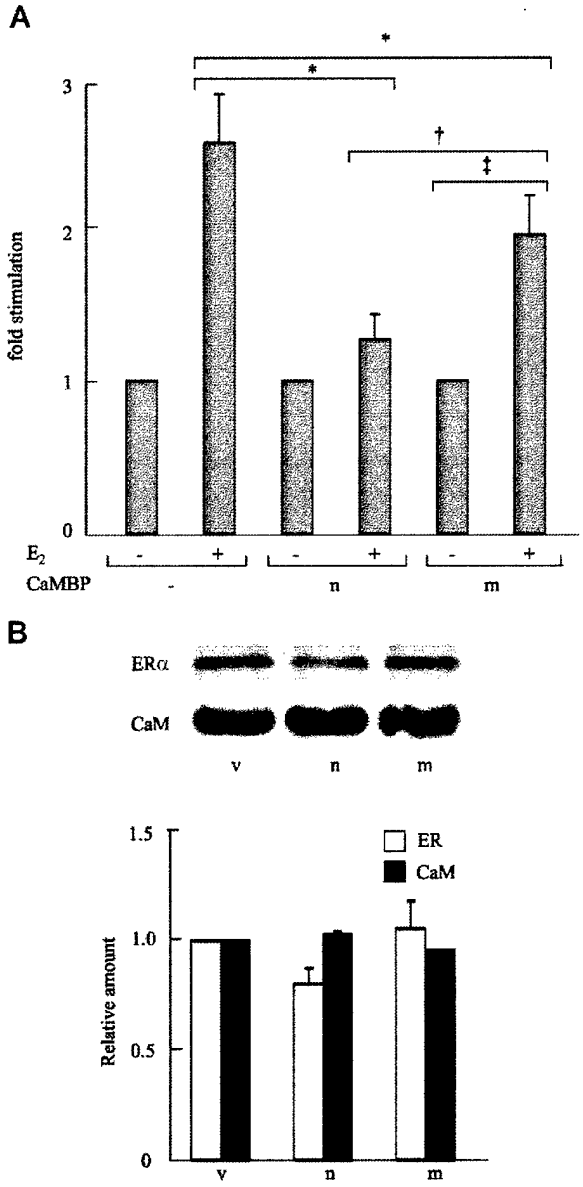

FIG. 6. **CaMBP reduced E_2-induced transcriptional activity without altering the amount of ER or calmodulin.** *A*, MCF-7 cells were transiently transfected with EYFP-Mem vector alone (−), CaMBP/n (*n*), or CaMBP/m (*m*), and cotransfected with ERE3-TK-Luc and pRL-TK. Cells were treated with vehicle or 10 nM E_2 for 16 h and assayed for luciferase activity as described under "Experimental Procedures." Results are expressed as fold stimulation produced by E_2 relative to vehicle control. Data represent the means ± S.E. of at least six separate experiments, each performed in triplicate. *, significantly different from vector ($p < 0.05$); †, significantly different from CaMBP/n ($p < 0.05$); ‡, significantly different from vehicle ($p < 0.05$). *B*, MCF-7 cells were transiently transfected with EYFP-Mem vector alone (*v*), CaMBP/n (*n*), or CaMBP/m (*m*) and lysed 48 h later. Equal amounts of protein were resolved by SDS-PAGE, transferred to PVDF, and probed for ERα and calmodulin. The positions of migration of ERα and calmodulin (*CaM*) are indicated. The relative amounts of ER and calmodulin were quantified by densitometry. The results, presented in the *graphs*, are expressed relative to vector and represent the mean and range of two independent experimental determinations. The error in the last calmodulin *bar* is too small to be visible.

always be exercised in interpreting results obtained with antagonists. Therefore, we adopted a complementary strategy to inhibit calmodulin. Transient transfection of an inhibitor peptide derived from muscle myosin light-chain kinase into mammalian cells blocks calmodulin function (15). The CaMBP was tagged with Flag and EYFP. To discriminate between the interaction of calmodulin and ER in the nucleus with the interaction in the plasma membrane, the EYFP-CaMBP-Flag construct was selectively targeted to subcellular regions. The constructs are termed CaMBP/m and CaMBP/n for membrane- and nuclear-targeted versions, respectively. The peptides were characterized before evaluation in ER transcription assays. To verify calmodulin binding, Flag-tagged CaMBP/m and CaMBP/n were transfected into cells and lysates were immunoprecipitated with anti-Flag affinity gel. Probing the resultant Western blots for calmodulin demonstrated that both CaMBP/n and CaMBP/m specifically bind endogenous calmodulin, with essentially the same affinity (Fig. 5*A*). Probing the immunoprecipitates for yellow fluorescent protein revealed that equal amounts of CaMBP are present (Fig. 5*A*). The EYFP-Mem vector is not seen on the blot (Fig. 5*A*, *top*) because it lacks Flag, but it was present in the lysates (data not shown).

The subcellular localization of CaMBP/m and CaMBP/n in MCF-7 cells was assessed by immunocytochemistry. EYFP-Mem vector (containing yellow fluorescent protein and the membrane-targeting sequence) was expressed at the plasma and intracellular membranes (Fig. 5*B*, *left*). CaMBP/m had a distribution virtually identical to that of the vector alone; it was expressed both at the plasma membrane and in the cytoplasm (Fig. 5*B*, *center*). By contrast, CaMBP/n was expressed almost exclusively in the nucleus (Fig. 5*B*, *right*). The merged images verify that the EYFP plasmids express the Flag-tagged peptides.

CaMBPs Attenuate ER Transcriptional Activation—We next examined the effect on ER transcription of neutralizing calmodulin function in different subcellular domains. As shown in Fig. 6*A*, transient transfection into MCF-7 cells of CaMBP/n (which neutralizes nuclear calmodulin) eliminated E_2-induced ER transcriptional activation. Inhibiting calmodulin function in the extranuclear regions of the cell with CaMBP/m had a much less dramatic effect. When membrane calmodulin function was neutralized, E_2 readily increased ER transcriptional activation, reaching a level only 24% below that attained in vector-transfected cells (Fig. 6*A*). Neither CaMBP/m nor CaMBP/n significantly altered basal ER transcriptional activity (data not shown). Importantly, in contrast to the reduction in ERα produced by CGS9343B, neither CaMBP/n nor CaMBP/m significantly changed the amount of ERα in MCF-7 cells (Fig. 6*B*). Similarly, the CaMBPs had no effect on the amount of calmodulin. Therefore, these data indicate that the effect of calmodulin in ER transcriptional activation is independent of its effect on ER stability.

Our results suggest that by blocking nuclear calmodulin function, CaMBP/n reduces transcriptional activation by ER. The attenuation of ER transcription by inhibiting the association of calmodulin with ER at the membrane was less anticipated. The mechanism is unknown. A membrane ER has been demonstrated, but this receptor is not believed to induce transcription (22), making it unlikely that this could account for the effect of CaMBP/m. Although CaMBP/m did not reduce total ER, the amount of ER in the nucleus could be lower. Alternatively, CaMBP/m could alter the cellular distribution of calmodulin, reducing the amount of nuclear calmodulin; this could decrease ER transcriptional activation. Studies are underway to identify the mechanism.

During the preparation of this manuscript, Pedrero *et al.* (21) showed that the calmodulin antagonist W7 reduced by 74% E_2-stimulated ER transcription in breast epithelial cells. However, no evidence was presented in that study that the inhibition of transcription was independent of the reduction in ER protein produced by calmodulin antagonists. Moreover, W7 lacks specificity and inhibits calmodulin-independent enzymes, such as protein kinase A and protein kinase C (23). Our study is not subject to these caveats. We inhibited calmodulin function by two independent strategies, namely with CGS9343B, believed to be a specific calmodulin antagonist (18), and a specific calmodulin target peptide. Importantly, we examined transcription under conditions in which the amount of ER was not significantly reduced. Together, our data document that disruption of the interaction between calmodulin and ER prevented the latter from activating transcription in response to E_2.

First reported almost 20 years ago (24), the participation of calmodulin in estrogen function has become the focus of renewed interest (Ref. 9, and references therein). Calmodulin binds to ER in intact cells independently of E_2, thereby modulating ER stability and steady state levels (9). Moreover, calmodulin is an integral component of the ER-estrogen response element complex (11, 25). The data presented here demonstrate that an interaction between calmodulin and ER in the nucleus is required for E_2-stimulated ER transcriptional activation. The molecular mechanism by which calmodulin facilitates ER transcription is unknown. Calmodulin has been shown to modulate the activity of a number of nuclear proteins, several of which are involved in transcription. For example, Ca^{2+}/calmodulin-dependent kinases regulate gene transcription by altering coactivator function (26). Furthermore, calmodulin binds to members of the basic helix-loop-helix transcription factors, modifying their DNA binding (27). Recently, a family of calmodulin-binding transcription activators was identified (28). It is not known whether calmodulin directly binds a transcription activator or has another role in ER transcription. Our previous results imply that calmodulin alters the tertiary conformation of ER (9). One could envisage that this would alter the ability of ER to interact with coactivators and/or corepressors, altering transcription. Regardless of the mechanism, our data contribute to deciphering the intricate meshwork of ER signaling pathways. In addition, they further explain the prior observations that calmodulin antagonists inhibit the growth of breast cell lines (29) and synergistically amplify antiestrogen therapy (30).

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REFERENCES

1. Tsai, M. J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63,** 451–486
2. Dickson, R. B., and Stancel, G. M. (2000) *J. Natl. Cancer. Inst. Monogr.* 135–145
3. McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) *Endocr. Rev.* **20,** 321–344
4. Xu, L., Glass, C. K., and Rosenfeld, M. G. (1999) *Curr. Opin. Genet. Dev.* **9,** 140–147
5. MacGregor, J. I., and Jordan, V. C. (1998) *Pharmacol. Rev.* **50,** 151–196
6. Cohen, P., and Klee, C. (1988) *Calmodulin*, Elsevier, New York
7. Chun, K. Y., and Sacks, D. B. (2000) in *Calcium: The Molecular Basis of Calcium Action in Biology and Medicine* (Pochet, R., Donato, R., Haiech, J., Heizmann, C., and Gerke, V., eds), pp. 541–563, Kluwer Academic Publishers, Dordrecht, The Netherlands
8. Chin, D., and Means, A. R. (2000) *Trends Cell Biol.* **10,** 322–328
9. Li, Z., Joyal, J. L., and Sacks, D. B. (2001) *J. Biol. Chem.* **276,** 17354–17360
10. Bouhoute, A., and Leclercq, G. (1992) *Biochem. Biophys. Res. Commun.* **184,** 1432–1440
11. Biswas, D. K., Reddy, P. V., Pickard, M., Makkad, B., Pettit, N., and Pardee, A. B. (1998) *J. Biol. Chem.* **273,** 33817–33824
12. Migliaccio, A., Rotondi, A., and Auricchio, F. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 5921–5925
13. Strobl, J. S., and Peterson, V. A. (1992) *J. Pharmacol. Exp. Ther.* **263,** 186–193
14. Sacks, D. B., Porter, S. E., Ladenson, J. H., and McDonald, J. M. (1991) *Anal. Biochem.* **194,** 369–377
15. Wang, J., Campos, B., Jamieson, G. A., Jr., Kaetzel, M. A., and Dedman, J. R. (1995) *J. Biol. Chem.* **270,** 30245–30248
16. Briggs, M. W., Li, Z., and Sacks, D. B. (2002) *J. Biol. Chem.* **277,** 7453–7465
17. Kim, S. H., Li, Z., and Sacks, D. B. (2000) *J. Biol. Chem.* **275,** 36999–37005
18. Norman, J. A., Ansell, J., Stone, G. A., Wennogle, L. P., and Wasley, J. W. (1987) *Mol. Pharmacol.* **31,** 535–540
19. Joyal, J. L., Burks, D. J., Pons, S., Matter, W. F., Vlahos, C. J., White, M. F., and Sacks, D. B. (1997) *J. Biol. Chem.* **272,** 28183–28186
20. Li, Z., Kim, S. H., Higgins, J. M., Brenner, M. B., and Sacks, D. B. (1999) *J. Biol. Chem.* **274,** 37885–37892
21. Garcia Pedrero, J. M., Del Rio, B., Martinez-Campa, C., Muramatsu, M., Lazo, P. S., and Ramos, S. (2002) *Mol. Endocrinol.* **16,** 947–960
22. Levin, E. R. (1999) *Trends Endocrinol. Metab.* **10,** 374–377
23. Sugimura, M., Sato, T., Nakayama, W., Morishima, Y., Fukunaga, K., Omitsu, M., Miyamoto, E., and Shirasaki, Y. (1997) *Eur. J. Pharmacol.* **336,** 99–106
24. Flandroy, L., Cheung, W. Y., and Steiner, A. L. (1983) *Cell Tissue Res.* **233,** 639–646
25. Bouhoute, A., and Leclercq, G. (1995) *Biochem. Biophys. Res. Commun.* **208,** 748–755
26. Corcoran, E. E., and Means, A. R. (2001) *J. Biol. Chem.* **276,** 2975–2978
27. Onions, J., Hermann, S., and Grundstrom, T. (1997) *J. Biol. Chem.* **272,** 23930–23937
28. Bouche, N., Scharlat, A., Snedden, W., Bouchez, D., and Fromm, H. (2002) *J. Biol. Chem.* **277,** 21851–21861
29. Wei, J. W., Hickie, R. A., and Klaassen, D. J. (1983) *Cancer Chemother. Pharmacol.* **11,** 86–90
30. Newton, C. J., Eycott, K., Green, V., and Atkin, S. L. (2000) *J. Steroid Biochem. Mol. Biol.* **73,** 29–38

#3194 Calmodulin regulates the transcriptional activity of estrogen receptors. Lu Li, Zhigang Li, and David Sacks. *Brigham and Women's Hospital, Harvard Medical School, Boston, MA.*

ABSTRACT The steroid hormone estrogen elicits biological effects in cells by binding to and activating the estrogen receptor (ER). Estrogen binding induces a conformational change in the receptor, inducing nuclear translocation and transcriptional activation of ER. We previously demonstrated that the ubiquitous Ca^{2+}-binding protein calmodulin interact directly with ER and enhance its stability (J Biol. Chem, 2001; 276 (20):17354-60). To further elucidate the functional sequelae of the association between calmodulin and ER, in this study we examined the effect on ER transcriptional activation of specifically inhibiting calmodulin. The cell-permeable calmodulin antagonist CGS9343B prevented estrogen-induced transcriptional activation by ER both in MCF-7 and T47D ER positive breast carcinoma cell lines, without altering basal transcription. The inhibition was dose-dependent and independent of the time of estrogen stimulation. To validate these findings, calmodulin function was also neutralized by targeted expression of a specific inhibitor peptide. By inserting localization signals, the inhibitor peptide was selectively targeted to different subcellular domains. Inactivation of calmodulin function in the nucleus virtually eliminated estrogen-stimulated ER transcriptional activation. By contrast, when membrane calmodulin was specifically neutralized, estrogen-stimulated transcriptional activation by ER was only slightly attenuated. Importantly, the inhibitor peptides did not significantly reduce the amount of ER in the cells. Together these data demonstrate that calmodulin is a fundamental component of ER transcriptional activation and imply that calmodulin antagonists may value in the treatment of breast carcinoma.