0888-8809/02/$15.00/0
*Printed in U.S.A.*

Molecular Endocrinology 16(5):947–960


# Calmodulin Is a Selective Modulator of Estrogen Receptors

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**In the search for differences between ERα and ERβ, we analyzed the interaction of both receptors with calmodulin (CaM) and demonstrated that ERα but not ERβ directly interacts with CaM. Using transiently transfected HeLa cells, we examined the effect of the CaM antagonist *N*-(6-aminohexyl)-5-chloro-naphthalene sulfonilamide hydrochloride (W7) on the transactivation properties of ERα and ERβ in promoters containing either estrogen response elements or activator protein 1 elements. Transactivation by ERα was dose-dependently inhibited by W7, whereas that of ERβ was not inhibited or even activated at low W7 concentrations. In agreement with these results, transactivation of an estrogen response element containing promoter in MCF-7 cells (which express a high ERα/ERβ ratio) was also inhibited by W7. In contrast, transactivation in T47D cells (which express a low ERα/ERβ ratio) was not affected by this CaM antagonist. The sensitivity of MCF-7 cells to W7 was abolished when cells were transfected with increasing amounts of ERβ, indicating that the sensitivity to CaM antagonists of estrogen-responsive tissues correlates with a high ERα/ERβ ratio. Finally, substitution of lysine residues 302 and 303 of ERα for glycine rendered a mutant ERα unable to interact with CaM whose transactivation activity became insensitive to W7. Our results indicate that CaM antagonists are selective modulators of ER able to inhibit ERα-mediated activity, whereas ERβ actions were not affected or even potentiated by W7. (*Molecular Endocrinology* 16: 947–960, 2002)**

THE DISCOVERY OF a second ER, the ERβ (1), and the development of ERα and ERβ single and double knockout mice (2) made it possible to show that ERα and ERβ mediate different biological actions. The knockout mice show different phenotypic alterations as a consequence of their ER deficiency. ERα and ERβ are encoded by different genes and show different expression in target tissues. ERβ appears to be the quantitatively dominant ER and predominates over ERα in bone, the cardiovascular system, urogenital tract, central nervous system, immune system, kidney, and lung. Many of these tissues were not recognized as estrogens targets until recently. On the other hand, ERα has a significant role in the reproductive system in both males and females (3, 4). Because ERα is overexpressed in more than 50% of breast cancers, it was the first ER to be identified.

The activation of both receptors by 17β-E2 induces ERα and ERβ binding to the same DNA sequence and activates the transcription of genes regulated by estrogen response elements (ERE) (1). However, only ERα mediates E2-dependent transcription of genes regulated by activator protein 1 (AP1) (5) and genes regulated by Sp1 (6). In contrast, it has been shown in some cell lines that ERβ specifically mediates JNK inhibition (7), an alternative mode whereby steroids conduct their immunosuppressive, antiinflammatory, and antineoplasic pharmacological actions (8). For all the reasons outlined above, the finding of selective inhibitors of ERα and ERβ is of paramount importance, to identify the roles of each receptor and to eliminate the deleterious effects of estrogen therapy. Novel ligands that function as selective estrogens or antiestrogens for ERα or ERβ have been previously reported (9, 10), and studies providing a basis for some differential transcription activities between both receptors have also been published (11). Here, we show striking differences between ERα and ERβ. The fact that only ERα binds to calmodulin (CaM) provides new ways of searching for ERα selective inhibitors. The interaction of CaM with the ER was demonstrated by Castoria *et al.* (12) who purified the only known ER at that time, ERα. These same authors also postulated a CaM binding domain based on the homology with other CaM binding proteins. Since then, several reports have indicated that antiestrogens and anti-CaM drugs stop MCF-7 cells at the G1 phase of the cell cycle (13) and that CaM is essential both for the interaction of

Abbreviations: AP1, Activator protein 1; CaM, calmodulin; ECL, enhanced chemiluminescence; ERE, estrogen response element; GST, glutathione *S*-transferase; h, human; Luc, luciferase; mut, mutant; OHT, 4-hydroxytamoxifen; p, plasmid; PLB, passive lysis buffer; PMSF, phenylmethylsulfonyl fluoride; pRL, *Renilla* luciferase plasmid; sFCS, 0.5% charcoal/dextran-treated FCS; TK, thymidine kinase; W7, *N*-(6-aminohexyl)-5-chloro-naphthalene sulfonilamide hydrochloride; wt, wild-type.

ERα with DNA and for the activation of responsive promoters (14). In this study, we demonstrate very relevant differences between ERα and ERβ. ERα is tightly bound to CaM; its transactivation capacity is inhibited by the CaM antagonist *N*-(6-aminohexyl)-5-chloro-naphthalene sulfonilamide hydrochloride (W7). In contrast, ERβ does not interact with CaM and, interestingly, its transactivation capacity is activated by low concentrations of W7. Furthermore, we have been able to convert the W7-sensitive ERα into a W7-insensitive receptor by mutating amino acids located at the putative calmodulin binding site. Remarkably, these lysines have been recently reported as targets for acetylation by p300 (15). Moreover, K303 is mutated to R in 34% of premalignant breast lesions (16). These findings, together with our results, strongly suggest that these residues (K302, K303) might play a crucial role in the ERα regulation *in vivo* and provide new ways of searching for ERα inhibitors which do not inhibit ERβ-mediated functions.

## RESULTS

### ERα But Not ERβ Interacts with CaM

Previous reports have shown that CaM interacts with ERα (12) and that the CaM antagonist W7 inhibits ERα-dependent transactivation at ERE-containing promoters (14). We analyzed the interaction of both ERα and ERβ with CaM using three different constructs (Fig. 1). ERα (1–595) (Fig. 2A), a truncated ERα (280–595) lacking the AF-1, the DNA binding domain and part of the hinge region (Fig. 2B) but containing the postulated CaM binding site and the HBD/AF-2 (12), and ERβ (1–530) (Fig. 2C) were [$^{35}$S]-labeled by *in vitro* translation and immunoprecipitated with anti-ER, anti-CaM or anti-Pho4 as unrelated polyclonal antibody (Pho4 is a transcription factor implicated in phosphate starvation signals in yeast). Both the full-length ERα and the truncated receptor ERα (280–595) were coimmunoprecipitated with anti-CaM and polyclonal anti-ER antibodies (Fig. 2, A and B, lanes 1 and 2). No immunoprecipitation was observed when no antibodies or an unrelated polyclonal antibody (anti-Pho4) were used (Fig. 2A, lanes 3 and 4, respectively). The addition of E2, purified CaM, or HeLa nuclear extracts had no effect on immunoprecipitation because CaM was present in the reticulocyte extract used for *in vitro* synthesis as detected by Western blot analysis (data not shown).

In contrast, [$^{35}$S]-labeled ERβ was not coimmunoprecipitated with the anti-CaM antibodies (Fig. 2C, lane 1) even after addition of exogenous CaM (Fig. 2C, lane 2), thus indicating that only ERα interacts with CaM. The presence of ERβ was confirmed by immunoprecipitation with rabbit polyclonal anti-ER antibodies (Fig. 2C, lane 3).

To demonstrate *in vivo* whole cell interactions between CaM and ERα, whole cell extracts from MCF-7 cells were immunoprecipitated as described in *Materials and Methods* with anti-CaM antibodies, monoclo-

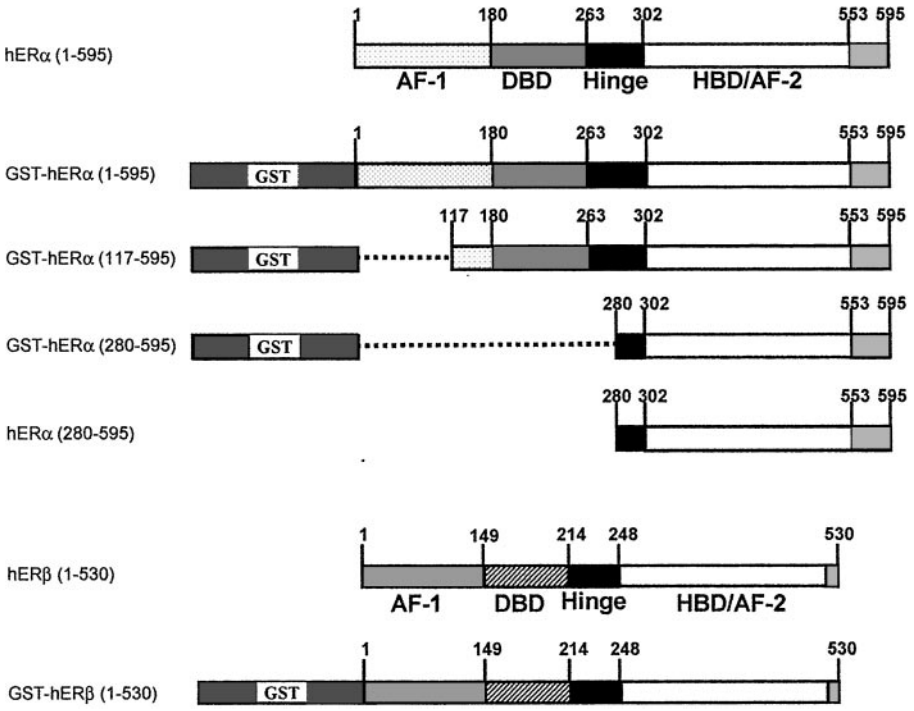


**Fig. 1.** ERα and ERβ Various Functional Domains and the Different Constructs Used in this Study
AF-1, Activating function 1; DBD, DNA binding domain; HBD/AF-2, hormone binding domain/activating function 2.

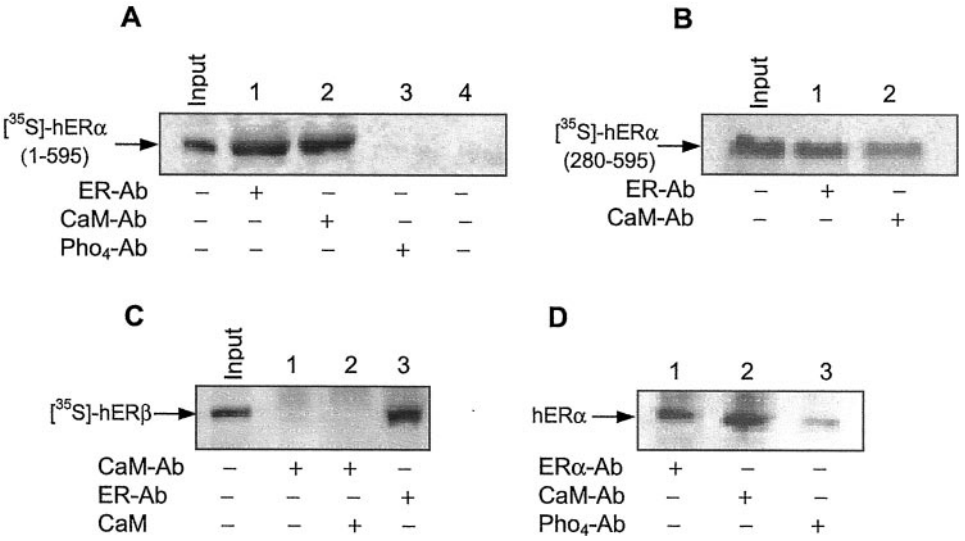


**Fig. 2.** Differential Immunoprecipitation of [35S]-Labeled hERα (A) and hERβ (B) with Anti-CaM Antibodies

A, [35S]-Labeled full-length ERα produced by *in vitro* transcription and translation was immunoprecipitated with polyclonal anti-ER (ER-Ab, lane 1), anti-CaM (CaM-Ab, lane 2), anti-Pho4 (Pho4-Ab, lane 3) or no antibodies (lane 4). The input lane contains 10% of the total amount of [35S]-labeled receptor used in the immunoprecipitations. B, [35S]-Labeled ERα (280–595) obtained by *in vitro* transcription and translation was immunoprecipitated with polyclonal anti-ER (lane 1) or anti-CaM antibodies (lane 2). C, [35S]-Labeled ERβ obtained by *in vitro* transcription and translation was immunoprecipitated with polyclonal anti-ER (lane 3) or anti-CaM (lanes 1 and 2). A quantity of 0.5 μg of CaM was added before inmunoprecipitation to the transcription-translation system mix in lane 2. D, 5 mg of total protein extracted from MCF-7 cells were immunoprecipitated with anti-CaM antibodies (CaM-Ab, lane 2), with monoclonal anti-ERα antibodies (C-314, Santa Cruz Biotechnology, Inc.) (ERα-Ab, lane 1) or with anti-Pho4 antibodies (Pho4-Ab, lane 3) as indicated. Immunoprecipitated proteins were analyzed by SDS-PAGE and detected by autoradiography in A–C. In D, proteins were resolved by SDS-PAGE and ERα was detected by Western blot with polyclonal anti-ER antibodies.

nal anti-ERα antibodies, or with unrelated rabbit polyclonal antibodies (Anti-Pho4). Immunoprecipitates were analyzed by SDS-PAGE and ERα was detected by Western blot with rabbit polyclonal anti-ER antibodies. We found that ERα (67 kDa) immunoprecipitates to the same extent with both anti-CaM or anti-ERα antibodies (Fig. 2D, lanes 1 and 2), whereas the immunoprecipitation with nonspecific antibodies was negligible (Fig. 2D, lane 3).

The results shown until now strongly suggest that: 1) ERα but not ERβ interact with CaM; and 2) the carboxi terminus (280–595) region of ERα also interacts with CaM, indicating that this truncated ERα contains the CaM binding site as previously predicted (residues 298–310) (12).

Additional evidence of direct CaM-ERα interaction was obtained in another set of experiments using purified glutathione *S*-transferase (GST)-ERs hybrid proteins and dansyl-CaM. Emission spectra of dansyl-CaM ($\lambda_{333}$nm) was determined before and after addition of the indicated GST-proteins or equivalent amounts of buffer in the controls. In the presence of GST-ERα (1–595) an enhancement of the fluorescence of dansyl-CaM was observed, as expected when the dansyl group bound to CaM reached a more hydrophobic environment as a consequence of CaM interaction with ERα (Fig. 3A). A similar approach was previously reported by Zuhlke *et al.* (17) to demonstrate the interaction of CaM with the IQ motif of the $Ca^{2+}$-channel subunit $\alpha_{1c}$. Moreover, the fluorescence of the ERα-dansyl-CaM complex was decreased by 40% upon addition of $10^{-8}$ M E2, thus indicating that the conformational change induced by E2 in ERα affects the ERα-CaM interaction pattern (Fig. 3A). The results obtained with GST-ERβ and GST-ERα (280–595) hybrid proteins were, however, completely different. Thus, neither GST-ERβ (Fig. 3B) nor GST-ERα (280–595) (Fig. 3C) altered the fluorescence of dansyl-CaM independently of the presence of E2. The results shown in Fig. 3 confirm that of the two complete ERs only GST-ERα (1–595) interacts with calmodulin.

The interaction of the same hybrid proteins with CaM was also determined by far-western experiments using biotin-CaM. For this purpose GST-ERα (117–595), GST-ERβ and GST-ERα (280–595) were resolved by SDS-PAGE and electroblotted onto nitrocellulose filters. Immobilized proteins were denatured, renatured, and incubated with biotin-CaM as described in *Materials and Methods*. Finally, CaM binding proteins were visualized with streptavidin-peroxidase and the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Barcelona, Spain). Figure 3D shows that biotin-CaM only interacts with a protein of about 94 kDa (lane 1), which corresponds to the GST-ERα (117–595), and a smaller protein that is probably a degradation product of the

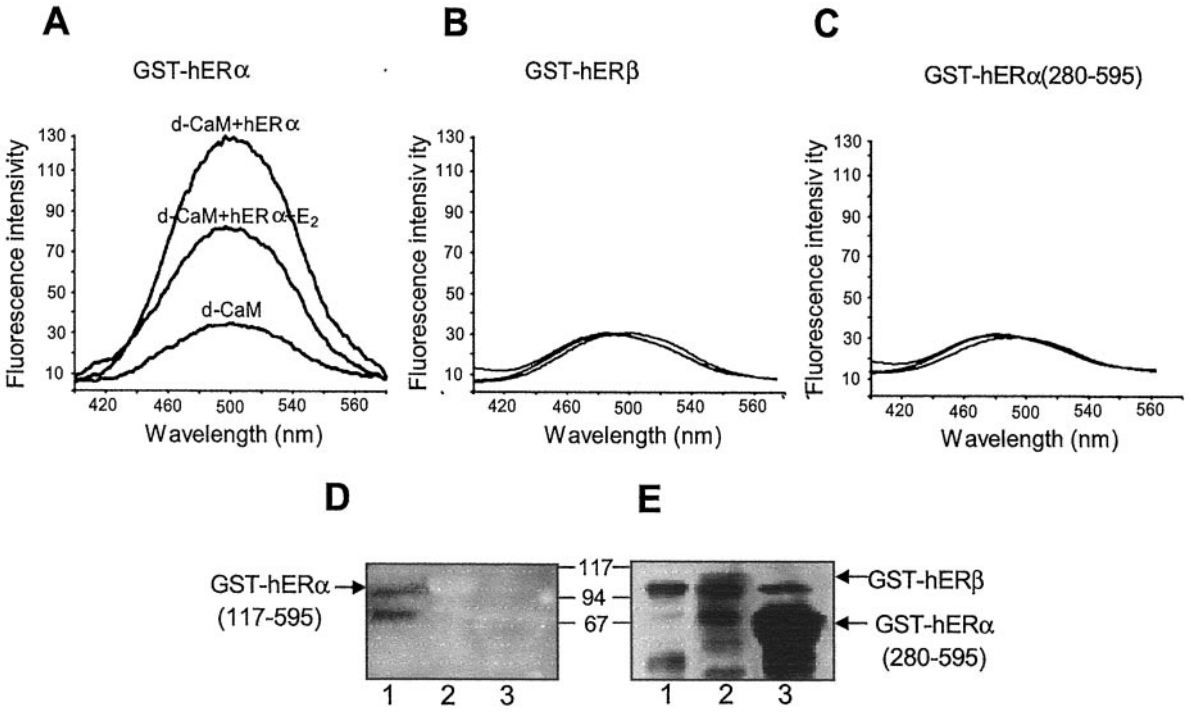


**Fig. 3.** Interaction of GST-ERα and GST-ERβ Hybrid Proteins with Dansyl-CaM and Biotin-CaM

A, Emission fluorescence spectra of 25 μM dansyl-CaM in 10 mM MOPS (pH 7.2), 1 mM $MgCl_2$, 100 mM KCl, and 1 mM $CaCl_2$ was determined before (d-CaM) and after addition of 1.5 μg of GST-ERα hybrid protein (d-CaM + hERα) in the absence or presence of E2 ($10^{-8}$ M) (d-CaM + hERα + E2). B, Similar experiments were performed with 4.5 μg of GST-ERβ (1–530) and (C) with 10 μg of GST-ERα (280–595). The amount of added buffer was maintained constant in all the experiments performed. D and E, Three micrograms of GST-ERα (117–595) (lane 1), 6 μg of GST-ERβ (1–530) (lane 2) and 15 μg of GST-ERα (280–595) (lane 3) were resolved by SDS-PAGE and electroblotted onto nitrocellulose filters. After denaturation/renaturation, proteins were detected with biotin-CaM as a probe. CaM binding proteins were visualized with streptavidin-peroxidase and ECL detection (D) or polyclonal anti-ER antibodies and ECL detection (E).

hybrid protein. A similar interaction was observed when GST-ERα (1–595) was assayed (data not shown). In contrast, no interaction was observed when GST-ERβ and GST-ERα (280–595) were analyzed (Fig. 3D, lanes 2 and 3). The presence of these hybrid proteins on the filters were confirmed by Western blot analysis using polyclonal anti-ER antibodies (Fig. 3E).

The results presented up to now confirm that CaM directly interacts with the endogenous ERα present in MCF-7 cells (Fig. 2D), with the *in vitro* translated ERα (1–595) (Fig. 2A) and with the truncated ERα (280–595) containing part of the hinge and the HBD/AF2 regions of ERα (Fig. 2B). However, neither the *in vitro* translated ERβ (Fig. 2C) nor GST-ERβ (Fig. 3, B and D) were able to bind to CaM. The lack of interaction of ERβ with CaM may be explained because the postulated CaM binding site is not conserved in ERβ.

Strikingly, GST-ERα (280–595), which does contain the CaM binding site (as demonstrated by site directed mutagenesis; see Fig. 7B) does not interact with dansyl-CaM or biotin-CaM (Fig. 3, C and D). The lack of interaction of GST-ERα (280–595) with CaM has been observed by other authors (18). This might be a consequence of the proximity of GST to the hinge region of the receptor, as suggested by the fact that ERα (280–595) can be immunoprecipitated with anti-CaM antibodies (Fig. 2B).

### Specific Inhibition of ERα-Mediated Transcriptional Activation by W7

To further understand the regulation of ERα and ERβ by CaM, we studied the effect of the CaM antagonist W7 on E2-dependent transactivation of each receptor isoform. To do this, we transiently transfected HeLa cells with ERα or ERβ expression vectors, together with the plasmid (p)ERE-thymidine kinase (TK)-luciferase (Luc) reporter plasmid. In both ERα and ERβ transfected cells, E2 ($10^{-7}$ M) stimulated transcription about 2.5-fold. The presence of W7 at $10^{-6}$ M inhibited E2-ERα-induced transactivation by 95%. However, E2-ERβ-induced gene expression was not affected by this concentration of W7 (Fig. 4A). The inhibition caused by W7 in ERα-transfected cells was dose dependent (Fig. 4B) with an $IC_{50}$ of $10^{-8}$ M. In contrast, E2-ERβ transcriptional activation was dose dependently activated by W7, reaching almost 2-fold stimulation at $10^{-7}$ M W7. This activation was only observed in a weak promoter context (pERE-TK-Luc). When transfection experiments were performed with

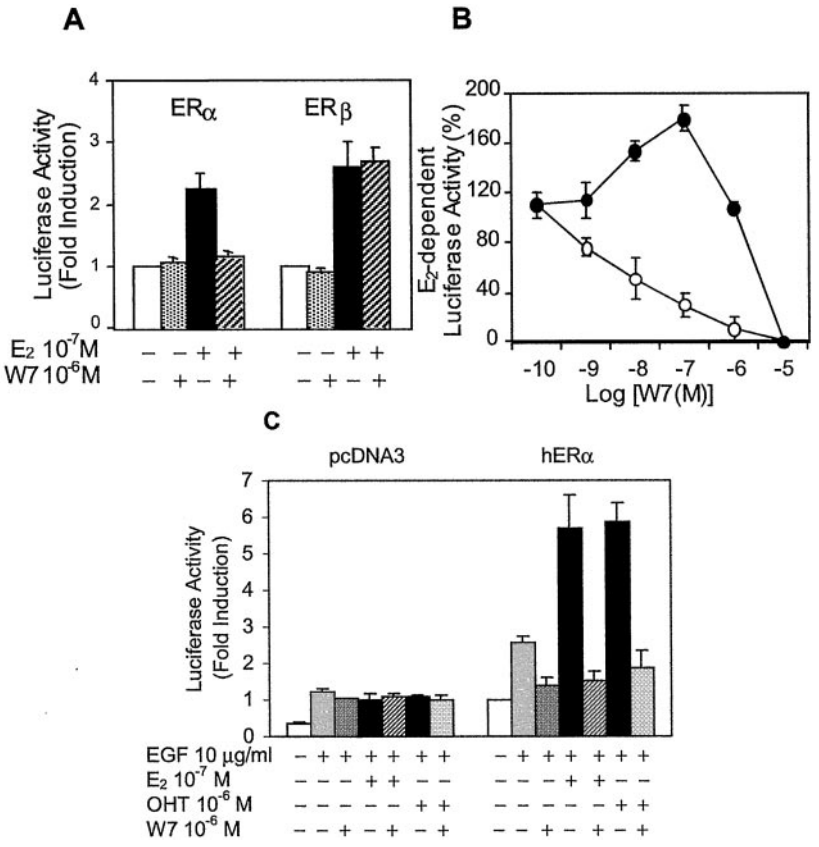


**Fig. 4.** Differential Effect of W7 on the Transactivation Properties of ERα and ERβ

A, HeLa cells were transfected with 0.5 μg of reporter plasmid pERE-TK-Luc, 0.1 μg of the indicated receptor expression vector and 50 ng of internal control plasmid pRL-TK, using FuGENE 6. After 18–24 h, medium was renewed and cells were stimulated for 24 h with either vehicle, $10^{-7}$ M E2 alone, $10^{-6}$ M W7 alone or both E2 + W7 as indicated. Luciferase activities were normalized to the *Renilla* luciferase activities. The data are reported as fold induction with respect to untreated cells, which were arbitrarily assigned as 1. The *bars* represent the means ± SD of three independent experiments run in duplicate. B, Dose response to W7 of E2-induced ERα (o) or ERβ (●) transactivation. HeLa cells were transfected as above and stimulated for 24 h with $10^{-7}$ M E2 alone or with the indicated concentrations of W7. E2-dependent luciferase activity is expressed as the percentage of E2 stimulation. C, Effect of W7 on E2- and OHT-dependent hERα transactivation at an AP1 element. HeLa cells were transfected with either 0.1 μg of the ERα expression vector or empty pcDNA3 as indicated, 50 ng of internal control plasmid pRL-TK and the 0.5 μg of AP1-containing reporter plasmid (Δcoll 73-Luc). Cultures were stimulated for 48 h with 10 μg/ml EGF, $10^{-7}$ M E2, $10^{-6}$ M OHT, and $10^{-6}$ M W7 as indicated. Luciferase activity was determined as in Fig. 4A.

stronger promoters such as 3x-ERE-TATA-Luc, neither inhibition nor stimulation by W7 on E2-ERβ was observed (data not shown). High concentrations of W7 resulted in a decrease of the activity (Fig. 4B). The inhibition of ERα and ERβ by W7 concentrations higher than $10^{-6}$ M might indicate that other CaM-dependent processes are affected by these concentrations of W7, for example, the synthesis or stability of coactivators necessary for ER-mediated transactivation could be impaired or alternatively, corepressors might be up-regulated by these high concentrations of W7 ($10^{-5}$ M). However, it is clear from these experiments that a wide range of W7 concentrations ($10^{-10}$ to $10^{-6}$) specifically inhibit E2-ERα-mediated transactivation, whereas ERβ is not inhibited or even stimulated at these concentrations of W7.

We next examined the ability of W7 to inhibit ERα transcriptional activity mediated through AP1-responsive genes by transiently transfecting HeLa cells with Δcoll 73-Luc reporter plasmid. Δcoll 60-Luc reporter plasmid (5) lacking the AP1 binding site was included as a negative control, and no activation was observed with any of the treatments used in these set of experiments (data not shown). The AP1 pathway has been proposed to account for some of the cell-specific agonist effects of tamoxifen (19). Even though E2-dependent ERα-mediated AP1 activation in HeLa cells and other cell lines have been described (5, 19), we, similar to other authors (20, 21), found it necessary to prime the cells with EGF, IGF, or PMA to observe this effect (Fig. 4C, *right panel*, lane 4). Growth factors and PMA stabilize the

levels of c-*jun* and c-*fos* family proteins allowing a synergistic effect of these factors with E2-ERα dependent on AP1 transcription activation (20, 21). We found that AP1 activity was increased by EGF in an ERα-independent fashion (Fig. 4C, *left panel*). This effect was strengthened when cells were transfected with ERα (Fig. 4C, *right panel*). Moreover, E2 significantly potentiated the activity of AP1 in ERα-transfected cells. These results agree with results previously reported (20, 21), indicating that EGF synergize with E2 but only in cells expressing ER (compare Fig. 4C, lane 4 in *left* and *right panels*). Similar results were also obtained with 4-hydroxytamoxifen (OHT) (Fig. 4C, *right panel*, lane 6).

Very importantly, the synergistic effect of EGF and either E2 or OHT was sensitive to W7. Both the activation by E2 and OHT and the inhibition by W7 are statistically significant. We can infer from the transfection experiments shown in Fig. 4 that CaM is a regulator of ERα/ERE and ERα/AP1 pathways because the transcription

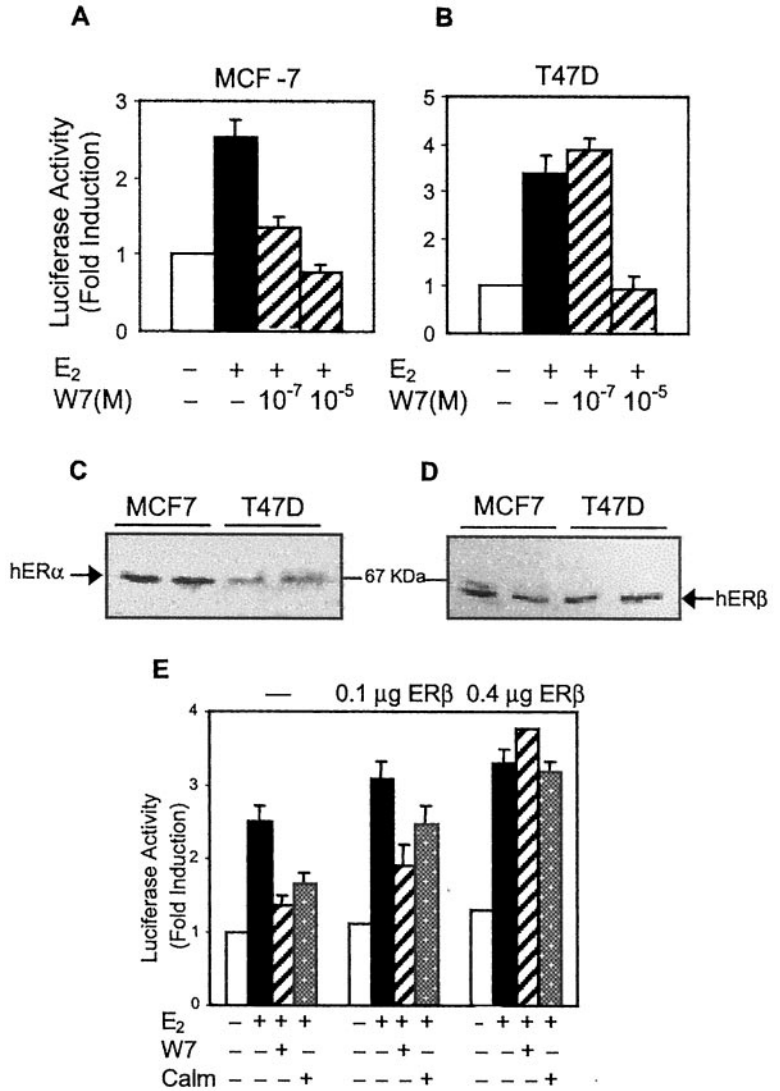


**Fig. 5.** Effect of CaM Antagonists on E2-Dependent Transcriptional Activation in Human Breast Cancer Cell Lines

MCF-7 (A) and T47D cells (B) were transiently transfected with 0.5 μg of reporter plasmid pERE-TK-Luc and 50 ng of control reporter vector pRL-TK. Cells were treated for 24 h with either vehicle or $10^{-7}$ M E2, in the absence or presence of the indicated concentrations of W7. Luciferase activity was determined as in Fig. 4. C and D, Protein levels of both ERα and ERβ receptors were determined by Western blot analysis of MCF-7 and T47D nuclear extracts obtained from synchronized cells treated for 40 min. with $10^{-8}$ M E2. Two samples of each cell line containing 50 μg of protein from nuclear extracts were resolved by SDS-PAGE and the presence of ERs was determined by Western blot analysis with rabbit polyclonal anti-ER antibodies (C) or specific anti-ERβ (H-150) rabbit polyclonal antibodies (D). E, MCF-7 cells were transfected with 0.5 μg of pERE-TK-Luc, 50 ng of pRL-TK plasmid and the indicated amounts of pCMX-mERβ expression vector. The total amount of DNA was held constant to 0.95 μg per well by addition of empty expression vector. After transfection, cells were treated for 24 h with $10^{-7}$ M E2, $10^{-7}$ M W7 and $10^{-6}$ M calmidazolium as indicated. Luciferase activity was determined as in Fig. 4.

mediated by both complexes is sensitive to CaM antagonists.

### MCF-7 and T47D Breast Cancer Cells Show Different Sensitivity to CaM Antagonists

The effect of CaM antagonists on E2-dependent transactivation was also determined in MCF-7 and T47D cell lines derived from human breast cancer. These two cell lines that express endogenous ERα and ERβ (22) were transiently transfected with pERE-TK-Luc reporter plasmid and stimulated with E2 ($10^{-7}$ M). E2 induced an almost 3-fold increase in luciferase expression in both cell lines (Fig. 5, A and B). The activation was inhibited (74%) by $10^{-7}$ M W7 in MCF-7 cells (Fig. 5A). However, E2-induced transactivation in T47D cells was unaffected by the same concentration of W7 (Fig. 5B). To check whether the different sensitivity to W7 was due to a differential expression of ERα and ERβ in both cell lines, we determined the levels of protein expression of both receptors by Western blot analysis in nuclear extracts (50 μg of protein) from cells that were synchronized and then treated for 40 min with $10^{-8}$ M E2.

In MCF-7 cells, anti-ER antibodies detected a protein with a molecular mass of about 67 kDa corresponding to the canonic ERα. In T47D cells, this 67-kDa band was also detected but the signal was much weaker than that observed in MCF-7 (Fig. 5C), indicating that ERα expression in T47D is much lower than in MCF-7 cells. With this antibody, ERβ was almost undetectable in both cell lines. When the same filter was probed with specific anti-ERβ antibodies (SC 8974), a protein of about 60 kDa was detected. The amount of this protein was approximately the same in both cell lines (Fig. 5D) and this was considerably increased in T47D cells when E2 treatment was maintained for 24 h (data not shown). This agrees with the 40-fold increase reported in the levels of ERβ mRNA in this cell line (22). On the other hand, ERβ mRNA levels are extremely low (about 200-fold lower than that of ERα) in MCF-7 cells (Lykkesfeldt, A., Institute of Cancer Biology, Danish Cancer Society, Copenhagen, Denmark, personal communication, December 2001).

The detection of ERα and ERβ with different antibodies allowed us to make an estimation of the ERα/ERβ ratio on each cell line. These results show that the ratio ERα/ERβ is much higher in MCF-7 cells than in T47D, which might explain the lack of sensitivity of the later cell line to $10^{-7}$ M W7.

To test this hypothesis, MCF7 cells were transfected with the pERE-TK-Luc reporter plasmid and increasing amounts of ERβ expression vector to determine whether ERβ overexpression affects the sensitivity of these cells to CaM antagonists. As expected, in cells transfected with no ERβ expression vector, both W7 and calmidazolium inhibited E2-stimulated transcription activation. Interestingly, the inhibitory effects of both CaM antagonists were abolished by increasing ERβ expression (Fig. 5E). These results are in agreement with those obtained in HeLa cells independently transfected with each ER isoform (Fig. 4), and once again indicate that CaM antagonists can be considered as selective ER modulators able to specifically prevent ERα-mediated estrogenic response while preserving ERβ activity. Furthermore, these results indicate that the sensitivity to CaM antagonists of estrogen target tissues correlates with a high ERα/ERβ ratio and could be used as a tool to specifically define the role of ERα and ERβ in these tissues.

### CaM Antagonists Cause E2-ER-ERE Complex Destabilization in MCF-7 Nuclear Extracts

The results presented above show that W7 inhibits the E2-induced transactivation in MCF-7 cells. W7 has been reported to inhibit the binding of ER to the ERE (14). E2-dependent binding of ER to the ERE of the *Xenopus* vitellogenin A2 gene was evaluated in nuclear extracts from MCF-7 cells using a gel retardation assay. Cells were synchronized by serum and estrogen deprivation and stimulated for 40 min in 0.5% charcoal/dextran-treated FCS (sFCS) plus $10^{-8}$ M E2 as previously described (23). Nuclear extracts were obtained and assayed for ERE binding as described in *Materials and Methods*. The treatment of nuclear extracts with $10^{-8}$ M E2 increased the binding to the ERE 3-fold. This binding was 90% inhibited when W7 ($10^{-4}$ M) was added during the assay (Fig. 6A). The binding was specific because 1) it was competed by a 50 and 100 molar excess of unlabeled ERE, and 2) addition of monoclonal anti-ERα antibody resulted in a supershift. The results shown in Fig. 6A are similar to those previously reported by Biswas *et al.* (14). To test whether W7 affected the binding affinity of the E2-ER complex for ERE, we performed another set of experiments in which the rate of dissociation of the E2-ER-ERE complex formed in the absence and in the presence of $10^{-4}$ M W7 was determined. For this purpose, a 200-fold molar excess of unlabeled ERE was added once the complex was formed, and the stability of the complex monitored. Figure 6, B and C, shows that the rate of dissociation of the E2-ER-ERE complex formed in the presence of $10^{-8}$ M E2 plus $10^{-4}$ M W7 is much faster than that of the complex formed in the presence of $10^{-8}$ M E2 alone. Thus, at 30 min more than 90% of the later complex remained, whereas only 20% of the former was detected. These results indicate that the presence of W7 destabilizes the binding of E2-ER complex to DNA.

### An ERα Mutant Unable to Bind CaM Becomes Insensitive to CaM Antagonists

Sequence analysis of the postulated CaM binding site in ERα (amino acids 298–310) revealed that this region is highly conserved between human and mouse ERα, and that there are important differences between human (h) ERα and hERβ. Particularly important, the residues K302 and K303 of hERα are both substituted

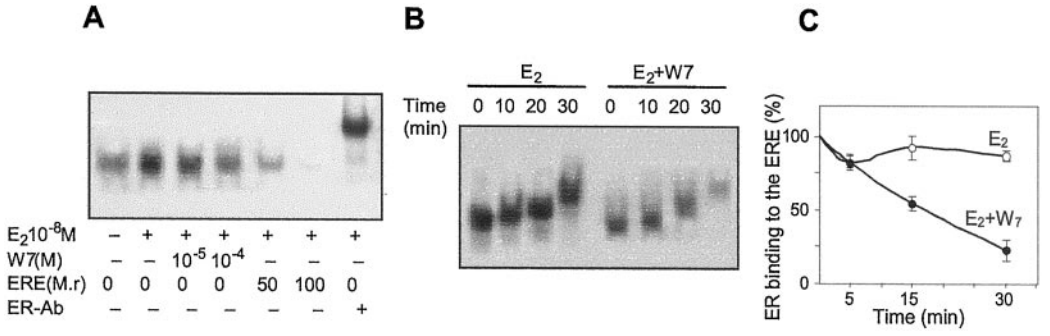


**Fig. 6.** Effect of W7 on E2-Dependent Binding of MCF7 Nuclear Extract to ERE

A, Synchronized MCF-7 cells in 0.5% sFCS media were stimulated for 40 min with $10^{-8}$ M. E2 and nuclear extracts were prepared. Equal amounts of nuclear proteins (5 μg) were treated during the assay with $10^{-8}$ M E2 plus or minus the indicated concentration of W7. The control received an equal volume of vehicle. ER binding to ERE was assayed by EMSA as described in *Materials and Methods*. When indicated, 0.2 μg of monoclonal anti-ER antibody (NCL-ER-LH1) was added before probe addition. The retarded band was competed by 50- and 100-fold molar ratio (M.r) unlabeled ERE oligonucleotide. B, Effect of W7 on the rate of dissociation of E2-ER-ERE complex. Nuclear extracts were assayed for ERE binding in the presence of $10^{-8}$ M E2 plus or minus $10^{-4}$ M W7. After the E2-ER-ERE complex was formed, a 200-fold excess of unlabeled ERE was added followed by incubation at 20 C. At the indicated times, 20-μl samples were withdrawn and loaded on a preelectrophoresed gel. The different mobility of the retarded band is due to the different times during which samples were run. C, Quantification of the remaining binding of nuclear extracts treated with E2 (○) or E2 plus W7 (●). The results represent the mean ± SD of three independent experiments, taking the binding at 0 time as 100%.

for glycine residues in hERβ (Fig. 7A) because lysine residues have been previously described to be crucial in the interaction of other proteins with CaM (24). We tested whether these amino acids located in the postulated ERα calmodulin-binding site (12) were essential for CaM binding. For this purpose, K302 and K303 were substituted for G in the receptors ERα (1–595) and ERα (280–595). Both wild-type (wt) ERα and the mutant ERα (K302G, K303G) [hereafter named as ERα (mut-CaM)] were *in vitro* labeled with [$^{35}$S]-methionine followed by immunoprecipitation with anti-CaM or anti-ER antibodies. The results presented in Fig. 7B show that ERα (wt) was almost equally immunoprecipitated with anti-ER antibodies and with anti-CaM antibodies (Fig. 7B, lanes 2 and 3). Contrary to the wt ERα, only 18% of ERα (mut-CaM) was immunoprecipitated with anti-CaM antibodies (Fig. 7B, lanes 5 and 6). Similar results were obtained when both the wt and mut-CaM ERα (280–595) were analyzed. Thus, anti-CaM antibodies immunoprecipitated 89% of ERα (280–595) as compared with anti-ER antibodies (Fig. 7C, lanes 2 and 3), whereas only 21% of the ERα (mut-CaM) (280–595) coimmunoprecipitated with anti-CaM antibodies (Fig. 7C, lanes 5 and 6). Figure 7 shows one representative IP experiment of many performed with similar results. The right panels in this figure represent the quantification of all data obtained. These data strongly indicate that substitutions of K302 and K303 for G in ERα (mut-CaM) render a receptor with a notably reduced affinity for CaM.

Transcriptional activation studies further demonstrated that these two critical residues for ERα binding to CaM are not essential for ERα transcriptional activation. Thus, when HeLa cells were transiently transfected with ERα (wt) and compared with those transfected with ERα (mut-CaM), both showed similar levels of both basal and E2-induced transcriptional activation. Once again, we observed a striking difference when cells were treated with W7. HeLa cells transfected with wt-ERα were 80% inhibited by $10^{-6}$ M W7, whereas those transfected with ERα (mut-CaM) were completely insensitive to the same concentration of W7 (Fig. 8A). These results indicate that W7 induces conformational changes in CaM, which prevent ERα-dependent transcription probably by destabilization of the E2-ERα-CaM-ERE complex. This destabilization neither occurs in the mutant receptor nor in ERβ due to its inability to bind CaM.

Since the submission of this report, it has been reported by Wang *et al.* (15) that K302 and K303 of hERα are targets for acetylation by p300. These authors have obtained mutants in which K302 and K303 were substituted by A, T, R, or Q. These mutants, like our ERα (mut-CaM), were able to mediate E2 transcriptional activation. In their report, ERα (K302A, K303A) shows a 2.5-fold reduced basal activity while the rest of the mutants were similar to the wt. In their case, all the mutants show enhanced ligand sensitivity in such a way that the activation by $10^{-7}$ M E2 was 2- to 4-fold higher than that of the wt. They suggest that these lysines within the ERα hinge region may play a role in normally repressing ligand-dependent ERα activity. However, we have not observed this enhanced ligand sensitivity in our mutant. In our case, the activation of ERα (mut-CaM) by $10^{-7}$ M E2 is not significantly higher than that of the wt when we used the reporter pS2-Luc (Fig. 8A).

To test this discrepancy, we have performed transient transfection experiments using a stronger reporter (3x-ERE-TATA-Luc). Once again, we have ob-

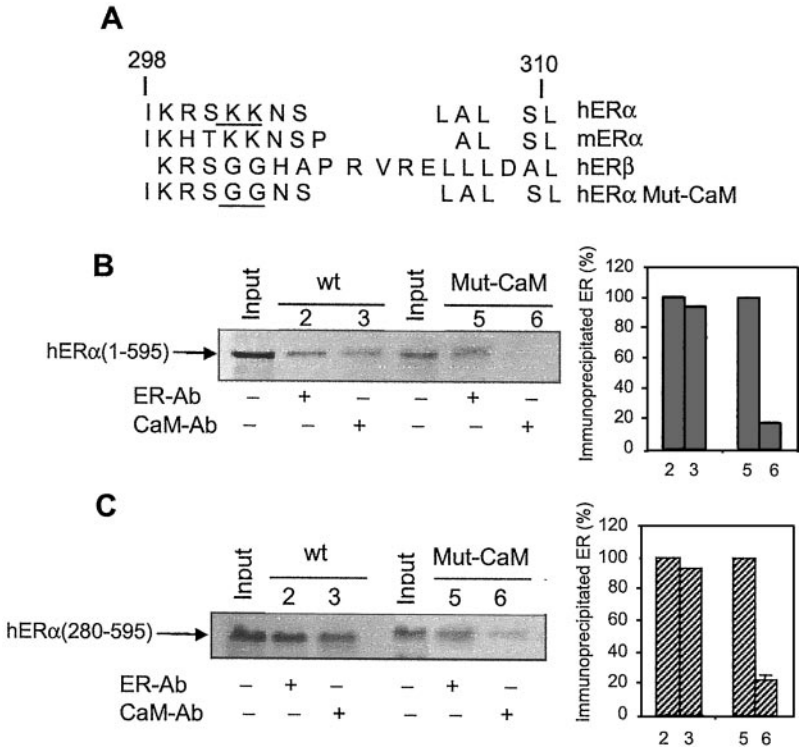


**Fig. 7.** Mutagenesis of ERα CaM-Binding Domain

A, Sequence alignment of the region containing the postulated CaM binding site in human (hERα) and mouse (mERα) ERα, human ERβ (hERβ) and the mutant hERα (mut-CaM) in which K302 and K303 were replaced by G. B, Immunoprecipitation of wt or mut-CaM [$^{35}$S]-hERα (1–595) proteins with polyclonal anti-ER antibodies (ER-Ab) (lanes 2 and 5, respectively) or anti-CaM antibodies (CaM-Ab) (lanes 3 and 6, respectively). The input lanes contain 10% of the total amount of [$^{35}$S]-labeled receptor used in the immunoprecipitations. C, Immunoprecipitation of wt or mut-CaM [$^{35}$S]-hERα (280–595) proteins with anti-ER antibodies (ER-Ab) (lanes 2 and 5, respectively) or anti-CaM antibodies (lanes 3 and 6, respectively). The input lanes contain 10% of the total amount of [$^{35}$S]-labeled receptor used in the immunoprecipitations. Graphs correspond to the quantification of radioactivity and represent the percentage of ERα immmunoprecipitated taking as 100% the ERα immunoprecipitated with anti-ER antibody.

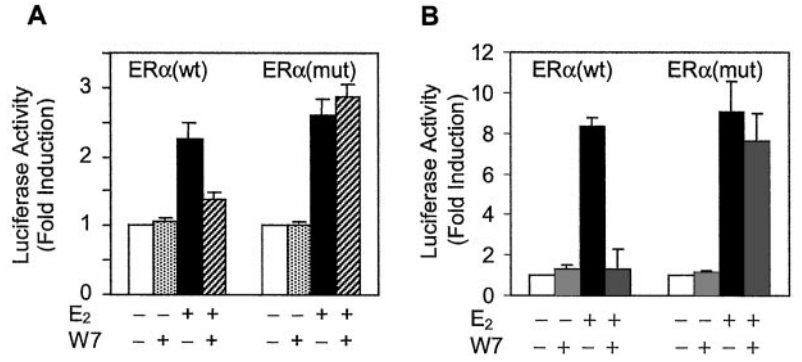


**Fig. 8.** Transactivation Activity of ERα (wt) and ERα (mut-CaM): Sensitivity to the CaM Antagonist W7

A, HeLa cells were transfected with 0.5 μg of pS2-Luc reporter plasmid, 0.1 μg of pcDNA-hERα wt, or pcDNA-hERα (mut-CaM) expression vectors and 50 ng of internal control plasmid pRL-TK. Cells were treated and luciferase activity determined as in Fig. 4A. B, HeLa cells were transfected with 0.5 μg of 3x-ERE-TATA-Luc reporter plasmid, 0.1 μg of pcDNA-hERα (wt) or pcDNA-hERα (mut-CaM) expression vectors and 50 ng of control plasmid pRL-TK. Cells were treated and luciferase activity determined as in Fig. 4A.

tained similar activations of the wt ERα (8.4-fold) and the mutant (9.1-fold) (Fig. 8B). Importantly, the wt ERα activations that we report in Fig. 8, A and B, are in agreement with the results of (25) and are slightly higher than those reported by Wang *et al.* (15). The difference in transactivation in our experiments and those reported by these authors might be due to the fact that our mutant contains different amino acid sub-

stitutions and that we have used different reporter plasmids as well as different culture conditions during transfection and activation.

## DISCUSSION

In this work, we describe for the first time CaM antagonists as specific inhibitors of ERα transcriptional activity. We have demonstrated that: 1) ERα but not ERβ has a CaM binding site and interacts with calmodulin; 2) CaM antagonists act as specific inhibitors of ERα in a dose-dependent manner but show no inhibitory effect over ERβ-mediated transcription; in fact, at certain concentrations there is even an enhancement of the activity of the β isoform in a promoter-dependent fashion; 3) ERα-mediated transcription is sensitive to CaM antagonists, both in ERE and AP1 driven promoters; 4) the effect of CaM antagonists on ERα could be explained by the conformational change that W7 cause on CaM (26, 27) decreasing the stability on the ERα-CaM-ERE complex; and 5) we have generated a mutant ERα (mut-CaM) that can promote E2-dependent transcriptional activation in the same fashion as the wt receptor. This receptor, however, is unable to bind CaM and, as a consequence, becomes insensitive to CaM antagonists. Our findings suggest that CaM might be a specific modulator of ERα functions.

In a previous report (23), we proposed that calmodulin could be a potential candidate for mediating the antiestrogenic effects of melatonin. The interaction of this calcium-regulated protein with ER has been known for several years, and a putative CaM binding site on ER has been postulated (12). After employing different approaches to show that ERα but not ERβ interacts with CaM, we predicted that calmodulin antagonists might function as specific inhibitors of ERα. This is an extremely important possibility because overexpression of ERα has been reported to occur in more than 50% of breast cancers. Moreover, the ratio ERβ/ERα, which is high in normal tissues, decreases during breast and ovarian tumor progression (28, 29) and in both male and female malignant transformation of the colon (30, 31). Together, these data support the idea of ERβ playing a role in preventing the deleterious effects of ERα as previously proposed (4) and favor the concept that CaM antagonists could be of therapeutical importance in tumors with high ERα/ERβ ratio, that is, in advanced tumors. In addition, calmodulin antagonists alone or in combination with antiestrogens decrease the viability and induce apoptosis of breast cancer cells (32–34). Thus, vinorelvine, a cytostatic drug effective against breast cancer and currently in clinical trial phase IV (35–37), is also a potent antagonist of CaM (38). Both the reported data and the results presented in this work, strongly suggest that CaM antagonists may have the potential to act as inhibitor agents of ERα with antitumoral effects on advanced breast, ovarian and colon cancer.

We attempted to address whether our *in vitro* observations reflected the *in vivo* situation. We therefore analyzed the response to CaM inhibitors in cell lines expressing no endogenous ERs and transfected with both receptor isoforms. When ERα is transfected in HeLa cells, it is clear that the transactivation properties of ERα are dependent on the native conformation of CaM, and thus, sensitive to the CaM antagonists (Fig. 4), which act by decreasing the stability of the E2-ERα-ERE complex (Fig. 6, B and C). In contrast, when ERβ is transfected in HeLa cells, its transactivation capacity is not inhibited by a wide range of W7 concentrations able to inhibit ERα (Fig. 4B). Remarkably, the effect of the calmodulin antagonist W7 over the stability of the E2-ERα-ERE complex is very similar to that of melatonin, as we have previously reported (23). W7, as well as melatonin, impairs ER activation by destabilizing the ternary complex in a saturable, specific, and E2-dependent fashion. Recent results in our laboratory point to melatonin as another specific modulator of ERα but not ERβ trancriptional activity (unpublished data).

We next tested the effect of CaM antagonists in breast cancer cell lines expressing ERs. The E2-dependent transcriptional activation in T47D cells, which show a low ERα/ERβ ratio, was not affected by CaM antagonists. On the other hand, in MCF-7 cells, which show a high ERα/ERβ ratio, E2-dependent transactivation was inhibited by W7 and calmidazolium. Interestingly, the inhibition by anti-CaM agents was abolished when ERβ was overexpressed in these cells (Fig. 5D). These *in vivo* results confirm those obtained *in vitro*. The most promising and encouraging result arising from these experiments is that CaM antagonists reduce the transcriptional activation on breast cancer-derived cell lines with a high ERα/ERβ ratio. These results support the notion that novel therapeutical approaches might be developed in the near future, specifically targeting the transcriptional activation by ERα. It should be pointed out that not every cell line responds in the same fashion to CaM antagonists. Thus, calmidazolium did not inhibit E2-dependent transcriptional activation in HeLa cells transfected with ERα (data not shown). We do not know the reason for this resistance, but it is likely that is due to the low permeability of HeLa cells to calmidazolium.

Another important finding here is that CaM antagonists also inhibit ERα-dependent AP1 transcriptional activation (Fig. 4C). It has been previously observed that AP1 activity correlates with acquired tamoxifen resistance in ER+ breast tumors (39) and the ability of tumors to switch from recognizing tamoxifen as an antagonist to recognizing it as an agonist has been proposed as the most likely cause of resistance (40). Our results demonstrate the ability of W7 to abolish the E2-induced ERα/AP1 activation (Fig. 4C). This can explain the synergistic effects of CaM inhibitors and tamoxifen reported elsewhere (32) and provide a novel mechanism to block the mitogenic activity of ERα in both antiestrogen-responsive and antiestrogen-resis-

tant breast cancer cells. It is therefore feasible that CaM antagonists could be developed to be used as alternative pharmacological treatments for ER+ tamoxifen-refractory tumors.

Finally, we have obtained a mutant in the ERα region that has been proposed as a CaM binding site. The two lysine residues (K302, K303) were good candidates to mutagenize because lysines have been reported as critical residues in the interaction of CaM with other proteins. The capability of the mutant receptor ERα (mut-CaM) to interact with CaM is almost completely abolished (Fig. 7). However, the receptor is fully functional as E2-dependent transcriptional activator (Fig. 8). Interestingly, this mutant receptor is not inhibited by CaM antagonists and behaves in a similar fashion to that of the ERβ receptor. The characterization of this mutant receptor will provide another experimental approach for better understanding the mechanisms by which the ER activities are modulated.

Lysines 302 and 303 have been recently reported as targets for acetylation and substitutions by different residues have resulted in enhanced ligand sensitivity (15). These authors suggest that the lysine residues (302, 303) within the ERα hinge region may play a role in normally repressing ligand-dependent ERα activity and indicate that lack of acetylation of these lysines might result in enhanced recruitment of coactivators or loss of binding of transcriptional repressors (15). We have not observed this enhanced ligand sensitivity in our mutant. The basal activity of ERα (K302G, K303G) is not significantly higher than that of the wt, even though we have tested E2-mediated transactivation by means of two different reporter plasmids (Fig. 8). The difference in transactivation in our experiments and those reported by Wang *et al*. (15) might be due to the fact that our mutant contains different amino acid substitutions and the fact that we have used different reporter plasmids as well as different culture conditions during transfection and activation.

Further evidence exists to indicate that these residues (K302, K303) play a crucial role in the ERα regulation *in vivo*. For example, it has been reported that K303 is mutated in 34% of premalignant breast lesions (16). This finding suggests that lysine 303 might be somehow involved in tumorigenesis. Data from both our work and that of Wang *et al.* (15) indicate that these lysines seem to be important for acetylation and for calmodulin binding, although calmodulin binding does not seem to be essential for the receptor activity. Calmodulin might play a role as a regulator in the ERα complex. Because CaM is not essential for transcriptional activity, we therefore ask whether it is important for the acetylation of these residues *in vivo* or, alternatively, whether the acetylation is important for ERα interaction with CaM. These and other questions should be addressed in future studies. It is likely that this region of ERα is extremely important *in vivo* and further investigations will be required to fully understand the regulatory mechanisms that govern the function and biology of ERs.

In summary, CaM antagonists might therefore provide a powerful tool to analyze the distinct roles of ERα and ERβ. To date, the search for specific ERα and ERβ inhibitors has been conducted according to the different affinities of both receptors for various estrogenic substances (9, 10, 41) and more recently the development of antagonist peptides targeting ERα or ERβ has proven to be useful (26, 42). The results presented here might be the starting point of new ways to search for ERα inhibitors that do not inhibit ERβ. Our results indicate a differential mode of regulating the transcriptional activity of ERα an ERβ, and open the possibility for designing new drugs with lower toxicity than W7 and calmidazolium, but with a stronger specific inhibitory effect on ERα based upon the interaction of this receptor with CaM.

## MATERIALS AND METHODS

### Plasmids

The mutant ERα (mut-CaM) was generated by substitution of hERα K302 and K303 for G with the QuikChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA). Plasmids pcDNA-hERα (1–595) and pcDNA-hERα (280–595) were used as templates. The mutant cDNAs were amplified by PCR with *Pfu* turbo DNA polymerase using the oligonucleotides, ERMut1: 5′-CGCTCATGATCAAACGCTCTGGGGGGAACAG-CCTGGCCTTGTCCC-3′ and ERMut2:5′-GGGACAAGGCCAG-GCTGTTCCCCCCAGAGCGTTTGATCATGAGCG-3′ (mutagenic sequence is *underlined*). PCR products were digested with *Dpn*I according to the manufacturer's instructions. Mutations were confirmed by sequencing.

The ERα (1–595) and ERα (280–595) cDNAs were amplified by RT-PCR from MCF-7 cells using the following primers: 5′-CGGGATCCATGACCATGACCCTC-3′ (at positions 291–307), 5′-CGGGATCCGGGTCTGCTGGAGAC-3′(at positions 1133–1147) and 5′-GCGAATTCTCAGACTGTGGCAGGG-3′(at positions: 2065–2082). DNA sequences were confirmed and subcloned as *Bam*HI/*Eco*RI fragments into pcDNA3 (Invitrogen, San Diego, CA) and pGEX-2TK (Amersham Pharmacia Biotech) vectors. pERE-TK-Luc, pS2-Luc and pCMX-mERβ were kindly provided by Dr. V. Giguere from the R. W. Johnson Pharmaceutical Research Institute (Don Mills, Ontario, Canada). pCXN2-hERβ (1–530), GST-hERα (117–595) and GST-hERβ (1–530) (43), *Renilla* luciferase plasmid (pRL)-TK (Promega Corp., Madison, WI), Δcoll 73-Luc and Δcoll 60-Luc (5) were also used in this work. The plasmid 3x-ERE-TATA-Luc was kindly provided by Dr. S. Safe from the Department of Veterinary Physiology and Pharmacology, Texas A&M University (College Station, TX).

### Cell Culture and Transient Transfection Assays

HeLa cells were propagated in phenol-red DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 100 μg/ml gentamicin and 10% heat-inactivated FBS (Roche Molecular Biochemicals, Mannheim, Germany) in a humidified atmosphere of 5% $CO_2$ at 37 C.

Before transfection, HeLa cells were seeded in 12-well plates and incubated 12–18 h at 37 C. Then, cells were transferred to phenol-red free DMEM containing sFCS and maintained for 3 d. At 60–80% confluency, cells were transfected with 0.5 μg of ERE-driven or AP1-driven reporter plasmids, 0.1 μg of ER expression vector and 50 ng of an internal control *Renilla* luciferase plasmid, pRL-TK (Promega

Corp.) using FuGENE 6 Transfection Reagent from Roche Molecular Biochemicals as recommended by the manufacturer's protocol/instructions. After 18–24 h, medium was renewed and cells were stimulated during 24 h with different chemicals, as indicated.

MCF-7 cells and T47D cells were propagated in RPMI 1640 medium containing 25 mM HEPES/NaOH, pH 7.3, and synchronized cells were transfected as above. When indicated, ERβ expression vector or the empty vector were included in the transfection.

For luciferase assays, cells were washed with PBS and lysed by the addition (per well) of 200 μl of passive lysis buffer (PLB) 1× as recommended by Dual Luciferase System (Promega Corp.). After centrifugation, supernatants were saved as extracts. Luciferase activities were determined in 20 μl of each cellular extract and experimental values were normalized to *Renilla* luciferase activity, to correct for differences in transfection efficiency.

The results represent the means ± SD of three independent experiments performed at least in duplicate. All data presented in this work show statistically significant differences ($P < 0.05$). The *t* test was used for the statistical analysis.

Transactivation experiments were performed with both mouse and human ERβ, and identical trends in ligand behavior were shown by both ERβs in HeLa cells.

### EMSA and Determination of the Stability of the E2-Dependent ER Binding to DNA

Nuclear extracts were obtained from MCF-7 cells maintained for 3 d in phenol-red free RPMI media with 0.5% of sFCS, as we have previously described (23).

Nuclear extracts (2 μl), containing 5 μg of protein, were mixed with 10 μl of buffer B (20 mM HEPES-KOH, pH 7.9; 10 mM $MgCl_2$; 1 mM EDTA; 10% (vol/vol) glycerol; 100 mM KCl; 0.2 mM phenylmethylsulfonyl flouride (PMSF); 0.2 mM dithiothreitol, 0.5% Nonidet P-40; and protease inhibitors) and incubated with 1 μg of poly(deoxyinosine-deoxycytidine) in a total volume of 20 μl. Mixtures were preincubated at 0 C for 15 min, followed by incubation with the indicated hormones at 0 C for 10 min. Ethanol hormone solutions were diluted at the required hormone concentrations in 1% BSA containing 10% ethanol keeping the ethanol concentration at 0.1% during the assay. The binding reaction was then initiated by adding a [$^{32}$P] 5′ end-labeled synthetic ERE double-stranded oligonucleotide 5′-TCGAAAAGTC***AGGTCA***CAG***TGACCT***-GATCAATCGA-3′ (10 fmol containing 3–5 × $10^4$ dpm), which corresponds to sequence −338 to −312 of the promoter upstream element of the *Xenopus* vitellogenin A2 gene (23). The reaction with the probe was allowed to bind for 1 h at 0 C, followed by incubation for 30 min at 20 C. The samples were analyzed in a preelectrophoresed (10 mA) 5% polyacrylamide gel (acrylamide to bisacrylamide ratio of 40:1) in 45 mM Tris-borate and EDTA 1 mM at 11 mV/cm. After 2–3 h, suitable separation was achieved, and the gel was vacuum-dried. Gels were exposed to obtain autoradiographies and were quantified with an Instantimager (Packard). For specificity assays, different concentrations of unlabeled competitor ERE oligonucleotide were mixed with the labeled probe before adding them to the binding reaction. When indicated, monoclonal anti-ER antibodies NCL-ER-LH1 (Novocastra Laboratories, Newcastle-upon-Tyne, UK) were added before probe addition.

To determine the effect of W7 on the stability of E2-ER-ERE complex, nuclear extracts were assayed for ERE binding in the presence of $10^{-8}$ M E2 with or without $10^{-4}$ M W7. After the E2-ER-ERE complex was formed (20 min at 20 C), a 200-fold excess of unlabeled ERE was added and incubation at 20 C was pursued. At the indicated times, 20-μl samples were withdrawn and loaded on the electrophoresis gel.

### *In Vitro* Transcription and Translation and Immunoprecipitation Assays

Plasmid DNA (1 μg) containing the indicated ER cDNA was used to produce [$^{35}$S]-methionine-labeled ERs. DNA was added to 40 μl of a coupled transcription-translation system (Promega Corp.) and 10 μCi of Pro-mix (14.3 mCi/ml, >1000 Ci/mmol; Amersham Pharmacia Biotech). The reaction was performed for 90 min at 30 C. After this time, aliquots (4.5 μl) of reticulocyte lysate were mixed with 45 μl of immunoprecipitation buffers containing either HEPES-KOH 10 mM (pH 7.9), 5 mM $MgCl_2$, 0.5 mM EDTA, 100 mM KCl, 0.2 mM PMSF, 0.2 mM dithiothreitol, 0.5% Nonidet P-40, and protease inhibitors or PBS (pH 7.2), NaCl 125 mM, 0.25% Nonidet P-40 and 0.1% SDS. The samples were incubated for 10 min at 0 C with the indicated additions and then immunoprecipitated with 1 μg of goat anti-CaM antibodies (SC-1988), 5 μl of rabbit polyclonal anti-ER antibodies raised in our laboratory against the C-terminal (residues 280–595) of human ERα (23), or 5 μl of rabbit polyclonal anti-Pho4 antibodies. After 60 min at 4 C, the immunocomplex was collected with 20 μl of drain protein G-Sepharose (30 min at 4 C), and washed three times with either HEPES-KOH 10 mM (pH 7.9), 5 mM $MgCl_2$, 0.5 mM EDTA, 100 mM KCl, 0.2 mM PMSF, 0.2 mM dithiothreitol, 0.5% Nonidet P-40, and protease inhibitors or PBS (pH 7.2), 250 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxicolic acid, 0.1% SDS, and 0.05% BSA. Proteins were analyzed by SDS-PAGE, and [$^{35}$S]-labeled ERs were detected by autoradiography.

*In vivo* immunoprecipitation from MCF-7 cells was performed as follows: cells (10 × $10^7$ were lysed in 1 ml buffer containing Tris-HCl 50 mM (pH 7.4), NaCl 150 mM, Nonidet P-40 0.5%, NaF 50 mM, $Na_3VO_4$ 0.1 mM, PMSF 1 mM and leupeptin 10 μg/ml. Five milligrams of protein from the lysates was incubated with 1 μg of goat polyclonal anti-CaM antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 5 μl of monoclonal anti-ERα (C-314 Santa Cruz Biotechnology, Inc.) antibodies or 5 μl of rabbit polyclonal anti-Pho4 antibodies for 2 h at 4 C. Immunocomplexes were then incubated with Protein G-Sepharose for 1 h at 4 C, collected by centrifugation, washed three times in buffer A and solubilized in Laemmli sample buffer. Immunoprecipitated proteins were then analyzed by electrophoresis and Western blot with rabbit polyclonal anti-ER antibodies.

### Western Blot Analysis

Western blot analysis was carried out as described (23) using rabbit polyclonal anti-ER antibodies raised in our laboratory against the C-terminal (residues 280–595) of human ERα for ERα detection. ERβ was detected using the specific rabbit polyclonal anti ERβ antibody (SC-8974) raised against the N terminal (residues 1–150) of ERβ. Goat antirabbit IgG antibodies coupled to horseradish peroxidase (Sigma) were used as secondary antibodies. Immunoreactive bands were visualized with the ECL detection system (Amersham Pharmacia Biotech).

### *In Vitro* Interaction of GST-ER Hybrid Proteins with CaM

Expression and purification of recombinant proteins was essentially as described by Frangioni (44). Fluorescence experiments were performed in a Perkin-Elmer Corp. (Foster City, CA) fluorimeter using a 100 μl cuvette. A quantity of 2.5 nmol of dansyl-CaM (Sigma) were dissolved in 100 μl of 10 mM morpholinopropane sulfonic acid (pH 7.2), 1 mM $MgCl_2$, 100 mM KCl, and 1 mM $CaCl_2$. Emission fluorescent spectra were obtained ($\lambda_{Ex}$ 333 nm) before and after addition of 1.5 μg of GST-ERα (1–595) and E2 ($10^{-8}$ M), successively. Similar experiments were performed with 4.5 μg of GST-ERβ (1−530)

and 10 μg of GST-ERα (280–595). Equivalent amounts of buffer were added to the controls.

Interaction of purified GST-proteins with biotin-CaM was determined by far Western experiments. 3.0 μg of GST-ERα (1−595), 6.0 μg of GST-ERβ (1–530) and 15 μg of GST-ERα (280–595) were subjected to SDS-PAGE and electroblotted onto nitrocellulose filters. Proteins were denatured and renatured as described (45) and filters were blocked for 60 min with a buffer containing: 50 mM Tris-HCl, pH 7.5; 0.2 M NaCl; 50 mM $MgCl_2$; 0.5 mM $CaCl_2$; and 5% of BSA. Next, biotin-CaM (Calbiochem, San Diego, CA) was added up to 100 ng/ml and incubated for 1 h. Then, filter was washed and CaM binding proteins were visualized with horseradish peroxidase-linked streptavidin and ECL detection (Amersham Pharmacia Biotech) (46). After stripping filters, ER immunoreacting proteins were detected by Western blot with rabbit polyclonal antibodies (23).

## Acknowledgments

We wish to thank Dr. Ana Aranda for critically reading this manuscript and Ana Corao Trueba for technical assistance. We also thank Dr. Vincent Guiguere for providing us with pERE-TK-Luc, pS2-Luc and pCMX-mERβ, Dr. Stephen Safe for providing us with 3x-ERE-TATA-Luc plasmid, and Dr. Colin Goding for Anti-Pho4 antibodies.

Received May 4, 2001. Accepted January 17, 2002.

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This work was supported by grants from CICYT SAF/96–0132, Fondo de Investigaciones Sanitarias 00/1086, and CICYT SAF/98–0174. J.M.G.P. was recipient of a fellowship from Junta Provincial de Asturias de la Asociación Española Contra el Cáncer, B.R.L. was the recipient of a fellowship from Fundación Científica de la Asociación Española Contra el Cáncer. C.M.-C. was recipient of a fellowship from IUOPA (Instituto Universitario de Oncología del Principado de Asturias, Obra Social Cajastur).

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