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Ca^{2+}-dependent Binding of Tamoxifen to Calmodulin Isolated from Bovine Brain[1]

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ABSTRACT

The interaction of the antiestrogen tamoxifen (Tx) with calmodulin (CaM) was investigated by cross-linking between the protein and [^{3}H] tamoxifen aziridine. We observed that CaM binds Tx in a Ca^{2+}-dependent manner and that two components are involved in the binding, with apparent dissociation constants (K_d) of about 6 nM and 9 μM. The high affinity binding site has a maximal capacity of 25 pmol/mg protein, whereas the low affinity binding site has a B_{max} value of 120 nmol/mg protein. The stimulatory effect of Ca^{2+} is maximal at the pCa value of 5, and it is noncompetitively inhibited by Mg^{2+}. In the micromolar range, the cation-dependent interaction of Tx with CaM exhibits positive cooperativity ($n_H = 1.4$) and it is specific in the sense that it is inhibited by unlabeled Tx and by the CaM antagonist trifluoperazine. In contrast, no specificity was observed for the Tx binding, which is cation independent. Tx in the nanomolar range forms complexes with CaM which can be visualized by fluorography after electrophoretic separation in a polyacrylamide gel. Furthermore, CaM antagonism of Tx was observed with respect to inhibition of the CaM effect on the RBC membrane (Ca^{2+} + Mg^{2+})-ATPase. The results indicate that Tx may alter Ca^{2+}-dependent processes by interacting directly with CaM.

INTRODUCTION

Tx,[3] a synthetic nonsteroidal antiestrogen, has been successfully used in the therapy of human breast cancer (1–3). However, the mechanism by which Tx inhibits mammary cancer cell proliferation is not well understood. It has been suggested that Tx antagonizes the growth-enhancing activity of estradiol by competing with the hormone for binding to estrogen receptors (4–7). However, it has been reported that antiestrogen binding sites, distinct from the estrogen receptors, exist in the cell and bind Tx with high affinity (8–13). Furthermore, some of the biological effects of Tx are not reversed by estrogen (6, 14), which indicates that Tx acts on target molecules other than the estrogen receptor.

CaM, which is a regulatory protein of many cellular processes dependent on Ca^{2+}, has been suggested as a good candidate for mediating estrogen-independent processes involved in cell proliferation (15–17). Indeed, some authors reported that Tx inhibits the activation of cAMP phosphodiesterase by CaM through a process similar to that of the phenothiazines (16, 18). It has been suggested that the structural similarities between triphenylethylene antiestrogens and phenothiazines form the basis for the CaM antagonism of Tx, since both types of drugs contain an hydrophobic region of aromatic rings and an hydrophilic cationic alkyl amino side chain (19, 20), which interact with the CaM domain exposed in the presence of Ca^{2+} (21). These observations support the idea that Tx may interact directly with CaM. However, a detailed analysis of this interaction has not yet been reported.

In this work, we studied the characteristics of Tx binding to CaM isolated from bovine brain and we investigated the involvement of the Tx-CaM complex in the inhibition of the CaM-modulated (Ca^{2+} + Mg^{2+})-ATPase of the RBC membrane.

MATERIALS AND METHODS

Materials. [^{3}H]Tamoxifen aziridine (26 Ci/mmol), nonradiolabeled tamoxifen, and Amplify were purchased from Amersham (London, England); dextran T70 from Pharmacia (Uppsala, Sweden); *N*,*N*-dimethylformamide from Merck (Darmstadt, Germany); charcoal from Norit, monothioglycerol, and *N*-tris[hydroxymethyl]-2-amino-ethane sulfonic acid from Sigma (St. Louis, MO).

Binding of [^{3}H]TAZ to CaM Isolated from Bovine Brain. CaM was isolated from bovine brain cortex as described by Alface and Pires (22). The purified CaM (10–20 μg) was incubated 3 h, at 25°C, in a medium containing 10 mM *N*-tris[hydroxymethyl]-2-aminoethane sulfonic acid (pH 7.0), 12 mM monothioglycerol, 250 mM sucrose, 5–25 nM [^{3}H] TAZ, 5 mM EDTA (if present), 0.5 mM $CaCl_2$ or 5 mM $MgCl_2$ or $CaCl_2$ plus $MgCl_2$ (0.5 mM plus 5 mM), in a total volume of 200 μl. Aliquots of 50 μl of charcoal-dextran suspension (2.5% charcoal Norit A and 0.25% dextran T70 in 10 mM *N*-tris[hydroxymethyl]-2-aminoethane sulfonic acid, pH 7.0, 12 mM monothioglycerol, 250 mM sucrose) were added to each sample. The tubes were then transferred to an ice-water bath and kept for 15 min with occasional vortexing. Then, the tubes were centrifuged 10 min at 10,000 rpm in an Eppendorf microfuge. Finally, the radioactivity was measured in 150 μl of the supernatant fluid.

Some experiments were carried out in the presence of different Ca^{2+} concentrations adjusted with 1 mM ethylene glycol bis(*N*,*N*′-tetraacetic acid).

The optimal concentrations of CaM and [^{3}H]TAZ, as well as the optimal temperature and incubation time, were tested to achieve maximal (Ca^{2+} + Mg^{2+})-dependent binding.

Following the precautions of Katzenellenbogen *et al.* (23), [^{3}H]TAZ was diluted from the stock (in ethanol) into dimethylformamide just before the experiment. Then, it was added directly to the incubation medium to yield a final concentration of 5–8 nM [^{3}H]TAZ and about 5% dimethylformamide.

In the experiments using micromolar concentrations of Tx, we used 8 nM [^{3}H]TAZ and we added unlabeled Tx to obtain the desired Tx concentration in the reaction medium.

Assay of Competition between [^{3}H]TAZ and Nonradioactive Tx or Trifluoperazine for Tx Binding Sites of CaM. CaM (10 μg) was incubated with 8 nM [^{3}H]TAZ, at 25°C for 1 h, in the presence of $CaCl_2$ (0.5 mM), $MgCl_2$ (5 mM), and nonradioactive Tx or trifluoperazine (0.1–260 μM). Bound and unbound ligand were separated by the DCC technique described above, and the radioactivity was counted in the resulting supernatant.

Fluorography of CaM Labeled with [^{3}H]TAZ. CaM (100 μg) was incubated 3 h, at 25°C, with [^{3}H]TAZ (40 nM) in the presence or absence of cations. The assay was performed in the same experimental conditions as described above. The unbound [^{3}H]TAZ was removed by the DCC technique and about 60 μg of the CaM, contained in the supernatant, were electrophoresed in a 10% Laemmli one-dimension slab gel (24). The gel was stained with Coomassie blue, soaked in Amplify 30 min in the dark, and dried in a gel slab drier (Pharmacia, model GSD-4). Then, it was fluorographed at −80°C using a Du Pont Cronex 2D X-ray film.

Assay of ATPase Activity. CaM-depleted RBC plasma membrane was isolated by the method of Buckley (25).

The (Ca^{2+} + Mg^{2+})-ATPase activity was measured at 25°C in a medium containing 2 mM Tris, 40 mM KCl, 20 mM NaCl, 50 μM $CaCl_2$,

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[3] The abbreviations used are: Tx, tamoxifen; CaM, calmodulin; [^{3}H]TAZ, [^{3}H] tamoxifen aziridine; DCC, dextran-coated charcoal.

and 3 mM $MgCl_2$, at pH 7.2. To 183 μl of this buffer were added 15 μl of CaM solution (10 μg), 5 μl of different concentrations of Tx in dimethylformamide, and 50 μl of the RBC ghost suspension (250 μg of protein). The reaction was started by adding 2 mM ATP. The ATP hydrolysis was determined by measuring the continuous release of H^+ for 10–15 min, as previously described (26, 27). In some experiments, (Ca^{2+} + Mg^{2+})-ATPase purified by CaM-affinity chromatography was used instead of native membranes.

Control experiments were performed to measure the effect of dimethylformamide in the CaM-stimulated Ca^{2+}-ATPase activity.

RESULTS

Binding of [^{3}H]TAZ to CaM. Tx binds to CaM in a cation-dependent manner. Fig. 1 shows that Ca^{2+} has a strong stimulatory effect on the binding of [^{3}H]TAZ to CaM. At 5 nM Tx, CaM binds about 21 pmol of [^{3}H]TAZ/mg of CaM in the presence of Ca^{2+}, whereas only 7 pmol of drug are bound in the absence of cations (EDTA present). Added Mg^{2+} has a slight stimulatory effect on the [^{3}H]TAZ binding by CaM (≈11 pmol/mg CaM), but it reduces the Ca^{2+} effect from 21 to 18 pmol [^{3}H]TAZ/mg CaM when both cations are simultaneously added to the reaction medium. The effect is in agreement with the results depicted in Fig. 2, where it is evident that Mg^{2+} reduces the [^{3}H]TAZ binding of CaM, even at high Ca^{2+} concentrations.

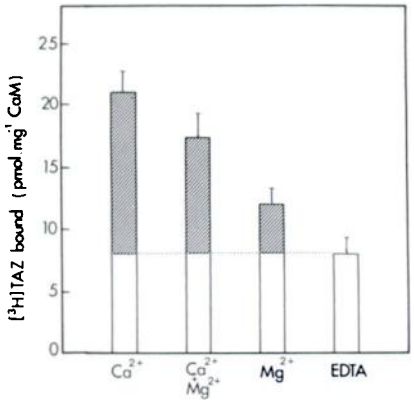

Fig. 1. Effect of divalent cations on the binding of [^{3}H]TAZ to purified CaM. CaM (10 μg) was incubated with 5 nM [^{3}H]TAZ in the presence of Ca^{2+} (0.5 mM), $MgCl_2$ plus $CaCl_2$ (0.5 mM plus 5 mM), $MgCl_2$ (5 mM), or EDTA (5 mM) in a medium containing 10 mM *N*-tris[hydroxymethyl]-2-aminoethane sulfonic acid, pH 7.0, 250 mM sucrose, and 12 mM monothioglycerol. The unbound [^{3}H]TAZ was removed by the DCC technique, as described in "Materials and Methods." The radioactivity was counted in the resulting supernatant, and the amount of bound [^{3}H]TAZ was expressed per mg of protein. □, cation-independent binding of [^{3}H]TAZ to CaM; ■, cation-dependent binding of [^{3}H]TAZ to CaM.

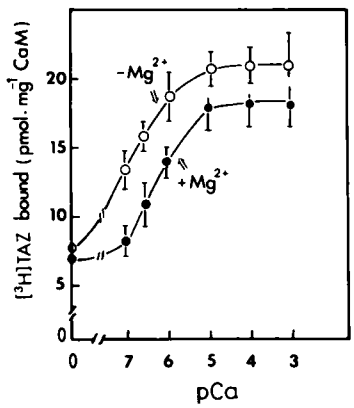

Fig. 2. Effect of pCa on the interaction of [^{3}H]TAZ with CaM. CaM (10 μg) was incubated in the presence or absence of Mg^{2+} (5 mM) with [^{3}H]TAZ (5 nM), at different free calcium concentrations (pCa values between 7 and 3), as described in "Materials and Methods." ●, presence of Mg^{2+}; ○, absence of Mg^{2+}.

Above 10^{-5} M Ca^{2+}, about 22 pmol [^{3}H]TAZ/mg CaM are bound to CaM in the absence of Mg^{2+}, whereas in its presence the [^{3}H]TAZ binding to CaM is about 18 pmol/mg CaM.

The stimulatory effect of Ca^{2+} increases as the Ca^{2+} concentration in the medium increases, reaching a maximal value at about 10^{-5} M (pCa = 5) (Fig. 2). It appears that the optimal pCa value for the [^{3}H]TAZ binding to CaM is not altered by the presence of Mg^{2+}, suggesting that Mg^{2+} acts by a mechanism other than simple competition.

The Ca^{2+}-dependent binding of Tx to CaM is observed at concentrations of Tx as low as 5–25 nM and it is saturable (Fig. 3, *left*), with a K_d of about 6 nM and a B_{max} of 25 pmol/mg CaM (Fig. 3, *right*). On the other hand, we found that, in the micromolar range of Tx concentrations, there is another class of Ca^{2+}-dependent binding sites with a K_d of about 10 μM and a B_{max} of 120 nmol/mg CaM (Fig. 4). The second component of the graph represents nonspecific Tx binding, which is Ca^{2+} independent. We observed that, at concentrations above 80 μM, no significant difference exists between Tx binding (≈175 nmol/mg CaM) in the presence or absence of Ca^{2+} (Fig. 4). It appears that Ca^{2+}-independent binding continuously increases as Tx concentration in the medium increases, whereas Ca^{2+}-dependent binding saturates and is masked by the Ca^{2+}-independent component at high Tx concentrations (Fig. 4). We also observed that, in the micromolar range Tx concentrations, the

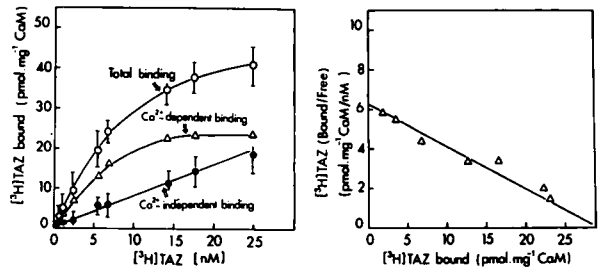

Fig. 3. Characterization of high affinity Tx binding to CaM. CaM (20 μg) was incubated with various concentrations of [^{3}H]TAZ in the presence of Ca^{2+} (0.5 mM) or EDTA (5 mM) in a buffer solution, as described in Fig. 1. The bound and unbound Tx were separated by the DCC technique, as described in "Materials and Methods." *Left*, saturation curve; *right*, Scatchard analysis. ○, total binding; △, Ca^{2+}-dependent binding; ●, Ca^{2+}-independent binding. The Ca^{2+}-dependent binding was obtained by subtracting the Ca^{2+}-independent binding from the total binding.

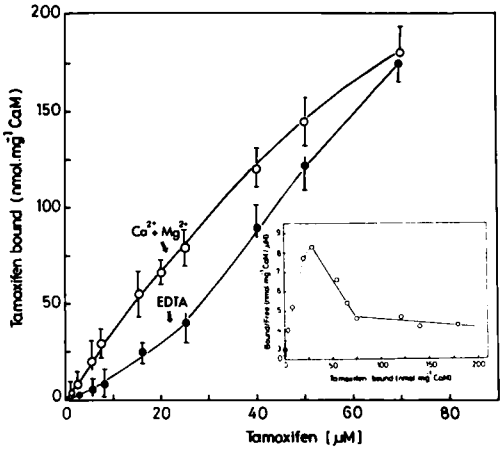

Fig. 4. Characterization of low affinity Ca^{2+}-dependent binding of Tx to CaM. CaM (20 μg) was incubated with various concentrations (0.5–80 μM) of Tx in the presence of Ca^{2+} plus Mg^{2+} (0.5 mM plus 5 mM) or EDTA (5 mM), in a buffer solution containing 8 nM [^{3}H]TAZ and the components described in Fig. 1. ○, Tx binding in the presence of Ca^{2+}; ●, Tx binding in the presence of EDTA. *Inset*, Scatchard plot of the same data. Only those points where 8 μM < [Tx] < 25 μM were used in the determination of B_{max}.

Ca^{2+}-dependent binding of Tx to CaM is cooperative, with an Hill coefficient of 1.4 (Fig. 5), in agreement with the irregular Scatchard analysis observed between 0.5 and 7 μM Tx (Fig. 4, *inset*). Furthermore, we found that the Ca^{2+}-dependent binding of Tx to CaM is specific, as judged by the competition studies depicted in Fig. 6. It is shown that unlabeled Tx or trifluoperazine reduces the amount of [^{3}H]TAZ bound to CaM in a Ca^{2+}-dependent manner but the inhibition occurs only above 7 μM concentrations of the unlabeled drugs. Below this concentration there is no drug competition, since cooperative effects occur (Fig. 6), in agreement with the observation of Fig. 4, *inset*. In contrast, Tx binding was not decreased by the competitors when the assay was performed in the absence of cations. It appears, therefore, that Ca^{2+}-dependent Tx binding is CaM specific, whereas Ca^{2+}-independent binding is nonspecific in the sense that it cannot be displaced by an excess of Tx or trifluoperazine.

The IC_{50} values found for Tx and trifluoperazine competition were about 40 μM (Fig. 6*A*) and 50 μM (Fig. 6*B*), respectively, which indicates that, in the micromolar range, Tx and trifluoperazine are similarly bound to CaM.

Fluorography of CaM Labeled with [^{3}H]TAZ. The effect of cations on the interaction of Tx with purified CaM can be visualized by fluorography after gel electrophoresis of CaM previously cross-linked with [^{3}H]TAZ (Fig. 7). Under these conditions, only covalently bound [^{3}H]TAZ remains bound to CaM. The nonspecific binding of Tx to CaM is disrupted by urea (8 M)-containing buffer before the electrophoresis, so that it is not visualized in the gel.

In the presence of Ca^{2+}, CaM appears strongly labeled by [^{3}H]TAZ (Fig. 7*B*, *lanes 1* and *2*), whereas, in the absence of cations (EDTA present), no labeling is observed (Fig. 7*B*, *lane 4*). In contrast to the strong stimulatory effect of Ca^{2+}, Mg^{2+} promotes a negligible interaction between CaM and [^{3}H]TAZ (Fig. 7*B*, *lane 3*). As a control (Fig. 7*A*), we observed that neither treatment of CaM with [^{3}H]TAZ (Fig. 7*A*, *lane 1*, *2*, *3*, and *4*) nor treatment with DCC, used to remove unbound [^{3}H]TAZ (Fig. 7*A*, *lane 5*), alters the normal electrophoretic mobility or the amount of CaM recovered in the gel (Fig. 7*A*, *lane 6*).

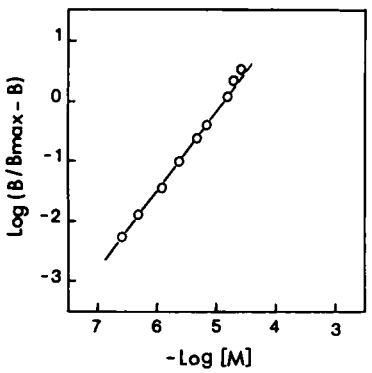

Fig. 5. Hill plot analysis of Tx binding to CaM. The plot was performed with the results (Ca^{2+}-dependent binding) depicted in Fig. 4.

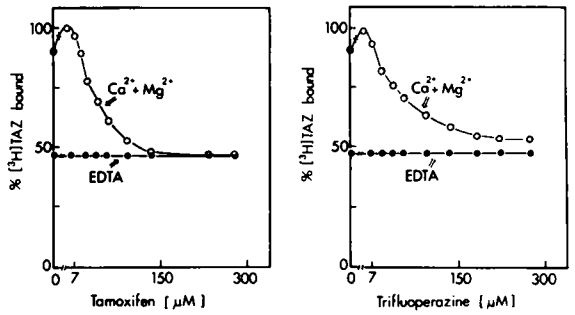

Fig. 6. Competition between [^{3}H]TAZ and nonradiolabeled Tx or trifluoperazine for Ca^{2+}-dependent Tx binding sites of CaM. CaM (15 μg) was incubated 1 h at 25°C with various concentrations (0.1–260 μM) of Tx or trifluoperazine, in a medium containing Ca^{2+} plus Mg^{2+} (0.1 mM plus 5 mM) or EDTA (5 mM) and 8 nM [^{3}H]TAZ, in a buffer solution as described in Fig. 1. The unbound ligands were removed by the DCC technique as described in "Materials and Methods." *Left*, competition between [^{3}H]TAZ and unbound Tx; *right*, competition between [^{3}H]TAZ and trifluoperazine. ○, presence of Ca^{2+}, ●, absence of cations (EDTA present).

Effect of Tx and Trifluoperazine on the (Ca^{2+} + Mg^{2+})-ATPase Activity of RBC Membranes. RBC membranes, previously depleted of endogenous CaM by treatment with EDTA, exhibit an ATPase activity which is significantly stimulated by addition of exogenous CaM. Fig. 8 shows that concentrations of Tx up to 50 μM inhibit the stimulatory effect of CaM on the ATPase (40 nmol of ATP hydrolyzed/mg of protein), whereas the basal ATPase, measured in the absence of CaM, is not affected.

The IC_{50} value (≈15 μM) obtained for Tx inhibition of the ATPase is similar to that (≈18 μM) obtained for trifluoperazine inhibition of the enzyme (Fig. 8), which indicates that, in the micromolar range, both drugs have similar CaM antagonism, in agreement with the observations depicted in Fig. 6.

We also observed that the inhibitory effect of Tx on the RBC membrane (Ca^{2+} + Mg^{2+})-ATPase activity can be reversed by adding an excess of CaM (Fig. 9). At about 30 μM Tx, the (Ca^{2+} + Mg^{2+})-ATPase activity was recovered from about 40 to 60 nmol ATP hydrolyzed/mg protein/min by adding 80 μg excess of endogenous CaM. This observation suggests that the effect of Tx is probably mediated by CaM rather than by a direct effect on the enzyme. Interestingly, we observed that, using purified (Ca^{2+} + Mg^{2+})-ATPase, the CaM stimulation of the enzyme is inhibited by 50 nM Tx (results not shown), which indicates that CaM inactivation occurs in the nanomolar range of Tx concentrations. Presumably, when native membranes are used, most of the Tx added (micromolar range) is bound to membrane lipids, so that the actual free Tx concentration available to the Tx target molecule (CaM) is less than expected.

The results indicate that CaM has two classes of Ca^{2+}-dependent binding sites for Tx and that it is functionally inactivated by interaction with the antiestrogen drug.

DISCUSSION

The binding of Tx to CaM isolated from bovine brain was studied by cross-linking between [^{3}H]TAZ and the protein. [^{3}H]TAZ behaves as Tx except that it is irreversibly bound, whereas Tx binding is reversible (23, 28). We observed that the interaction of Tx with CaM is influenced by Ca^{2+} and Mg^{2+}. Ca^{2+} and Mg^{2+} increase the binding of the drug to CaM, but the stimulatory effect of Ca^{2+} is more potent than that of Mg^{2+} (Fig. 1). When both cations are present in the medium, we found that Mg^{2+} reduces the stimulation by Ca^{2+}, but the effect is not due to simple competition between these cations. Indeed, we observed the same amplitude of the Mg^{2+} effect even at high concentrations of Ca^{2+} in the reaction medium (Fig. 2). It appears that Mg^{2+} modifies the Ca^{2+}-dependent CaM conformations, so that it is differently recognized by several ligands. Thus, whereas Mg^{2+} inhibits the Ca^{2+}-dependent binding of the CaM antagonist Tx, it enhances the binding of CaM to its receptors in various types of biomembranes (29–31). These observations are supported by those of Tanokura and Yamada (32), who suggested that the overall structure of the Ca^{2+}-CaM complex is loosened in the presence of Mg^{2+} and tightened in

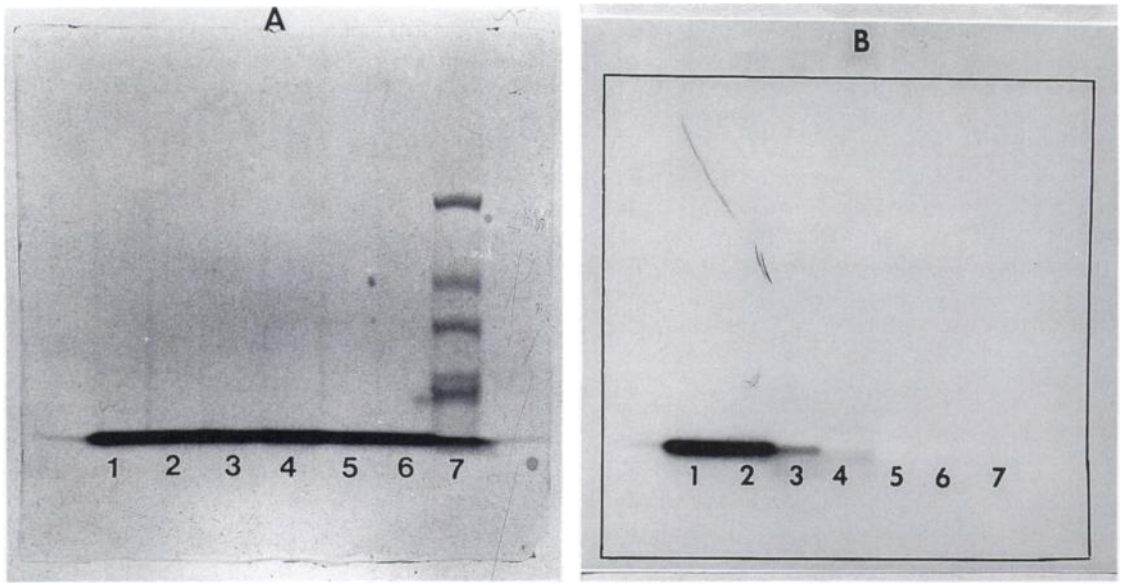

Fig. 7. Interaction of [^{3}H]TAZ with CaM. CaM (100 μg) was incubated 3 h at 25°C with [^{3}H]TAZ (40 nM) in the presence or absence of cations, as described in "Materials and Methods." The unbound [^{3}H]TAZ was removed by the DCC technique and about 60 μg of the CaM, contained in the resulting supernatant, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *A*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *B*, fluorogram of the gel electrophoresis. The reaction was carried out in the presence of: *lane 1*, Ca^{2+} (0.5 mM); *lane 2*, Ca^{2+} plus Mg^{2+} (0.5 mM plus 5 mM); *lane 3*, Mg^{2+} (5 mM); or *lane 4*, EDTA (5 mM). *Lane 5*, control performed in the presence of Ca^{2+} plus Mg^{2+} (0.5 mM plus 5 mM) but without [^{3}H]TAZ; *lane 6*, stock CaM solution (≈60 μg); *lane 7*, molecular mass markers:bovine albumin, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa; calmodulin, 17 kDa.

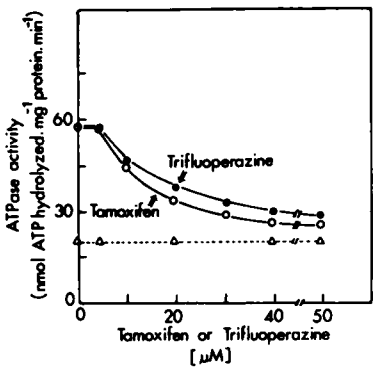

Fig. 8. Effect of Tx and trifluoperazine on the CaM-stimulated (Ca^{2+} + Mg^{2+})-ATPase of RBC plasma membranes. The activity of (Ca^{2+} + Mg^{2+})-ATPase was determined in the absence or presence of Tx or trifluoperazine (1–50 μM) and 10 μg of CaM, as described in "Materials and Methods." ——, CaM-stimulated ATPase; - - -, basal ATPase. ○, presence of Tx, ●, presence of trifluoperazine. The points represent the mean values obtained from three experiments.

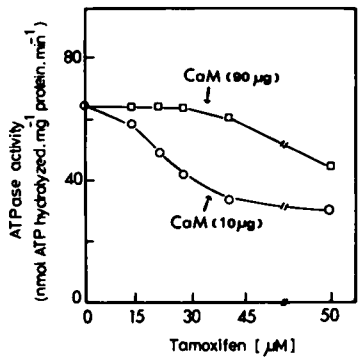

Fig. 9. Reversal of the inhibitory effect of Tx on the ATPase by an excess of CaM. The ATPase activity was determined in the presence of 10 or 90 μg of CaM and various concentrations of Tx, as described in Fig. 8. ○, presence of 10 μg of CaM; □, presence of 90 μg CaM. The points represent the mean values obtained from two experiments.

its absence. Furthermore, it was reported that CaM contains four Mg^{2+} binding sites and that, at high concentrations of both cations, a CaM-Ca_4-Mg_4 species is formed (33). Probably these Ca^{2+}-dependent Mg^{2+}-regulated conformers of CaM are responsible for the different effects of Mg^{2+} on the interaction of CaM with its ligands, such as receptor proteins (30, 31), and with CaM antagonists (32).

The results described here indicate that CaM conformations induced by Ca^{2+} in the absence of Mg^{2+} appear to be more favorable for Tx binding. The free Ca^{2+} concentration for maximal binding of the drug is about 10^{-5} M, which corresponds to a concentration of Ca^{2+} which activates CaM in the cell (34). This activation involves exposure of an hydrophobic domain of the CaM molecule, which serves as the acceptor site either for the CaM-modulated enzymes or for the CaM antagonist drugs (21, 35, 36). Hydrophobic compounds, such as phenothiazines, R-24571, compound 48/80, and others, have been described as good antagonists of the CaM modulatory effects. However, it has been reported that anticalmodulin properties are determined not only by hydrophobic interactions but also by electrostatic interactions (37, 38). Prozialeck and Weiss (37) noticed that all of the most potent CaM inhibitors have several common structural characteristics, namely a large hydrophobic region composed of aromatic rings and an amino side chain group, which accounts for those molecular forces, respectively. The triphenylthylene antiestrogen Tx has a structure with these hydrophobic and hydrophilic portions (19, 20), so it can easily interact with CaM. This interaction is demonstrated in the fluorogram reported here, which clearly shows that, even at very low Tx concentrations (nanomolar range), the formation of the cation-dependent CaM-Tx adduct occurs (Fig. 7).

Two distinct classes of cation-dependent binding sites were found; one of them saturates in the nanomolar range of Tx concentration (Fig. 3), whereas the other one saturates in the micromolar range (Fig. 4). Under these conditions, the Tx binding appears to be specific, in the sense that both labeled and unlabeled Tx or trifluoperazine compete for the same

binding sites of CaM. Furthermore, in the micromolar range of Tx concentrations, we observed positive cooperativity for the Tx binding to CaM (Figs. 4, 5, and 6). These results are consistent with previous studies on the interaction of other hydrophobic CaM antagonists such as trifluoperazine (39), felodipine (40, 41), and [^{3}H]norchlorpromazine isothiocyanate with CaM (42).

Although a significant Ca^{2+}-dependent binding of Tx to CaM occurs at nanomolar concentrations, the inhibition of the (Ca^{2+} + Mg^{2+})-ATPase is only observed in the micromolar range, in agreement with previous results obtained using other CaM antagonists (43, 44). These observations probably are related to the large partitioning of the drugs within the lipid phase of the membranes (45), so that the real concentration of the drug at its target molecule (CaM) is less than that expected from the added concentration.

The plasma membrane (Ca^{2+} + Mg^{2+})-ATPase is not the only CaM-regulated enzyme which is inhibited by Tx. It has been demonstrated that Tx is a CaM antagonist in the activation of the cAMP phosphodiesterase, a key enzyme for the metabolism of cyclic nucleotides (16, 18). Furthermore, protein kinase C, which is a crucial enzyme in tumor promotion, has been reported to be inhibited by Tx (46, 47), suggesting that the antiproliferative effects of the drug may involve several components of the second messenger system. Indeed, the cellular effects of Tx cannot be explained solely by estrogenic blockage, since some estrogen receptor-negative breast cancer cells respond to Tx (48). High affinity antiestrogen binding sites, distinct from the estrogen receptors, have been reported by several investigators (8–13), but their nature is not known. In this work, the results indicate that CaM may be part of these antiestrogen binding sites, which have an affinity for Tx (6 nM) higher than that exhibited by the estrogen receptors (≈80 nM) (1). It has been suggested that CaM appears to be involved in the estrogen action by stimulating tyrosine phosphorylation of the estrogen receptor (49), which is converted from a nonbinding to a hormone-binding state (50). Therefore, CaM antagonism may be the primary event responsible for the antiestrogen cell growth inhibition, even in the case of estrogen-reversible action of antiestrogens. Recently, Musgrove *et al.* (51) also reported that there is an indistinguishable mechanism for the action of pure estrogen antagonists, nonesteroidal antiestrogens, and calmodulin antagonists on breast cancer cell proliferation.

On the other hand, the Ca^{2+}-dependent binding sites of CaM, which we observed to bind Tx with low affinity (K_d, 9 μM), appear to be ineffective at the levels of Tx found in the cytosol of breast tumor cells (≈1 μM) (52) and in human blood serum (0.6 μM) (53). Great divergence exists among the values reported for blood levels of Tx (0.5 nM to 0.5 μM) (3, 53–56), probably due to the efficiency of the methods used for extraction and detection of Tx and its metabolites. Since Tx is a lipophilic drug, it is trapped by cell membranes, so that the total amount of Tx should be higher than that measured in the fluid fraction (53–56), in agreement with the Tx concentrations (15 μM) required to inhibit CaM stimulation of the CaM-modulated enzyme tested in Fig. 8.

The results indicate that CaM inactivation by Tx involves Tx binding to the high affinity Ca^{2+}-dependent Tx binding sites of CaM, although we cannot rule out the possibility that inhibition of some CaM-modulated processes also requires the involvement of the Ca^{2+}-dependent and cooperative class of CaM-binding sites which bind Tx with low affinity.

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