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Tamoxifen is a Calmodulin Antagonist in the Activation of cAMP Phosphodiesterase

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The mechanism of action of tamoxifen, a triphenylethylene antiestrogen with antitumor activity, has not been fully established. In this paper, we present evidence that tamoxifen is an antigonist of calmodulin, a major cellular calcium receptor and calcium dependent regulator of many cellular processes. Our data showed that tamoxifen inhibited the activation of phosphodiesterase by calmodulin. This inhibition could be overcome by an increase in calmodulin concentration. Kinetic analysis demonstrated that tamoxifen is a competitive inhibitor of calmodulin in the activation of this enzyme. It could be speculated that the antagonism of calmodulin by tamoxifen may be one of the mechanisms responsible for its pharmacological actions.

Calmodulin (CaM) is a ubiquitous, acidic calcium binding protein which has been found in all eucaryotic cells. Intense studies on this protein has revealed its function as the major intracellular calcium receptor. It has been shown to be a Ca^{++}-dependent activator of many important cellular processes including the activation of certain forms of cyclic-nucleotide phosphodiesterase which is one of the key enzymes responsible for the metabolism of cyclic nucleotides in the second messenger system (1,2).

Many clinically useful drugs, including the phenothiazine antipsychotics (3) have been shown to bind to CaM and act as inhibitors of Ca^{++}-CaM dependent enzymes. Though inconclusive, the CaM antagonism of these drugs has been attributed at least partly to their mechanism of action.

Tamoxifen is one of the synthetic non-steroidal antiestrogens which has been proven to be useful clinical agents in the treatment of breast cancer (4,5).

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ABBREVIATIONS CaM - calmodulin; TAM-tamoxifen; EDTA-ethylene-diaminetetracetic acid; EGTA- ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid

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Binding of this agent to estrogen receptors and the modification of the processing of estrogen receptor is generally considered as its mechanism of action (6,7). As in phenothiazines, the molecule of tamoxifen also possesses a hydrophobic region of aromatic rings and a hydrophilic cationic alkyl amino side chain. In our search for the action sites of tamoxifen besides the estrogen receptor, the similarity in structure between the CaM antagonists phenothiazines and tamoxifen prompted us to investigate the interaction of tamoxifen with the CaM dependent cAMP phosphodiesterase.

MATERIALS AND METHODS

Tamoxifen-citrate was a generous gift from Stuart Pharmaceuticals, U.S.A. Other chemicals and biochemicals were the purest grade available and were obtained from Fisher Scientific Ltd., Winnipeg, Manitoba, or Sigma Company, St. Louis, Mo. DEAE-cellulose and Affi-gel Blue were purchased from Bio-Rad Laboratories, Mississauga, Ontario.

Bovine Brain Calmodulin

Bovine brain calmodulin was purified by the method of Charbonneau and Cormier (8). The preparation was homogeneous as judged by SDS-polyacrylamide gel electrophoresis.

Bovine Brain cAMP-Phosphodiesterase

A calmodulin-deficient bovine brain calmodulin-dependent cAMP phosphodiesterase was partially purified according to the procedure of Sharma et al (9). Bovine brain was homogenized in buffer A: Tris-HCl (0.1 M), EDTA (2 mM), mercaptoethanol (10 mM), pH 7.5. After centrifugation at 10,000 xg for 20 min., the supernatant was loaded to a column of DEAE-cellulose pre-equilibrated with buffer B: Tris-HCl (20 mM), imidazole (1 mM), $MgCl_2$ (1 mM), EGTA (0.1 mM) and 2-mercaptoethanol (10 mM), pH 7.0. The column was washed with 2 bed volumes of buffer B containing 0.05 M NaCl, then eluted with the same buffer containing 0.22 M NaCl. The 0.22 M NaCl fraction, which contained the calmodulin dependent cAMP phosphodiesterase, was dialysed against buffer B for 18 hrs. The protein solution after dialysis was passed through an Affi-gel Blue column pre-equilibrated with buffer B. This column was washed with 2.5 bed volume of buffer B containing 0.15 NaCl, then eluted with a linear gradient of NaCl from 0.15 M to 0.5 M in buffer B. The calmodulin deficient phosphodiesterase was recovered under a broad peak. This enzyme preparation usually showed 6-12 fold increase in activity with addition of exogenous calmodulin and calcium.

Assay of Calmodulin-dependent cAMP-phosphodiesterase

The procedure of Sharma and Wang (10) was used. Phosphodiesterase activity was assayed in an assay buffer containing (final concentration in the assay mixture): Tris-HCl (40 mM), imidazole (40 mM), $MgCl_2$ (5 mM), 5'-nucleotidase (0.33 U) and phosphodiesterase (0.012-0.016 U), pH 7.5. Enzyme reaction was started by adding the substrate cAMP (1.08 µmole). Incubation was carried out at 30°C for 30 min., and the reaction was stopped by adding trichloroacetic acid. The liberated inorganic phosphate in the reaction mixture was determined by the colorimetric method of Fiske and Subbarow (11). In the determination of the Ca^{++}-CaM activated activity, various concentrations of CaM and Ca^{++} were added to the assay mixture. Basal activity was determined in the assay buffer only. To determine the effects of a drug on the enzyme activity, the drug solution in dimethylformamide was added to the reaction mixture prior to the addition of cAMP. The final concentration of dimethylformamide in the assay mixture was 1%. At this concentration of dimethylformamide, the enzyme activity was suppressed by less than 5%.

RESULTS

Fig. 1 shows the inhibition of Ca^{++}-CaM activated phosphodiesterase activity by various concentrations of TAM. In contrast, the basal activity which is Ca^{++} and CaM independent was not affected by TAM, even at 6×10^{-5} M of TAM, the highest concentration in this experiment. The IC_{50} (the concentration of drug to inhibit 50% of the activable activity) of TAM is 2 μM.

In all experiments described in this paper, TAM was administered as a citrate salt. Since citrate may act as a sequestering agent to remove Ca^{++} from the assay mixture, we studied the effect of citrate on the phosphodiesterase activity in the assay system as described in the legend to Fig. 1. At a concentration of 1×10^{-4}M, which is higher than the highest concentration of TAM-citrate studied, citrate itself affected neither the activable nor the basal activity (data not shown). This indicates that TAM but not citrate was the inhibitor of phosphodiesterase.

The enzyme assay described in this paper is a two-enzyme coupling system. cAMP was hydrolysed to 5'-AMP by phosphodiesterase first, and then the 5'-AMP was further hydrolysed by 5'-nucleotidase to liberate inorganic phosphate which was quantified by colorimetric method. It is possible that the 5'-nucleotidase was the enzyme being inhibited. To test this question, we determined the hydrolysis of 5'-AMP (1.08 μmole) in the assay solution in the absence and presence of 6×10^{-5}M TAM. At this concentration, TAM showed no effect on the amount of

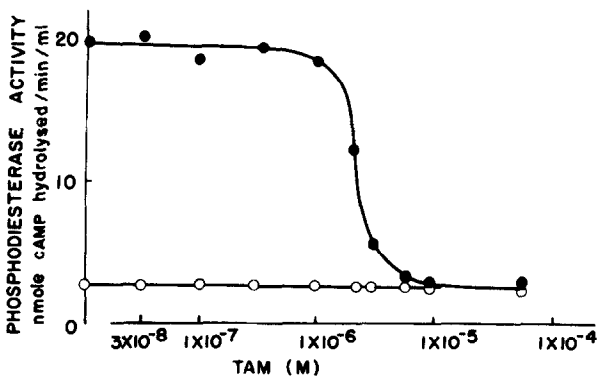

Fig. 1 Inhibition of CaM-stimulated phosphodiesterase activity by tamoxifen. cAMP phosphodiesterase activity was measured at a series of tamoxifen concentrations. The assay medium contained the assay buffer describe in Materials and Methods with (●) or without (○) 2 μg/ml CaM and 2.5 mM Ca^{++}. Each point represents the mean of 4 determinations, confidence interval was too small to be illustrated.

5'-AMP hydrolysed (not shown). This result provided evidence that the inhibitory action of TAM is on phosphodiesterase itself.

To further establish the direct interaction between phosphodiesterase and TAM, we studied the effect of CaM concentration on the TAM induced inhibition of Ca^{++}-CaM activated phosphodiesterase activity. Fig. 2 clearly shows that increases in CaM concentration gradually overcame the inhibition of TAM. A more detailed kinetic analysis of the competition between CaM and TAM is shown in the Lineweaver-Burk plot in Fig. 3. The V_{max} (y-axis intercept) value for the Ca^{++}-CaM activated phosphodiesterase activity in the presence and absence of 2 or 3 μM TAM were the same; the apparent K_m (slope = Km/V_{max}) of CaM decreased with increasing concentration of TAM. This suggests that TAM is a competitive inhibitor of CaM in phosphodiesterase activation. Ki of TAM was calculated to be 0.96 μM.

DISCUSSION

The mechanism of the antiestrogenic and the antitumor action of TAM is not fully understood. Most studies on this effective antitumor agent have been concentrated on its interaction with estrogen receptor (6,7). The recent discovery of the high affinity, saturable binding site of triphenylethylene antiestrogens in the microsomal fraction of many target and non-target tissues (12,13) as well as in the low density lipoprotein in rat serum (14) has received some attention.

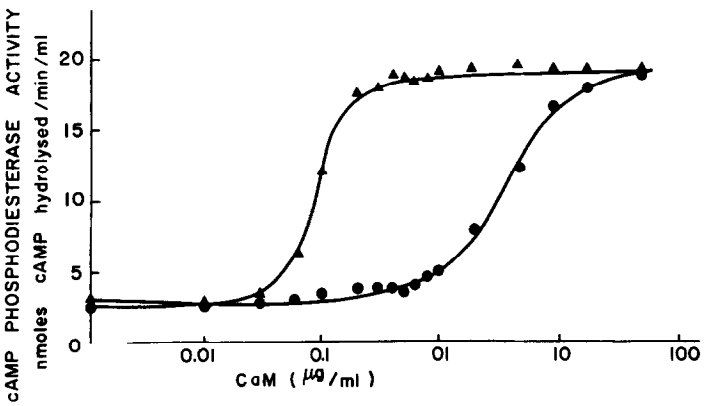

Fig. 2 Effect of various concentrations of CaM on the activity of phosphodiesterase. The enzyme assay procedure is described in Materials and Methods section. The assay medium contained the assay buffer plus 2.5 mM Ca^{++} and various concentration of CaM. The activity was measured in the presence (●) and absence (▲) of 6 μM tamoxifen.

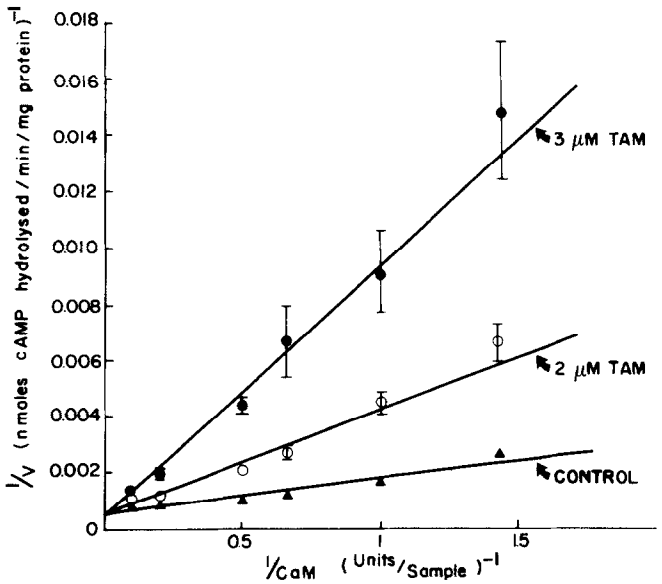

Fig. 3 Kinetic analysis of the tamoxifen-induced inhibition of activation of phosphodiesterase. Phosphodiesterase was measured in the presence of varying concentrations of tamoxifen and CaM in the assay buffer plus 2.5 mM Ca^{++}. The data were plotted as 1/V vs 1/CaM concentration. The velocity (v) is expressed as the nmole cAMP hydrolysed per min per mg protein of the partially purified CaM deficient phosphodiesterase. Concentration of CaM is expressed as the number of units of CaM per sample. 1 unit of CaM is defined as the amount of CaM necessary to produce 50% of the maximum activation of the phosphodiesterase preparation. Each point represents the mean of 4 determinations. Vertical brackets represents standard deviations. Lines are fitted by linear regression analysis.

The lack of correlation between the binding affinity of various antiestrogens and their antitumor activity suggested that these sites might not directly mediate the estrogen antagonism of antiestrogens but rather might alter the distribution and pharmcokinetics of this drug in vivo (15), though the possibility that these sites may represent some unknown pharmacological action sites of TAM can not be excluded.

In this paper, we present evidence that TAM is a potent antagonist of CaM in the activation of cAMP-phosphodiesterase (IC_{50}= 2μM, whereas trifluoperazine, the most studied phenothiazine CaM-antagonist, exhibited a IC_{50} = 5 μM under the same experimental conditions). The results of this paper do not elucidate the mechanism of this antagonism. The drug can bind directly to CaM or to a CaM binding site on the enzyme phosphodiesterase. Our more recent study demonstrated that [^{3}H]-TAM binds to CaM in a Ca^{++} dependent manner. The properties of CaM-TAM interaction is currently under investigation. Our findings suggested that there could be

other intracellular action sites of TAM besides the estrogen receptor. Whether the observed CaM antagonism by TAM and its interaction with the microsomal specific antiestrogen binding sites are related remains to be established.

It has been reported that several CaM antagonists also possess antitumor activity (16,17), though the CaM dependent cellular process(es) or key enzyme(s) responsible for the cytotoxicity is not yet identified. It is possible that TAM may also exert its antitumor activity via CaM antagonism. Above all, in view of the important role of CaM in the regulation of so many diverse vital cellular processes, future study of the interaction of TAM or other triphenylethylene antiestrogens with other calmodulin-dependent enzymes should be warranted.

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