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CHARACTERISTICS OF THE CYTOTOXIC EFFECTS OF THE PHENOTHIAZINE CLASS OF CALMODULIN ANTAGONISTS*

WILLIAM N. HAIT† and GARY L. LEE
Yale University School of Medicine, Department of Internal Medicine, Section of Medical Oncology, New Haven, CT 06510, U.S.A.

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Abstract—We have characterized the antiproliferative effects of the phenothiazines, a group of antipsychotic drugs possessing a wide range of pharmacological actions. The phenothiazines inhibited both the proliferation and clonogenicity of L1210 leukemic lymphocytes. This effect was dependent on both time of exposure and concentration of drug. Clonogenicity of cells in the logarithmic phase of growth was inhibited by greater than 99% at a concentration of drug that had no effect on cells in the plateau phase of growth. Human and murine cell lines, grown either in suspension or in monolayers, were equally susceptible. Calmodulin (CaM), purified from L1210 cells by preparative polyacrylamide gel electrophoresis, had sensitivity to inhibition by phenothiazines similar to that reported for CaM prepared from brain. The order of potency was trifluoperazine ≥ fluphenazine > chlorpromazine > chlorpromazine-sulfoxide. As a class, these drugs were less potent antagonists of CaM than was the bee venom polypeptide, melittin. The antiproliferative effects of phenothiazines were similar to the anticalmodulin effects. Thus, the same order of potencies was seen for both effects; the shapes of the dose–response curves were similarly steep and the effects of excess calcium on the inhibition of both were identical. These studies add pharmacological support for CaM being a potential intracellular target for the antiproliferative effect of the phenothiazines.

The phenothiazines are a group of antipsychotic drugs that have diverse pharmacological actions that include gangliolytic, adrenolytic, antifibrillatory, diuretic, antipyretic, anticonvulsant and antiemetic properties [1]. Although used clinically for their antipsychotic, antiemetic, and antihistaminic effects, their cytotoxic effects have been long appreciated. In fact, the first use of the phenothiazines were as antihelminthics, urinary antiseptics and insecticidals [2].

The discovery by Levin and Weiss [3, 4] that phenothiazines bind to and antagonize the action of calmodulin (CaM), a widely distributed, multifunctional, calcium-binding protein [5], offered a potential explanation for their spectrum of pharmacological actions. Examples of the enzymatic and functional processes mediated by CaM and antagonized by phenothiazines have been reviewed [6] and include adenylate cyclase, cyclic nucleotide phosphodiesterase, protein kinase, myosin light chain kinase, NAD-kinase, ATPase, phospholipase A_2, methyltransferase, cell secretion, and formation of the microtubular apparatus. However, phenothiazines also antagonize processes that are not dependent on CaM [7, 8] and may also have nonspecific membrane-destabilizing effects. Therefore, inhibition of CaM-mediated processes cannot unify the pharmacological properties of these drugs.

Recent evidence suggesting that CaM mediates the effects of calcium on normal and abnormal cellular proliferation has renewed interest in the antiproliferative effects of phenothiazines. For example, certain transformed cells have a greater content of CaM than their normal counterparts [9, 10] and may also require less calcium to sustain cellular proliferation [11]. Chafouleas *et al.* [12] and Sasaki and Hidaka [13] have shown that progression of cells into and through the cell cycle is associated with changes in CaM content and that the progression is blocked by CaM antagonists.

We [14–16] and others [17–19] have taken advantage of these earlier observations and have demonstrated the cytotoxic effects of phenothiazines to malignant cells and the ability of phenothiazines to augment the cytotoxicity of Adriamycin [20, 21] and bleomycin [22, 23]. Despite these observations, the pharmacological properties of this effect have been incompletely studied and remain poorly understood. Therefore, we have characterized the antiproliferative and cytotoxic effects of the phenothiazines in L1210 leukemic cells and have compared these effects directly to the pharmacological properties of CaM purified from this cell line. We also compared the phenothiazine class of CaM antagonists to a bee venom polypeptide, melittin, known to be one of the most potent antagonists of CaM.

MATERIALS AND METHODS

Drugs and reagents. Trifluoperazine, chlorpromazine, and chlorpromazine-sulfoxide were supplied by Dr. Alfred Brown of Smith, Kline and Beckman Co., Inc. (Philadelphia, PA). Fluphenazine was supplied by Dr. Fred Gorelick. Melittin and cyclic 3′,5′-

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† To whom correspondence should be addressed.

adenosine monophosphate were obtained from the Sigma Chemical Co. (St. Louis, MO). Myokinase and pyruvate kinase were obtained from Boehringer-Mannheim (Indianapolis, IN) and firefly luciferin-luciferase was from E.I. Dupont de Nemours & Co. (Wilmington, DL). Other reagents were of electrophoretic or reagent grade and were obtained from commercial sources.

Techniques of cell culture. L1210 and L5178Y leukemic lymphocytes were grown in suspension culture in Fischer's medium supplemented with 10% horse serum in 5 ml glass tubes.

In experiments designed to study the effect of exogenous calcium on cell growth, cells were grown in "calcium-free" RPMI-1640 medium supplemented with 100 μM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA).

HL-60 (human promyelocytic leukemia) cells were grown in suspension in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% non-essential amino acids and 1% sodium pyruvate in 5 ml glass tubes.

MIA-PACA (human pancreatic carcinoma) and HCT-8 (human colonic carcinoma) cells were grown in monolayers in RPMI-1640 medium supplemented with 10% fetal calf serum in 25 cm^2 plastic flasks.

All cell lines were maintained in culture at 37° in a humidified atmosphere of 5% CO_2 and 95% air. Cells were checked monthly and were found to be free of contamination with bacteria, mycoplasma or fungi.

The effect of drugs on cellular proliferation was determined by seeding cells in culture at a concentration of 1×10^4 cells/ml and then waiting 24–48 hr until they entered the logarithmic phase of growth. At that time the drugs were freshly prepared in sterile water and added to the cultures.

Clonogenic assay. L1210 cells were grown in media containing various concentrations of drugs or appropriate vehicle. Cells were washed by centrifuging at 100 *g* for 10 min and then resuspended in an equal volume of drug-free medium. One hundred to one thousand cells were added to culture tubes containing Fischer's medium supplemented with 15% horse serum and 0.3% agar [24]. After 14 days of incubation, colonies were stained with 2-(*p*-iodophenyl) - 3 - (*p*-nitrophenyl) - 5 - phenyltetrazolium chloride and counted 24 hr later using a dissecting microscope.

Preparation of L1210 CaM and analysis of CaM-dependent phosphodiesterase activity. CaM was prepared from L1210 cells by preparative polyacrylamide gel electrophoresis as previously described [15, 23]. Briefly, in this procedure, 1×10^9 cells were sonicated at 50 W for 10 sec in 2 vol. of a phosphate-buffered solution (pH 6.0) containing 88 mM Na_2HPO_4, 23 mM NaH_2PO_4, 640 mM glycine, 1 mM EGTA and 0.32 sucrose. Following heating in a boiling water bath for 5 min, the sonicate was centrifuged at 3600 *g* for 20 min. The supernatant fraction was removed, and the pH was adjusted to 6.0 and dialyzed for 12 hr against the sonicating buffer. Two milliliters of concentrated material was applied to a preparative polyacrylamide gel electrophoretic column (Shandon Southern Instruments, Inc., Sewickley, PA). Proteins were eluted with a solution of 0.1 M Tris buffer (pH 7.6) containing 1.0 mM $CaCl_2$. One hundred 1-ml fractions were collected and each fraction was assayed for CaM activity. Using this method, a single peak of CaM was obtained which eluted in fractions 35–50 and was found to be homogeneous by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis [25].

The activity of CaM was assayed by its ability to activate a CaM-sensitive phosphodiesterase prepared from rat cerebrum as previously described [3, 4]. Using our preparation of L1210 CaM, the activity of phosphodiesterase was increased 5-fold over basal activity. One unit of activity was defined as the amount of CaM required to achieve 50% of the maximum activation of phosphodiesterase. The antagonism of CaM by drugs was determined by their ability to inhibit the activation of phosphodiesterase in the presence of 10 units of CaM. The specificity of this assay was shown by determining the inhibition of phosphodiesterase in the absence of CaM. At the highest concentrations of drugs tested, there was less than 5% inhibition of basal enzymic activity.

RESULTS

Effect on cellular proliferation. Figure 1 demonstrates the effect of trifluoperazine on the proliferation of L1210 cells. At a concentration of 16 μM, no net increase in cell number was observed. At lower concentrations, after 24 hr of exposure, there was a dose-related decrease in the accumulation of cells exposed to the drug.

Effects of clonogenicity. Figure 2 demonstrates that there was a dose-related decrease in the clonogenicity of L1210 cells exposed to a range of concentrations of trifluoperazine for 48 hr. The con-

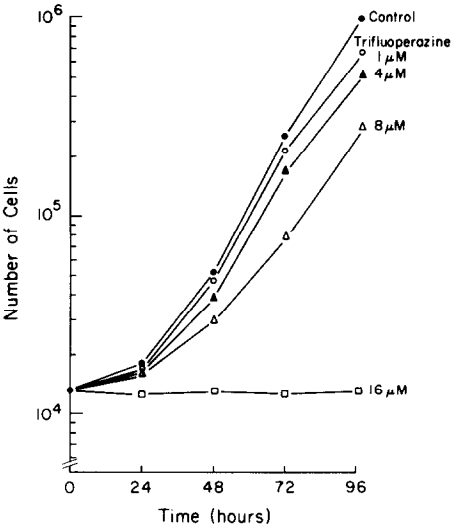

Fig. 1. Effect of trifluoperazine on the proliferation of L1210 cells. Cells were grown as described in Materials and Methods. Trifluoperazine was dissolved in water, sterilized, and added to the cultures 24 hr after the time of seeding. Cell counts were done daily using an electronic counter (Coulter). Each point represents the mean of three culture tubes. There was less than 5% variation among them.

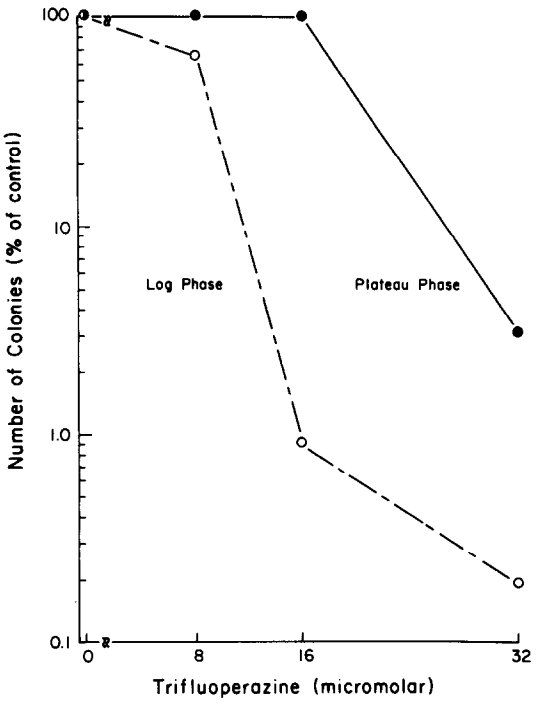

Fig. 2. Effect of trifluoperazine on the clonogenicity of L1210 cells. Cells were grown as described in Materials and Methods and those in the logarithmic phase (2×10^5 cells/ml) or plateau phase (2×10^6 cells/ml) were exposed to various concentrations of trifluoperazine for 48 hr then cloned in soft agar and compared to the cloning efficiency of untreated controls. Cloning efficiency was 80% and 60% respectively. Colonies were counted 14 days later. Each point represents the mean of three culture tubes with less than 10% variation among them.

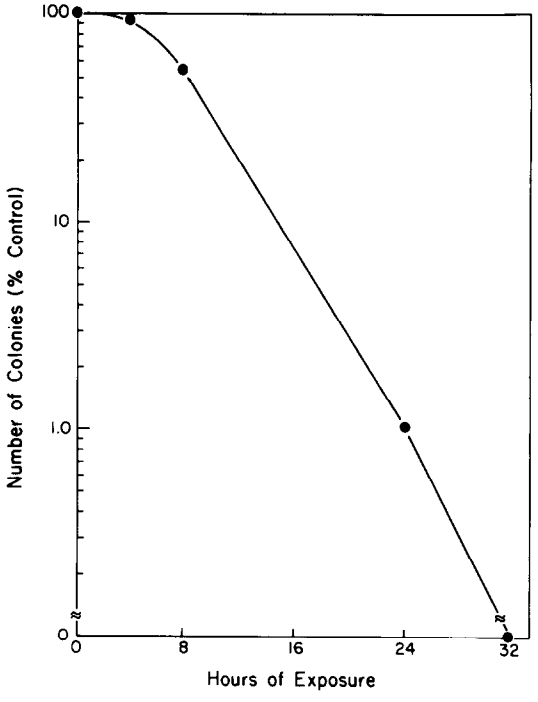

Fig. 3. Time-course of cytotoxicity of trifluoperazine to L1210 cells. Cells in the logarithmic phase of growth were exposed to 32 μM trifluoperazine, washed, and then cloned in soft agar as described in Materials and Methods and Fig. 2. Each point represents the mean of there culture tubes. There was less than 5% variation among them.

centration of drugs required to inhibit clonogenicity by 50% was 10 μM.

Influence of phase of growth on clonogenicity. The effects of trifluoperazine on the colonogenicity of L1210 cells in the logarithmic (2×10^5 cells/ml) and in the plateau (2×10^6 cells/ml) phases of growth are compared in Fig. 2. Cells were exposed to a range of concentrations of drug for 48 hr and then cloned in soft agar. At a concentration of 16 μM, trifluoperazine inhibited the clonogenicity of cells in the logarithmic phase of growth by 99% but had no inhibitory effect on cells in the plateau phase of growth.

Time-course of cytotoxicity. Figure 3 demonstrates that cytotoxicity was time-dependent and was first observed after 8 hr of exposure to a concentration of 32 μM trifluoperazine. No plateau in effect was seen, and the maximum demonstrable effect was reached by 32 hr of exposure.

Spectrum of antiproliferative effect. The effects of phenothiazines on several human and murine cell lines in the logarithmic phase of growth are shown in Table 1. These studies demonstrated that the phenothiazines were effective against all lines studied. The concentration of drug necessary to inhibit growth was similar for the various lines and was independent of whether they were grown in suspension (L1210, L5178Y, HL-60) or in monolayers (MIA-PACA, HCT-8) or whether their origin was human (HL-60, MIA-PACA, HCT-8) or murine (L1210, L5178Y). Melittin, the bee venom polypeptide inhibitor of CaM, was a more potent inhibitor of all cell lines than any of the phenothiazines. Fluphenazine was directly compared to the other phenothiazines against L1210 cells and was equipotent to trifluoperazine ($IC_{50} = 6$ μM).

Effects of drugs on the activity of CaM of L1210 leukemic lymphocytes. The effects of the phenothiazines on the activity of CaM prepared from L1210 leukemic cells are shown in Fig. 4. Among the phenothiazines, the concentration required to inhibit CaM-activated phosphodiesterase by 50% (IC_{50}, μM) was trifluoperazine ($IC_{50} = 10 \pm 3$) $\geq$ fluphenazine ($IC_{50} = 14 \pm 5$) $>$ chlorpromazine ($IC_{50} = 31 \pm 7$). Figure 4 also demonstrates that the most potent inhibitor of L1210 CaM was the bee venom polypeptide, melittin ($IC_{50} = 0.14 \pm 0.03$). Chlorpromazine-sulfoxide, the phenothiazine metabolite, had far less activity than the parent compound ($IC_{50} = 1200 \pm 140$ μM).

Effect of exogenous calcium on the inhibition of CaM and on the inhibition of growth of L1210 cells. Figure 5 demonstrates that the addition of excess calcium could overcome the antagonism of calmodulin by the calcium chelator, EGTA, as measured by the activation of cyclic nucleotide phosphodiesterase. Calcium could not overcome the antagonism of calmodulin by the phenothiazine, trifluoperazine.

Table 1. Effects of calmodulin antagonists on the growth of human and murine malignant cell lines*

Cell type	Cell line	IC_{50} (μM) Melittin	TFP	CPZ	CPZ S = O
Promyelocytic leukemia	HL-60†	1	4	8	100
Lymphocytic leukemia	L1210‡	1	5	7	80
Lymphocytic leukemia	L5178Y‡	1	5	7	90
Colonic carcinoma	HCT-8†	2	7	10	250
Pancreatic carcinoma	MIA-PACA†	1	8	10	220

* Cells were grown as described in Materials and Methods. The drugs were dissolved in water, sterilized, and then added to cells in the logarithmic phase of growth and counted 48 hr later. The IC_{50} value represents the concentration of drug (μM) necessary to inhibit the growth of cells by 50% compared to that of untreated controls and was determined from dose–response curves run in triplicate from at least two separate experiments. In a given experiment there was less than 10% variation among cultures. Abbreviations: TFP, trifluoperazine; CPZ, chlorpromazine; and CPZ S = O, chlorpromazine-sulfoxide.

† Human cell line.

‡ Murine cell line.

Figure 6 shows that excess calcium could also overcome the inhibition of cellular proliferation by EGTA, but calcium could not overcome the inhibition of cellular proliferation caused by trifluoperazine.

DISCUSSION

The present studies add pharmacological evidence to support the concept that phenothiazines may exert their antiproliferative effects through the inhibition of CaM. This evidence includes the correlation between the order of potency for inhibition of L1210 CaM and inhibition of cell growth; the similar steepness of the dose–response relationships for both processes; the preferential effect for proliferating cells; the broad-spectrum of antiproliferative activity; the lack of effects of the inactive metabolite of chlorpromazine, chlorpromazine-sulfoxide; and the failure of exogenous calcium to antagonize the pharmacological action.

The effect of phenothiazines as CaM antagonists was studied directly against CaM purified from L1210 cells rather than against standard preparations from brain. We found that the effect of phenothiazines on CaM isolated from leukemic cells was similar to their effect on CaM isolated from brain [3, 4]. The slight variations in potency may be explained by differences in preparation and/or amount of CaM used in the assay systems and do not necessarily imply differences in the L1210 CaM. Using our method of preparation, we were unable to identify the unique calcium-binding protein oncomodulin [26, 27] and, therefore, cannot comment on its existence in L1210

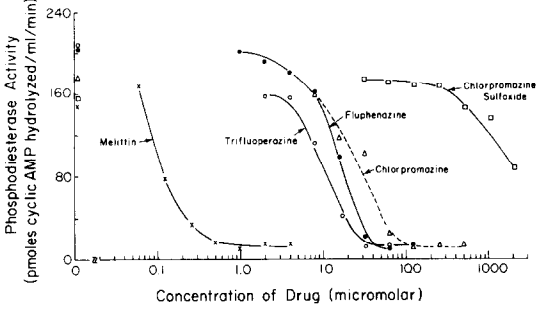

Fig. 4. Effects of drugs on calmodulin (CaM) from L1210 leukemic lymphocytes. CaM was purified and assayed as described in Materials and Methods by the activation of cyclic nucleotide phosphodiesterase. Drugs were dissolved in water, and their effects on CaM-activated phosphodiesterase were determined in the presence and absence of 10 units of CaM. In the absence of CaM, there was less than 5% inhibition of enzymic activity at the highest concentration of drug. Each point represents the mean of four determinations.

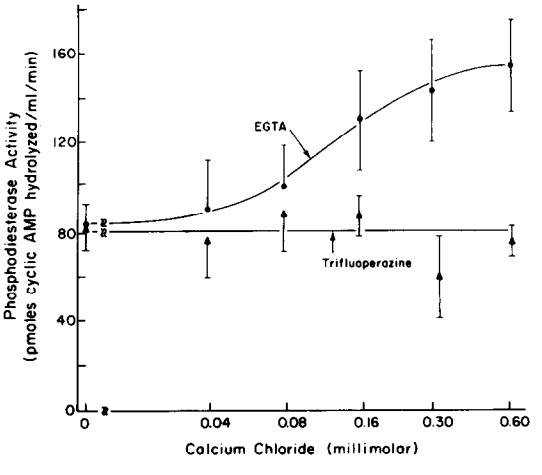

Fig. 5. Effect of calcium on the inhibition of L1210 CaM-activated phosphodiesterase by trifluoperazine or EGTA. Each reaction tube contained 50 mM glycyl-glycine buffer (pH 8.0), myokinase (0.025 units) pyruvate kinase (0.5 units) 25 mM ammonium acetate, 3 mM $MgCl_2$, 100 μM $CaCl_2$, 400 μM cyclic AMP, phosphodiesterase, 10 units of CaM and either 32 μM TFP or 400 μM EGTA. Values on the abscissa represent total added calcium. Each point represents the mean ± S.E.M. of three determinations.

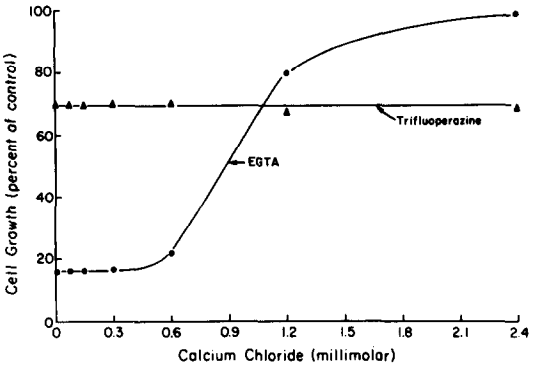

Fig. 6. Effect of calcium on the inhibition of L1210 cell growth by trifluoperazine or EGTA. Cells were grown in "calcium-free" RPMI-1640 medium. Either trifluoperazine or EGTA was added to cells in the logarithmic phase of growth to give a final concentration of 8 or 100 μM respectively. The effect of adding exogenous calcium chloride was determined after 48 hr. Values on the abscissa represent total added calcium. Each point represents the mean of three determinations. Control cultures contained 2.4×10^5 cells/ml.

cells, or the comparative sensitivity of this protein to inhibitors.

In all the cell lines studied, the order of potency of the drugs as CaM antagonists was similar to their order of potency as inhibitors of cellular proliferation (Table 1). The phenothiazines were less potent inhibitors of cell growth than the polypeptide, melittin. Chlorpromazine-sulfoxide, the phenothiazine metabolite, was a much less potent inhibitor of CaM and of cell growth. These data are consistent with our earlier work using the C_6 astrocytoma cell line which demonstrated an excellent correlation between inhibition of CaM and inhibition of cellular proliferation using structurally diverse CaM antagonists [16].

Within the phenothiazine class of calmodulin antagonists, trifluoperazine and fluphenazine had similar anticalmodulin and antiproliferative effects that were consistently greater than that of chlorpromazine (Fig. 4, Table 1, Refs. 14–16). Chlorpromazine-sulfoxide had the least activity in both systems. These data are in accord with the observations of Prozialeck and Weiss [28] who demonstrated that substitution of a $-CH_3$ for a -Cl moiety at position 2 of the phenothiazine nucleus and that modification of the side chain amino group on the molecule from a simple alkylamine to a piperazine enhances the anticalmodulin effect. Finally, oxidation of the -S moiety at position 5 of the phenothiazine nucleus to the sulfoxide derivative markedly reduced both the anticalmodulin and the antiproliferative effects of the drug.

These studies demonstrate that human colon carcinoma (HCT-8), human pancreatic carcinoma (MIA-PACA) and both human (HL-60) and murine leukemic (L5178Y, L1210) cells were inhibited by phenothiazines and that the polypeptide CaM antagonist, mellitin, was more potent than these drugs (Table 1). This broad-spectrum antiproliferative effect is consistent with the ubiquitous nature of CaM and with data from several laboratories which demonstrate the inhibition of different cell lines by antagonists of CaM [14–20]. In addition, the similar potencies of the drugs against the various cell lines may also reflect the fact that they were studied during the logarithmic phase of growth, and that their rates of growth were similar (see below).

By directly comparing the effects of phenothiazines on the profileration of L1210 cells to their effects on the antagonism of L1210 CaM, we have now shown a remarkable similarity between the dose–response relationships. For example, when studying trifluoperazine, the slope of the dose–response curves for both processes was steep, the greatest effect being seen between 8 and 16 μM (Figs. 1 and 4).

The cytotoxic effects of phenothiazines were found to be greater for proliferating cells than for quiescent cells (Fig. 2). This observation is similar to that of Chafouleas *et al.* [29, 30] and must be taken into account when the potency of a phenothiazine is compared between cell lines having different rates of proliferation. Furthermore, the preferential toxicity to proliferating cells is consistent with the hypothesis that CaM is important for the progression of cells through the cell cycle [12, 13], and that inhibitors of CaM can block this progression [12, 13].

The cytotoxicity of the phenothiazine was not demonstrable until 8 hr of exposure at a concentration of 32 μM (Fig. 3). This may explain the lack of cytotoxicity observed by others when lower concentrations of drug, or shorter periods of incubation, were used [20, 21]. At higher concentrations of trifluoperazine (> 100 μM), cytoxicity was seen after shorter periods of incubation (data not shown). However, at these concentrations the phenothiazines inhibit enzymic activity independently from their inhibition of CaM [3], making the significance of the cytotoxic effect at higher concentrations uncertain.

The phenothiazines inactivate CaM by binding to high affinity sites on the molecule in the presence of calcium and not by interfering with the binding of calcium to CaM [3, 4]. For this reason it was important to determine whether or not calcium could overcome the phenothiazine's inhibition of cellular proliferation. As previously shown with CaM purified from brain [3], calcium could not overcome the antagonistic effect of trifluoperazine on CaM purified from L1210 cells (Fig. 5). Furthermore, the current studies show that calcium was also incapable of overcoming the antiproliferative effect of phenothiazines (Fig. 6). Exogenous calcium could readily overcome the inhibitory effect of EGTA, a calcium chelator, on both CaM-activated phosphodiesterase (Fig. 5) and cellular proliferation (Fig. 6).

The inhibitory effect of trifluoperazine on CaM-activated enzymes can also be overcome by excess CaM [3]. Boynton *et al.* [31] have shown that exogenous CaM could overcome the inhibition of DNA synthesis by trifluoperazine in intact hepatoma cells. Although these studies appear to support the hypothesis that the phenothiazines exert their antiproliferative effect through interference with the action of CaM, there are alternative interpretations of this observation. For example, the exogenous CaM might merely act by binding the trifluoperazine and pre-

venting the drug from interacting with other target intracellular enzymes or structural proteins.

The actions of phenothiazines are not specific for CaM. Therefore, the similarities between inhibition of cellular growth and inhibition of purified calmodulin demonstrated in these studies may not be unique and, therefore, do not prove that the antiproliferative effect of phenothiazines is mediated through the inhibition of calmodulin. For example, protein kinase-C, a calcium-sensitive, phospholipid-activated kinase, believed to be activated by the tumor promoting phorbol esters [32], is also inhibited by phenothiazines and structurally similar drugs [7, 8]. Further studies of both protein kinase-C and CaM in cell lines that are resistant to killing by "CaM antagonists" are in progress.

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