MOLECULAR PHARMACOLOGY, 26:286–292

Effects of Calmodulin Antagonists on Immune Mouse Lymphocytes

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Received February 15, 1984; Accepted May 21, 1984

SUMMARY

The nature of the Ca^{2+} requirement of lymphocyte-mediated cytolysis (LMC) has been explored pharmacologically with a number of putative calmodulin antagonists. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), trifluoperazine, and chlorpromazine were found to inhibit LMC (IC_{50} values = 8.9, approximately 50, 7.4, and 9.4 μM, respectively) at concentrations which were not detectably toxic to either the effector or the target cell. Pimozide inhibited LMC by 50% at 15 μM but caused a substantial decrease in lymphocyte ATP content and viability at this concentration. 1-[Bis(*p*-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl]imidazolium chloride (R 24 571, calmidazolium), which has been reported to be the most potent antagonist of isolated calmodulin, caused a marked decrease in lymphocyte ATP content and viability at concentrations >4 μM and inhibited LMC only slightly at similar concentrations. Trifluoperazine sulfoxide and chlorpromazine sulfoxide were not inhibitory to LMC at ≤20 μM. LMC was inhibited in a sustained manner when cytolytic lymphocytes, but not target cells, were pretreated separately with W-7 or chlorpromazine at 37° and were then washed free of exogenous drug prior to the start of the LMC assay. The above cellular effects of the calmodulin antagonists were reduced in magnitude when the serum concentration in the culture medium was increased (from 5% to 20%). The inhibition of LMC by micromolar concentrations of W-7, trifluoperazine, and chlorpromazine, as well as the relative inactivities of W-5 versus W-7 and of the sulfoxide derivatives of trifluoperazine and chlorpromazine, are consistent with calmodulin's being a lymphocyte receptor whose occupancy by Ca^{2+} is required for the performance of this cytolytic function. However, this conclusion must be tempered by the finding that even W-7, trifluoperazine, and chlorpromazine can exert nonspecific effects on the energy metabolism and viability of the cytolytic lymphocytes at concentrations of drug severalfold higher than those required to inhibit LMC.

INTRODUCTION

LMC,[1] like many other cellular functions, is strongly dependent upon Ca^{2+} for its occurrence (1). This Ca^{2+} requirement has been shown to be restricted to the postrecognition stage of LMC, during which the cytolytic T lymphocyte programs its attached target cell for lysis (2); however, the biochemical basis for this Ca^{2+} requirement, like the molecular mechanism of LMC, remains unknown.

Calmodulin, the calcium-binding regulatory protein found ubiquitously in eukaryotic cells, has been implicated as the site of action of Ca^{2+} in many Ca^{2+}-dependent cellular phenomena (3). In an attempt to elucidate the Ca^{2+} requirement of LMC, we have investigated the effects of several putative calmodulin antagonists on the function and biochemistry of cytolytic T lymphocytes. The results obtained in this study demonstrate the difficulties encountered in attempting to use these putative calmodulin antagonists as pharmacological probes with which to implicate the involvement of calmodulin in a cellular process.

[1] The abbreviations used are: LMC, lymphocyte-mediated cytolysis; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; R 24 571, 1-[bis(*p*-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl] imidazolium chloride, or calmidazolium; TFP, trifluoperazine; TFPSO, trifluoperazine sulfoxide; CPZ, chlorpromazine; CPZSO, chlorpromazine sulfoxide; HPLC, high-performance liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

MATERIALS AND METHODS

Reagents and medium. W-5 and W-7 were purchased from Rikaken Company, Ltd. (Nagoya, Japan). Pimozide and R 24 571 were obtained from Janssen Pharmaceutica (Beerse, Belgium), and TFP and CPZ were obtained from Smith Kline & French Laboratories (Philadelphia, Pa.). TFPSO and CPZSO were donations of the Chemical Synthesis Program of the National Institute of Mental Health, and were obtained from Dr. Richard Mailman. [2,8-^{3}H]Adenine (34 Ci/mmol) was pur-

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chased from ICN Pharmaceuticals (Irvine, Calif.). Dulbecco's phosphate-buffered saline (Grand Island Biological Company, Grand Island, N.Y.), supplemented with 5% fetal calf serum (Sterile Systems Inc., Logan, Utah) (heat-inactivated), was used as the medium for all cell incubations, unless specified otherwise. Other materials were from sources identified elsewhere (4).

Cells. Target cells were EL4 leukemia cells, which were maintained by serial i.p. passage in C57BL mice (4). Target cells were labeled with $Na_2{}^{51}CrO_4$ (Amersham Corporation, Arlington Heights, Ill.) as described previously (4).

Cytolytic lymphocytes were obtained from CD-1 mice 10 or 11 days after i.p. injection of 3×10^7 EL4 cells (4). For the purpose of monitoring the effects of drugs on the release of ^{51}Cr from cytolytic lymphocytes, these cells were labeled with $Na_2{}^{51}CrO_4$ in the same manner as EL4 cells (4).

Where indicated, cell viability was assessed by the trypan blue exclusion method (5).

Cytolytic assay. The *in vitro* assay of cytolysis employed in this work has been described previously (4). Briefly stated, this assay monitors the release of ^{51}Cr during a 70-min incubation at 37° of 2.5×10^5 ^{51}Cr-labeled EL4 cells and an equal number of cytolytic lymphocytes. Under these conditions, 19%–36% specific lysis occurred in the control assays of different experiments.

W-7, W-5, CPZ, TFP, CPZSO, and TFPSO were added to cell suspensions as aqueous solutions. Stock solutions of R 24 571 and pimozide were prepared with ethanol as solvent and were diluted into medium so as to give ethanol concentrations of ≤0.2% (v/v) in the cell suspensions. In control assays, 0.2% (v/v) ethanol did not affect LMC significantly.

Lymphocyte ATP measurements. To determine the effects of drugs on the ATP content of cytolytic lymphocytes, cells (2.4 to 12.6×10^7) were first incubated with 300 μCi of [2,8-^{3}H]adenine for 30 min at 37° in 9 ml of medium supplemented with glucose (1 mg/ml). This cell suspension was then cooled in an ice bath for 10 min, and the cells were harvested by centrifugation and washed two times with cold medium. These washed lymphocytes were resuspended in cold medium to a density of 7.5×10^5 cells/2.7 ml, and 2.7-ml volumes of this cell suspension were supplemented with 0.3 ml of saline or drug and incubated for the specified times at 37°. These cell suspensions were then cooled in an ice bath for 10 min and the cells were harvested by centrifugation. Each cell pellet was extracted with 5.0 ml of cold 0.5 M perchloric acid containing 2.0 μM ITP as a recovery marker. These extracts were clarified by centrifugation, neutralized with KOH, filtered through glass wool to remove the insoluble potassium perchlorate, evaporated to dryness under reduced pressure (in a Buchler Evapo-Mix apparatus), and reconstituted with 300 μl of deionized water. These samples were stored at −20° until their analysis of [^{3}H]adenine nucleotides by anion exchange HPLC (4). The column effluent was monitored at both 254 and 280 nm with full-scale absorbance ranges of 0.02. Samples (100 μl) of each cell extract were injected into the liquid chromatograph, and the effluent was collected at 1.0-min intervals and monitored in a liquid scintillation spectrometer. The concentration of ITP present in the cell extracts was calculated from the response factor (ultraviolet peak area per nanomole of ITP) determined by injecting known amounts of ITP into the liquid chromatograph. The amount of ITP present in each extract was used to normalize each analysis to the original cell count. Each experiment was performed in duplicate. Extracts from lymphocytes labeled with [^{3}H]adenine in the above manner exhibited ratios for [^{3}H]ATP: [^{3}H]ADP: [^{3}H]AMP of 1.0:0.07:0.02, respectively, for the control cells.

In other experiments, larger numbers of cytolytic lymphocytes (1.5 to 1.8×10^7 cells/5.0 ml of medium) were incubated for the specified times in the absence or presence of drug, and acid-soluble extracts were prepared as above and analyzed by HPLC for their absolute content of ATP and ITP by direct measurement of the nucleotide peak areas present in the chromatograms (4). The concentration of ATP in control lymphocytes was 418–799 pmol/10^6 cells in different experiments.

RESULTS

Dependence of LMC on Ca^{2+}. As reviewed under introduction, Ca^{2+} has previously been reported to be an essential cofactor in the lysis of tumor cells by specifically sensitized mouse T lymphocytes (1, 2); however, as a prelude to the present investigation, this Ca^{2+} requirement was confirmed under the experimental conditions utilized in this laboratory for the *in vitro* assay of LMC.

Failure to add $CaCl_2$ to the medium ("calcium-deficient" medium) resulted in a 60% decrease in cytolysis, whereas the addition of 1.0 mM EGTA to complete medium caused total suppression of cytolytic activity. The addition of ≥1.0 mM $CaCl_2$ to EGTA (1.0 mM)-containing medium resulted in full restoration of cytolytic activity, thus indicating that the inhibitory effect of EGTA on LMC is an indirect effect due to the chelation of Ca^{2+} and not a direct inhibitory effect of EGTA itself (data not shown).

Effects of calmodulin antagonists on LMC assay. Eight putative calmodulin antagonists were examined for their effect upon the LMC assay: W-7 (6), W-5 (6), CPZ (7), TFP (7), R 24 571 (8), pimozide (7); CPZSO (7), and TFPSO (7). These agents were found to alter specific (i.e., lymphocyte-mediated) and spontaneous (i.e., lymphocyte-independent) lysis of ^{51}Cr-labeled EL4 cells in highly diverse manners (Figs. 1 and 2). W-7 (Fig. 1A), W-5 (Fig. 1B), CPZ (Fig. 1C), TFP (Fig. 1D), and pimozide (Fig. 2B) inhibited LMC by 50% at concentrations (8.9, approximately 50, 7.4, 9.4, and 15 μM, respectively) that did not increase appreciably the spontaneous release of ^{51}Cr from the radiolabeled EL4 target cells; however, at concentrations only slightly higher than these IC_{50} values, W-7, CPZ, TFP, and pimozide were observed to cause marked increases in the spontaneous release of radioactivity from the EL4 cells. By contrast with the other five agents tested, R 24 571 inhibited LMC only slightly (≤25%) at concentrations (≤6 μM) at which it did not cause an increase in the spontaneous ^{51}Cr release from EL4 cells (Fig. 2A). CPZSO and TFPSO were not inhibitory to LMC at ≤20 μM; at 100 μM, these agents inhibited LMC by 10 and 24%, respectively, without affecting spontaneous lysis of the EL4 cells (data not shown).

Reversibility and cell specificity of W-7 and CPZ in LMC. W-7 and CPZ were investigated in more detail in an attempt to determine whether these agents were acting upon the effector lymphocytes or the target cells in the LMC assay. Accordingly, cytolytic lymphocytes or ^{51}Cr-labeled EL4 cells were incubated separately for 60 min at 37° in the absence or presence of W-7 (15 μM) or CPZ (15 μM), and these cells were then washed free of drug and added to LMC assays containing either ^{51}Cr-labeled EL4 cells or cytolytic lymphocytes, respectively, which had not been pretreated with drug. Cytolytic lymphocytes which had been pretreated with W-7 or CPZ and washed free of exogenous drug were almost as fully inhibited in the LMC assay as when this same concentration of drug was added directly to the LMC assay mixtures containing non-drug-treated lymphocytes and target cells (Table 1). By contrast, prior treatment of ^{51}Cr-labeled EL4 cells with W-7 or CPZ was found to

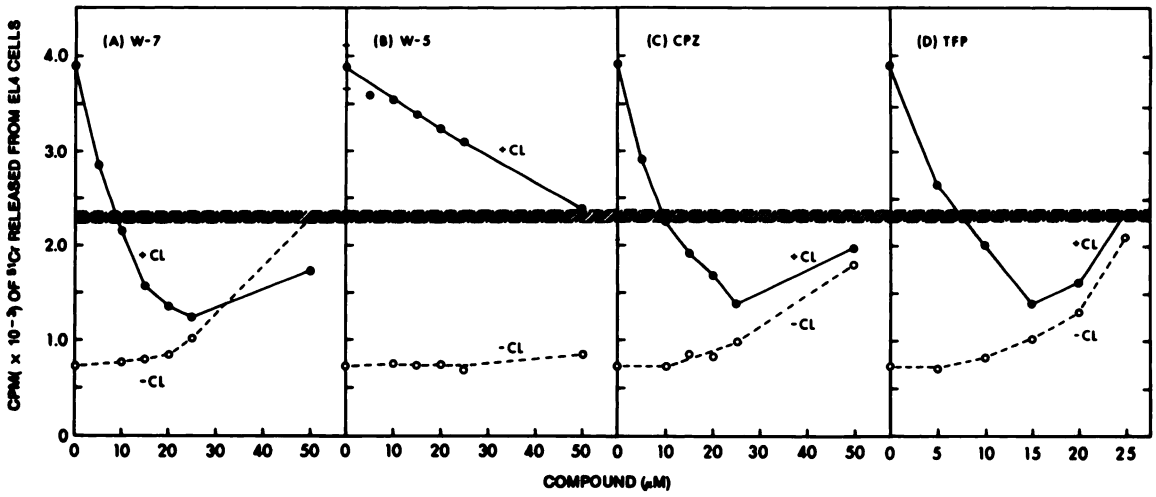

FIG. 1. *Concentration-response curves for the effects of several calmodulin antagonists on the specific and nonspecific release of radioactivity from ^{51}Cr-labeled EL4 cells*

The LMC assay were conducted in the presence (●) and absence (○) of cytolytic lymphocytes (*CL*) at the indicated concentrations of (A) W-7, (B) W-5, (C) CPZ, and (D) TFP. Of the total radioactivity released by freeze-thaw treatment (17,370 cpm) of the ^{51}Cr-labeled EL4 cells, 3887 cpm were released in the cytolysis assay in the presence of non-drug-treated cytolytic lymphocytes and 647 cpm were released nonspecifically when ^{51}Cr-labeled EL4 cells were incubated for 70 min at 37° in the absence of lymphocytes or drugs. Values represent the means for duplicate determinations. Results are representative of two or three experiments for each drug. The *striped zone* across the width of the figure represents the amount of released radioactivity corresponding to 50% inhibition of specific ^{51}Cr release in this particular experiment.

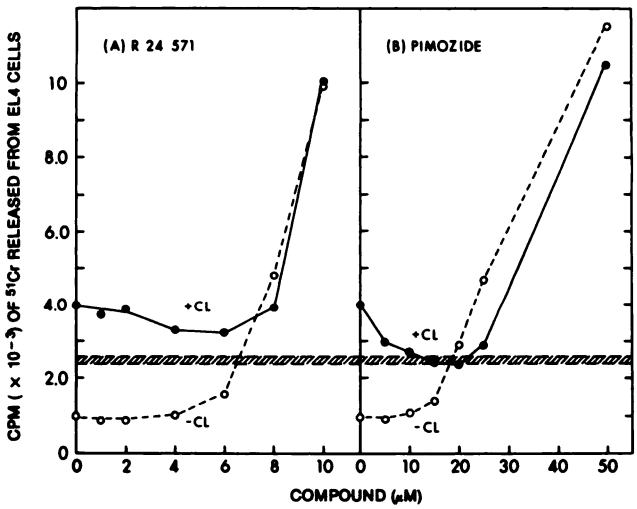

FIG. 2. *Concentration-response curves for the effects of R 24 571 and pimozide on the specific and nonspecific release of radioactivity from ^{51}Cr-labeled EL4 cells*

The LMC assays were conducted in the presence (●) and absence (○) of cytolytic lymphocytes (*CL*) at the indicated concentrations of (A) R 24 571 and (B) pimozide. Of the total radioactivity released by freeze-thaw treatment (13, 128 cpm) of the ^{51}Cr-labeled EL4 cells, 3985 cpm were released in the cytolysis assay in the presence of non-drug-treated cytolytic lymphocytes and 972 cpm were released nonspecifically when ^{51}Cr-labeled EL4 cells were incubated for 70 min at 37° in the absence of lymphocytes or drugs. Values represent the means of duplicate determinations. Results are representative for two experiments for each drug. Symbols and the *striped zone* have the same meaning as in Fig. 1.

enhance (by 28% and 19%, respectively) their subsequent lysis by cytolytic lymphocytes (Table 1). These findings demonstrate that W-7 and CPZ inhibit LMC solely as a result of their interaction with the effector lymphocytes and that this effect is not rapidly reversible upon washing the cells with drug-free medium.

In a separate experiment it was found that, when cytolytic lymphocytes were incubated for 60 min at 0° with either CPZ (15 μM) or W-7 (15 μM) and were then washed free of exogenous drug at 0° prior to their addition to LMC assays, no inhibition of cytolysis occurred (data not shown). This result is comparable to that reported by Nelson *et al.* (9).

Consistent with an earlier report (10), the data in Table 1 show that incubation of cytolytic lymphocytes for 60 min at 37° prior to the start of the LMC assay resulted in an increase in their cytolytic activity (36.3% versus 27.6% specific ^{51}Cr release); however, a similar preincubation of ^{51}Cr-labeled EL4 cells resulted in a decreased susceptibility of these cells to lysis (19.5% versus 27.6% specific ^{51}Cr release).

Effects of calmodulin antagonists on lymphocyte viability. The observation that many of these putative calmodulin antagonists cause an increase in the spontaneous (i.e., nonspecific) release of radioactivity from ^{51}Cr-labeled EL4 cells (Figs. 1 and 2) indicated that these agents may affect LMC as the result of a nonselective, toxic effect on the cytolytic lymphocytes. For this reason, these agents were investigated for their effects upon lymphocyte viability, as judged both by cellular exclusion of trypan blue and by cellular retention of radioactivity with $Na_2{}^{51}CrO_4$-labeled lymphocytes.

The ability of cytolytic lymphocytes to exclude trypan blue after a 60-min treatment with various concentrations of these calmodulin antagonists is presented in Table 2. W-7 (10 μM), W-5 (50 μM), CPZ (10 μM), and TFP (10 μM) had little or no effect upon dye exclusion by the lymphocytes at concentrations of each compound similar to their corresponding IC_{50} values in the LMC

TABLE 1

Reversibility and cell specificity of W-7 and CPZ effects in the LMC assay

Cytolytic lymphocytes or ^{51}Cr-labeled EL4 cells (3.6×10^5 cells/ml) were pretreated for 60 min at 37° with saline, W-7 (15 μM), or CPZ (15 μM). Other suspensions of cytolytic lymphocytes and ^{51}Cr-labeled EL4 cells were kept at 0° during this 60-min pretreatment period. The pretreated cells were harvested by centrifugation and washed one time with 10 ml of cold drug-free medium. These different cell samples were resuspended in drug-free medium and added to the specified LMC assays. Of the total radioactivity released by free-thaw treatment (26,794 cpm) of the ^{51}Cr-labeled EL4 cells, 10,213 cpm were released in the LMC assays in the presence of non-pretreated, control cytolytic lymphocytes and 2,824 cpm were released nonspecifically when ^{51}Cr-labeled EL4 cells were incubated for 70 min at 37° in the absence of lymphocytes. Each drug pretreatment was performed in duplicate and each resulting cell sample was tested in duplicate in subsequent LMC assays. Each value of percentage of specific ^{51}Cr release represents the mean ± standard error of the mean for four determinations.

Cytolytic lymphocyte pretreatment agent	^{51}Cr-Labeled EL4 cell pretreatment agent	Drug added to LMC assay	Cytolysis (% specific ^{51}Cr release)
Saline	None[a]	Saline	36.3 ± 1.5 (100)[b]
Saline	None	W-7 (15 μM)	5.6 ± 0.5 (15)
Saline	None	CPZ (15 μM)	13.4 ± 0.8 (37)
W-7 (15 μM)	None	Saline	7.4 ± 1.8 (20)
CPZ (15 μM)	None	Saline	19.4 ± 0.9 (53)
None[a]	Saline	Saline	19.5 ± 0.8 (100)
None	Saline	W-7 (15 μM)	0.2 ± 0.6 (3)
None	Saline	CPZ (15 μM)	6.0 ± 0.3 (31)
None	W-7 (15 μM)	Saline	24.9 ± 0.5 (128)
None	CPZ (15 μM)	Saline	23.2 ± 1.1 (119)
None[a]	None[a]	Saline	27.6 ± 0.2 (100)
None	None	W-7 (15 μM)	4.1 ± 0.7 (15)
None	None	CPZ (15 μM)	10.0 ± 0.2 (36)

[a] "None" means that these cells were kept at 0° during the 60-min pretreatment period.

[b] Values in parentheses represent the percentage of control cytolysis observed with each of the three different combinations of cells: pretreated lymphocytes plus non-pretreated EL4 cells, non-pretreated lymphocytes plus pretreated EL4 cells, and non-pretreated lymphocytes plus non-pretreated EL4 cells.

TABLE 2

Effects of calmodulin antagonists on the viability of cytolytic lymphocytes determined with the trypan blue exclusion test

Cytolytic lymphocytes (3.3×10^5 cells/1.0 ml of medium) were incubated for 60 min at 37° with the specified compound prior to determination of their uptake of trypan blue. Values represent the mean of duplicate determinations, and the results are representative of two similar experiments.

Compound (concentration)	Cellular dye uptake (% of total lymphocytes)
None (saline)	2.0
W-7 (10 μM)	2.5
W-7 (25 μM)	6.3
W-7 (50 μM)	14.9
W-5 (50 μM)	2.3
CPZ (10 μM)	4.3
CPZ (25 μM)	2.3
CPZ (50 μM)	21.9
TFP (10 μM)	4.1
TFP (25 μM)	66.7
R 24 571 (2.0 μM)	5.7
R 24 571 (10 μM)	96.8
Pimozide (10 μM)	13.4
Pimozide (15 μM)	77.2
Pimozide (25 μM)	83.5
Pimozide (50 μM)	95.0

assay (see Fig. 1); however, higher concentrations of W-7, CPZ, and TFP did increase the percentage of cells which took up trypan blue. R 24 571 had little effect upon this parameter at 2.0 μM, but caused essentially all of the cells to be stained with dye at 10 μM. Pimozide caused a large increase in trypan blue uptake by the lymphocytes at concentrations (10–25 μM) similar to those at which pimozide inhibits LMC (see Fig. 2B).

As another measure of cytotoxicity, these different calmodulin antagonists were examined for their ability to stimulate the release of radioactivity from $Na_2{}^{51}CrO_4$-labeled cytolytic lymphocytes (Fig. 3). W-7, W-5, CPZ, and TFP had little or no effect upon the cellular retention of ^{51}Cr during 60-min incubations of the lymphocytes with LMC-inhibitory concentrations of these four agents, although higher concentrations (i.e., ≥20 μM) of TFP markedly stimulated ^{51}Cr release. By contrast, pimozide stimulated ^{51}Cr release at concentrations (≥15 μM) similar to its IC_{50} value in the LMC assay. R 24 571 caused a large increase in ^{51}Cr release from the lymphocytes at concentrations of ≥5.0 μM.

Effects of calmodulin antagonists on lymphocyte ATP levels. Since metabolic energy is required for LMC (2), and since CPZ and TFP have previously been reported to depress cellular ATP levels (11), six calmodulin antagonists were examined for their effects upon the ATP content of the cytolytic lymphocytes. As shown in Fig. 4, a 60-min treatment of [^{3}H]adenine-pretreated lymphocytes with these various agents caused widely different effects upon the relative cellular content of [^{3}H]ATP. R 24 571 caused a marked depression in lymphocyte [^{3}H]ATP levels at a concentration as low as 4.0 μM. Pimozide was the next most active agent in this regard and caused a large decrease in lymphocyte [^{3}H]ATP above 10 μM. TFP was also quite effective in reducing the lymphocyte

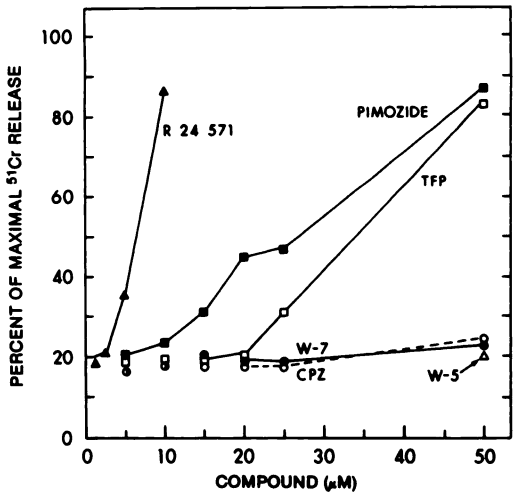

FIG. 3. *Concentration-response curves for the effects of calmodulin antagonists on the release of radioactivity from $Na_2{}^{51}CrO_4$-labeled cytolytic lymphocytes*

^{51}Cr-labeled lymphocytes (2.5×10^5 cells/1.0 ml of medium) were incubated for 60 min at 37° with the indicated concentrations of each compound. The amount of radioactivity released into the supernatant fluid was then determined after removal of the cells by centrifugation. Values represent the means for duplicate determinations. Results are representative of two similar experiments. Maximal ^{51}Cr release in these experiments is defined as the amount of radioactivity (4,872–26,656 cpm) released by freeze-thaw treatment of the $Na_2{}^{51}CrO_4$-labeled lymphocytes. ▲, R 24 571; ■, pimozide; □, TFP; ●, W-7; ○, CPZ; △, W-5.

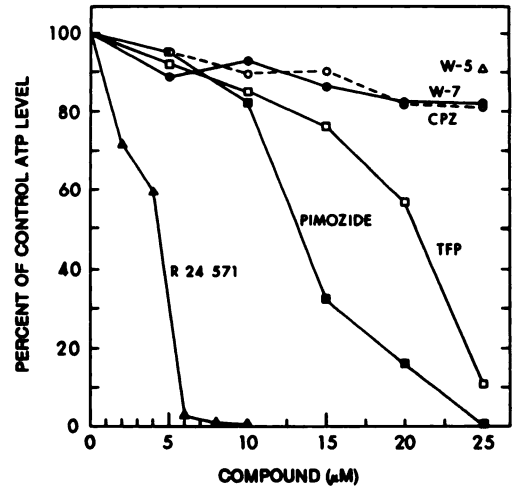

FIG. 4. *Concentration-response curves for the effects of calmodulin antagonists on the ATP content of cytolytic lymphocytes*

[^{3}H]Adenine-labeled lymphocytes (7.5×10^5 cells/3.0 ml of medium) were incubated for 60 min at 37° with the indicated concentrations of each compound. Acid-soluble extracts of these cells were then prepared and analyzed by anion-exchange HPLC in order to determine the cellular content of [^{3}H]ATP. Control cell [^{3}H]ATP levels ranged between 2,263,000 and 4,225,000 dpm/10^6 cells in these experiments. Values represent the means for duplicate determinations. Results are representative of two similar experiments. ▲, R 24 571; ■, pimozide; □, TFP; ●, W-7; ○, CPZ; △, W-5.

content of [^{3}H]ATP at drug concentrations >10 μM. By contrast, W-7, W-5, and CPZ caused relatively small (≤20%) decreases in lymphocyte [^{3}H]ATP levels at 25 μM drug, and W-7 and CPZ had even smaller effects at concentrations (approximately 10 μM) similar to their IC_{50} values in the LMC assay.

The results presented in Fig. 4, which depicts the effects of these different agents on the *relative* concentrations of lymphocyte ATP, have been confirmed by HPLC determination of the *absolute* concentrations of ATP in drug-treated cells in experiments employing much larger numbers of lymphocytes. In these latter experiments, W-7 (50 μM), CPZ (50 μM), TFP (50 μM), R 24 571 (10 μM) and pimozide (50 μM) were found to cause decreases in the absolute concentration of lymphocyte ATP of 29, 26, 94, 98, and 99%, respectively, after a 60-min drug treatment of the cells (data not shown).

In another experiment, [^{3}H]adenine-labeled lymphocytes were incubated for 15 min in the absence or presence of 10 μM R 24 571, and the radioactivity present, both within the cells and in the medium, was analyzed separately by anion exchange HPLC. During this 15-min incubation time, R 24 571 caused a 69% decrease in cellular [^{3}H]ATP. Within these same cells, R 24 571 caused a large buildup of [^{3}H]ADP and [^{3}H]AMP whose combined increment accounted for 56% of the concomitant decrease in [^{3}H]ATP. R 24 571 did not cause the appearance of [^{3}H]ATP or [^{3}H]ADP in the medium. The increased radioactivity found in the medium from the R 24 571-treated cells was in the form of monophosphorylated and nonphosphorylated compounds (results not shown). These results indicate that R 24 571 did not decrease lymphocyte [^{3}H]ATP content by making the cell membrane permeable to intact ATP but rather that R 24 571 interfered with cellular energy metabolism and that ATP breakdown products exited the cells.

Influence of serum concentration on effects of calmodulin antagonists. All of the above experimental results were obtained using as medium Dulbecco's phosphate-buffered saline supplemented with 5% fetal calf serum. During the course of these studies, it was observed that the calmodulin antagonists exhibited reduced cellular effects when they were tested in medium containing 20% serum. The IC_{50} values for W-7, CPZ, and TFP in the LMC assay were increased 1.5- to 1.7-fold when the LMC assays were carried out in medium containing 20% serum rather than 5% serum. This higher serum concentration was also observed to protect lymphocytes partially from the deleterious effects of the calmodulin antagonists on lymphocyte ATP levels and viability. W-7, CPZ, TFP, R 24 571, and pimozide exhibited reduced effects upon lymphocyte ATP content and/or cell viability in the medium containing the higher concentration (20%) of serum (results not shown).

DISCUSSION

The present study provides pharmacological evidence that calmodulin may be the molecular entity within the sensitized mouse T lymphocyte, which requires Ca^{2+} for the execution of its cytolytic function. The principal findings supporting this conclusion are the following:

1. W-7, W-5, CPZ, and TFP, all of which have been demonstrated to act as calmodulin antagonists *in vitro* (6–8), inhibited LMC at concentrations consistent with their published dissociation constants with calmodulin

(7, 12). Particularly noteworthy was the observation that W-7 was approximately 5-fold more potent than W-5 in inhibiting LMC, since this potency differential compares favorably with the reported relative potencies of these two agents as calmodulin antagonists *in vitro* (6). The relative inactivity toward LMC of CPZSO and TFPSO, derivatives of CPZ and TFP with greatly reduced affinities for calmodulin (7), provides further support for the involvement of calmodulin in the mechanism of LMC.

2. This inhibition of LMC occurred at concentrations of these four agents which did not cause detectable toxicity (i.e., altered cell viability or ATP depletion) to the effector lymphocytes. Previously, other investigators have demonstrated that calmodulin is present in lymphocytes from humans (13) and mice (9).

Two distinct but interrelated factors serve to obscure the interpretation of pharmacological studies such as the present one: (a) the existence of numerous metabolic sites at which Ca^{2+} possibly could be acting within the cytolytic lymphocytes, and (b) the recognized nonspecificity of virtually all of the putative calmodulin antagonists.

In addition to calmodulin, Ca^{2+}-activated phospholipid-dependent protein kinase (14), phospholipase A_2 (15), and phospholipase C (16) are examples of other cellular proteins which require Ca^{2+} for their normal function. Purified Ca^{2+}-activated phospholipid-dependent protein kinase has been reported to be inhibited by W-7 (17), W-5 (17), CPZ (18), and TFP (18); however, the concentrations of these four agents required to inhibit this purified protein kinase are generally higher than those required to inhibit LMC, and 100 μM TFP has been reported not to inhibit this enzyme in intact human platelets (19). Phospholipase A_2 has been reported to be inhibited by W-7 (20), W-5 (20), CPZ (21), and TFP (20, 21) in intact cells, but it is not known whether this is due to direct inhibition of the enzyme or to an indirect effect of these agents on calmodulin. Although phospholipase C present in soluble and particulate fractions of broken cells is inhibited by W-7 (20), CPZ (16, 20), and TFP (20), this enzyme is inhibited poorly within intact cells by these same three agents (19–21).

The principal, nonspecific side effects observed with several of the putative calmodulin antagonists was their interference with cellular energy metabolism. This phenomenon was particularly pronounced with R 24 571, pimozide, and TFP (Fig. 4). Although R 24 571 and pimozide did inhibit LMC to varying extents at micromolar concentrations (Fig. 2), it was not possible to dissociate this latter effect from the depression in lymphocyte ATP caused by similar concentrations of these two agents. With TFP, it was possible to demonstrate potent inhibition of LMC at drug concentrations (i.e., $\leq$10 μM) (Fig. 1D) which did not decrease substantially the lymphocyte ATP content (Fig. 4). This previously unreported effect of R 24 571 and pimozide on cellular ATP was somewhat disappointing, since these two agents are the most potent known antagonists of purified calmodulin (7, 8), and it was therefore anticipated that they would act in an especially specific manner toward calmodulin in experiments utilizing intact cells. On the basis of a study employing isolated mitochondria, it has been concluded that agents such as TFP and pimozide disrupt energy production by a calmodulin-independent mechanism (22). In general, the effects of these calmodulin antagonists on lymphocyte viability (see Figs. 3 and 4) paralleled their effects on lymphocyte ATP concentration (i.e., large decreases in cellular ATP content were accompanied by increased uptake of trypan blue and by decreased cellular retention of ^{51}Cr).

The ability of serum to decrease the potency of these calmodulin antagonists toward the lymphocytes is attributed to binding of these agents to serum proteins, thus resulting in reduced concentrations of free drug in the presence of higher serum concentations. Both CPZ (23) and TFP (24) have been shown to bind extensively to serum proteins, and preliminary experiments have provided evidence that W-7 also binds appreciably to serum proteins (results not shown). This influence of serum concentration upon the biological activity of these calmodulin antagonists is a factor which should be taken into account when comparing the potency of such agents in different *in vitro* cellular test systems.

If, indeed, calmodulin is the lymphocyte receptor whose occupancy by Ca^{2+} is necessary for cytolytic activity, one must then ask what metabolic event within these effector cells is modulated by the Ca^{2+}·calmodulin complex. Although a number of enzymes have been found to be regulated by the Ca^{2+}·calmodulin complex (3), such a question regarding LMC is particularly difficult to address because the molecular events involved in LMC are unknown. At the cellular level, it is known that LMC requires extensive membrane interaction between the cytolytic lymphocyte and its target cell (2), and that the area of attachment of cytolytic lymphocytes to their target cells shows high mobility and the polarization of actin but not of myosin (25). In addition, cytochalasins A and B, agents which interfere with actin polymerization, have been shown to inhibit LMC (26, 27). Thus, there are several types of indirect evidence suggesting the participation of lymphocyte contractile proteins in the molecular mechanism of LMC. With smooth muscle and nonmuscle cells, it has been found that the actin-myosin interaction is regulated by the phosphorylation/dephosphorylation of myosin and myosin kinase (28). The reversible phosphorylation of myosin by myosin kinase regulates the actin-activated MgATPase activity of myosin and allows the conversion of the chemical energy of ATP into the mechanical movement of myosin and actin filaments past each other to generate contractile activity. Myosin kinase is completely inactive in the absence of Ca^{2+} and calmodulin (28). These findings together indicate that the Ca^{2+} requirement of LMC may derive from the need for activation of myosin kinase by the Ca^{2+}·calmodulin complex in order to generate contractile activity within the sensitized lymphocytes, which is necessary for the cytolytic function of these cells. This hypothesis has another attractive feature: cyclic AMP-dependent protein kinase is known to catalyze the phosphorylation and consequent inactivation of myosin kinase (28); thus, an essential involvement of myosin ki-

nase in the mechanism of LMC would also provide an explanation for the observed inhibition of LMC by all agents that are known to raise lymphocyte cyclic AMP levels (29).

The results obtained in this study may also be pertinent to recent investigations in cancer chemotherapy. Calmodulin antagonists such as TFP have been found to enhance the cytotoxicity of vincristine (30–32) and Adriamycin (30–33), apparently because of the ability of the calmodulin antagonists to augment the cellular accumulation of these anticancer drugs by preventing their efflux from cells. Since efflux of vinca alkaloids from drug-resistant human leukemic lymphoblasts has been found to be an energy-dependent process (34), the observed therapeutic interaction between calmodulin antagonists and vincristine and Adriamycin may result, at least in part, from the ability of some of these putative calmodulin antagonists to depress cellular ATP levels. In a similar manner, this deleterious effect on cellular energy metabolism may contribute to the reported antiproliferative activity of some of these calmodulin antagonists when tested as single agents (35–38).

ACKNOWLEDGMENTS

We thank Robert L. Veasey and Robert D. Deeprose for their excellent technical assistance, and we express our gratitude to Ms. Linda de Leon for her preparation of the graphics and to Ms. Tonya Hales for typing the manuscript. We are grateful to Dr. Herman Van Belle, of Janssen Pharmaceutica, for generously providing the R 24 571 employed in this study, and to Dr. Richard Mailman, of the University of North Carolina, for his generous gifts of TFPSO and CPZSO.

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