0026-895X/90/050698-07$03.00/0

MOLECULAR PHARMACOLOGY, 38:698-704

Differential Inhibition of Calcium-Dependent and Calmodulin-Dependent Enzymes by Drug-Calmodulin Adducts

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Received February 13, 1990; Accepted August 15, 1990

SUMMARY

Most of the currently available calmodulin (CaM) antagonists inhibit the actions of CaM by binding directly to it. These CaM-binding drugs tend to be relatively nonselective, because they inhibit the interaction of CaM with most, if not all, of its target enzymes. In order to develop more selective CaM antagonists, we synthesized covalent adducts of CaM and several drugs, including chlorpromazine (CPZ), fluphenazine-*N*-mustard (FNM), and phenoxybenzamine (PBZ), and examined the effects of these adducts on various CaM and Ca^{2+}-dependent enzymes. One of the adducts (CPZ-CaM) selectively inhibited the CaM-induced activation of phosphodiesterase and myosin light chain kinase, without affecting the basal activity of either enzyme. The inhibition of these enzymes by CPZ-CaM was competitive with respect to CaM. CPZ-CaM did not inhibit CaM-sensitive Ca^{2+}-ATPase or CaM-dependent protein kinase or the CaM-insensitive enzyme protein kinase C. The FNM-CaM and PBZ-CaM adducts did not inhibit the effects of CaM on any of the enzymes, but they selectively activated two of the enzymes; FNM-CaM slightly activated the CaM-dependent protein kinase, and PBZ-CaM slightly activated phosphodiesterase. These results show that certain covalently linked drug-CaM adducts can differentially inhibit or activate various CaM-sensitive enzymes, and they provide further evidence that it may be possible to develop new classes of CaM antagonists that are directed against the CaM recognition sites on CaM-sensitive enzymes.

CaM is an important intracellular Ca^{2+}-binding protein that has been shown to regulate the activities of a variety of Ca^{2+}-sensitive enzymes (1–3). Each CaM molecule can bind up to four Ca^{2+} ions. Upon binding Ca^{2+}, the CaM molecule undergoes conformational changes that allow the Ca^{2+}-CaM complex to interact with specific recognition sites on various target enzymes.

Although there are a wide variety of approaches that have been used to modify CaM activity (4, 5), most efforts in this area have focused on agents such as the phenothiazine antipsychotics and structurally related cationic amphiphilic compounds, which inhibit the actions of CaM by binding to it in a reversible Ca^{2+}-dependent manner (6, 7) and preventing the interaction of the Ca^{2+}-CaM complex with its target enzymes. In general, these CaM-binding drugs are relatively nonselective, because they inhibit the interaction of CaM with most, if not all, of its target enzymes.

Thus far, relatively little attention has been given to developing CaM antagonists that act by binding to the CaM recognition sites on CaM-sensitive enzymes. However, several studies have shown that this may provide a useful approach for developing more selective CaM antagonists. For example, studies from our laboratories have shown that, upon irradiation with UV light, CPZ binds irreversibly to CaM (8). The resulting drug-CaM adduct (CPZ-CaM) was unable to activate the CaM-sensitive phosphodiesterase but did inhibit the activation of the enzyme by native CaM, apparently by competing with CaM for binding sites on the enzyme (9, 10). This same adduct had no effect on the activity of the CaM-sensitive Ca^{2+}-ATPase of human erythrocyte membranes.

Along similar lines, it was reported (11–13) that norchloropromazine isothiocyanate, an acylating phenothiazine, can bind irreversibly to CaM, even in the absence of UV irradiation. One of the resulting adducts ($CAPP_1$-CaM) was unable to activate CaM-sensitive phosphodiesterase or myosin light chain kinase but inhibited the activation of these enzymes by native CaM by competing with CaM for regulatory sites on the enzymes. This same adduct acted as a partial agonist for the CaM-sensitive phosphoprotein phosphatase calcineurin and as

This work was supported by Grant GM34334 awarded by the National Institute of General Medical Sciences and Grant MH42148 from the National Institute of Mental Health.

ABBREVIATIONS: CaM, calmodulin; CPZ, chlorpromazine; FNM, fluphenazine-*N*-mustard; PBZ, phenoxybenzamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′-tetraacetic acid; PIPES, piperazine-N,N′*-bis(2-ethanesulfonic acid); DTT, dithiothreitol; TFP, trifluoperazine; SDS, sodium dodecyl sulfate; $CAPP_1$, 2-chloro-10-(3-aminopropyl)phenothiazine.

a complete agonist for CaM-sensitive glycogen synthase kinase and phosphorylase kinase (12, 14).

Together, these preliminary studies indicate that covalently linked drug-CaM adducts can differentially inhibit various CaM-sensitive enzymes. In order to examine further the pharmacologic properties of drug-CaM adducts, we have prepared covalent adducts of CaM and several drugs, including CPZ, FNM (15, 16), and PBZ (17, 18), and examined the effects of these adducts (CPZ-CaM, FNM-CaM, and PBZ-CaM) on a variety of CaM-sensitive enzymes, including phosphodiesterase, myosin light chain kinase, Ca^{2+}-ATPase, and CaM-dependent protein kinase, and the CaM-insensitive enzyme protein kinase C.

Materials and Methods

Preparation and assay of CaM. CaM was purified from bovine testes by the method of Teo *et al.* (19). The purity of the CaM was assessed by SDS-polyacrylamide gel electrophoresis and by its ability to activate CaM-sensitive phosphodiesterase. At optimal concentrations, CaM increased the activity of the phosphodiesterase 10-fold. One-half maximal activation of the enzyme occurred at a CaM concentration of about 5 nM.

Preparation of the CPZ-CaM complex. CPZ was linked irreversibly to CaM by UV irradiation (8). Solutions of CaM (6–12 μM) and CPZ (100 μM) in 5 mM Tris·HCl buffer (pH 7.0), containing 100 μM $CaCl_2$, were stirred and irradiated from above, at a distance of 20 cm, with a long wavelength UV light (λ_{max} = 350 nm) for 1 hr. To remove unbound or reversibly bound drug, the sample was dialyzed for 6 hr against 5 mM Tris·HCl buffer (pH 7.0), containing 0.3 mM EGTA, and then for 12 to 24 hr against deionized water. Aliquots of the CPZ-CaM solution either were assayed immediately or were frozen and stored at −20° until used. Identical results were obtained using either fresh or frozen CPZ-CaM.

Preparation of the FNM-CaM adduct. Solutions of CaM (6–12 μM) and FNM (100 μM) in 5 mM Tris·HCl buffer (pH 7.0), containing 100 μM $CaCl_2$, were incubated at 37° for 2 hr. The samples were then dialyzed and frozen by the same procedure described above for the CPZ-CaM complex.

Preparation of the PBZ-CaM complex. Solutions of CaM (6 μM) and PBZ (60 μM) in 5 mM Tris·HCl (pH 7.0), containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$, were incubated at 37° for 2 hr. The samples were then dialyzed and frozen by the same procedure described above for the CPZ-CaM complex.

Preparation and assay of phosphodiesterase. CaM-sensitive phosphodiesterase was prepared from the soluble fraction of rat cerebrum by preparative polyacrylamide gel electrophoresis (20) and was stored at −20° until used. Cyclic AMP phosphodiesterase activity was measured by the luciferin-luciferase method (21). In this assay, the 5′-AMP formed from the hydrolysis of cyclic AMP is converted stoichiometrically to ATP, which is then quantified by the luciferin-luciferase technique using a Turner luminescence photometer.

Preparation of erythrocyte membranes and assay of Ca^{2+}-ATPase. The activity of the membrane-bound Ca^{2+}-ATPase was determined by a modification (10) of the procedure of Luthra (22). Briefly, blood from healthy male donors was collected in heparinized tubes. The blood was centrifuged at 4500 × *g* and the plasma and buffy coat were aspirated and discarded. The packed red blood cells were washed twice in 10 volumes of 3 mM histidine buffer containing 155 mM NaCl (isotonic buffer). To lyse the cells, saponin was added to give a final concentration of 0.1 mg/ml, and the cells were stirred gently at room temperature for 45 min. Erythrocyte membranes were isolated by centrifugation at 23,000 × *g*, and the membranes were washed twice with isotonic buffer. The membranes were resuspended in buffer, to give a final protein concentration of 1 to 2 mg of protein/ml, and then were frozen at −20° until used. The assay mixture contained 8.6 mM histidine (pH 7.5), 3.1 mM $MgCl_2$, 15 mM NaCl, 2 mM Tris-ATP, 0.1 mg/ml membrane, and either 5 μM $CaCl_2$ or 300 μM EGTA. Where indicated, the samples also contained varied amounts of CaM and/or the drug-CaM adducts. The reaction was started by addition of the ATP, and the samples were incubated at 37° for 2 hr. The reaction was stopped and inorganic phosphate was determined by the method of Baginski *et al.* (23). Ca^{2+}-ATPase activity was calculated by subtracting the amount of enzyme activity seen in the absence of Ca^{2+} (i.e., the presence of EGTA) from that seen in the presence of Ca^{2+}.

Preparation and assay of myosin light chain kinase. Myosin light chain kinase was purified from fresh chicken gizzards by DEAE-cellulose and CaM-Sepharose chromatography (24, 25). Enzyme activity was assayed by the method of Adelstein and Klee (25). The assay solution contained 20 mM Tris (pH 7.5), 1 mM DTT, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$ or 0.3 mM EGTA, 0.25 mg/ml myosin light chains, 240 nM CaM, 22 μg/ml myosin light chain kinase, and 0.1 mM ATP (0.5 μCi), in a final volume of 60 μl. The solution was preincubated at 30° for 5 min and the reaction was started by the addition of ATP. After 1 min, aliquots of 50 μl were transferred to phosphocellulose paper, which was placed in 75 mM phosphoric acid after 10 sec. The papers were then dried and counted. The activity of CaM-stimulated myosin light chain kinase and the basal activity of the enzyme were 2.37 and 0.53 μmol of P_i/min/mg of protein, respectively. The EC_{50} of CaM (one-half maximal activation of enzyme) occurred at a CaM concentration of about 120 nM.

Preparation and assay of protein kinase C. Protein kinase C was purified from rat brain by chromatography on DEAE-cellulose and Sephadex G-100 (26). The assay mixture contained 20 mM Tris·HCl (pH 7.5), 1 mM EGTA, 1.2 mM $CaCl_2$, 10 μM $MgCl_2$, 50 μg/ml phosphatidylserine, 5 μg/ml diolein, 62.5 μg/ml histone III-S, 10 μM [^{32}P] ATP (0.5 μCi), and 1 μg/ml protein kinase C, in a final volume of 60 μl. The reaction was started and stopped by the same procedure described above for the myosin light chain kinase. The activities of protein kinase C in the presence and absence of phosphatidylserine/diolein/Ca^{2+} were 39 and 9.8 μmol of P_i/min/mg of protein, respectively.

Preparation and assay of CaM-dependent protein kinase. Membrane-bound CaM-dependent protein kinase was partially purified from mouse brain, and the activity of the CaM-sensitive protein kinase in the membrane preparation was determined by measuring the effects of CaM on the phosphorylation of endogenous membrane proteins (27). The assay solution (60 μl) contained 50 mM PIPES (pH 7.0), 10 mM $MgCl_2$, 0.1 mM DTT, 0.12 mM EGTA, 0.25 mM $CaCl_2$, 0.5 μM CaM, 25 μM ATP (0.5 μCi/60 μl), and 0.2 mg of membrane protein/ml. The solution was incubated for 1 min at 30°. The reaction was started and stopped by the same procedure as described above for the myosin light chain kinase. The activities of the protein kinase in the presence and absence of CaM were 50 and 8 pmol of P_i/min/mg of protein, respectively. The EC_{50} of CaM was about 200 nM.

Statistical analyses. Statistical comparisons were done using analysis of variance, with Newman-Keuls *post hoc* analysis.

Materials. SDS, cAMP sodium salt, 5′-AMP, glycylglycine, phosphoenol pyruvate, histidine, saponin, PIPES disodium salt, histone III-S, phosphatidylserine, and diolein were obtained from Sigma Chemical Co. (St. Louis, MO); acrylamide from Bio-Rad Laboratories (Richmond, CA); ATP bioluminescence high sensitivity, DTT, pyruvate kinase, myokinase, and phenylmethylsulfonyl fluoride from Boehringer Mannheim Biochemicals (Indianapolis, IN); TFP, CPZ, PBZ, and FNM from SmithKline & French Laboratories (Philadelphia, PA); Sephadex G-100 from Pharmacia Fine Chemicals (Piscataway, NJ); DE-52 from Whatman (Kent, England); and [^{32}P]ATP (3000 Ci/mmol) from New England Nuclear Corporation.

Results

UV absorption spectra of CaM and the drug-CaM adducts. Concentrated aqueous solutions of the purified CaM (1 mg/ml) displayed characteristic absorption peaks at 276, 268,

265, and 253 nm (Fig. 1A). However, the intensity of absorption was quite low (extinction coefficient = 0.2/mg/ml/cm). At the low concentrations of CaM (6–12 μM) that were used to form the drug-CaM adducts, the absorbance was negligible.

In aqueous solutions, the spectra of both CPZ (Fig. 1B) and FNM (Fig. 1C) displayed sharp intense absorption peaks at 254 nm. In hydrophobic media such as octanol, these peaks were shifted to 260 nm. The absorption spectrum of PBZ differed from those of the phenothiazines. The main absorption peaks were located at 275, 268, and 263 nm and were much weaker than the phenothiazine absorbance peak (Fig. 1D).

Because the phenothiazine absorbance is much greater than that of CaM, it is possible to quantify the binding of phenothiazines to CaM by monitoring the major phenothiazine absorbance peak at 254–260 nm. The spectra of the CPZ-CaM (Fig. 2A) and the FNM-CaM (Fig. 2B) adducts each showed prominent phenothiazine absorption peaks at 260 nm, indicating that the phenothiazines were irreversibly bound to CaM. The fact that the peaks were located at 260 nm suggests that the phenothiazine chromophore is associated with a hydrophobic region of CaM.

The spectrum of the PBZ-CaM adduct showed an increased absorbance at 260–280 nm, when compared with that of native CaM (Fig. 2C). However, the stoichiometry for the binding of PBZ could not be accurately determined from this spectrum because the extinction coefficient of PBZ was rather low (only about 800/M/cm).

By using the known molar extinction coefficients for CPZ and FNM (each about 33,000/M/cm), the amount of drug bound to CaM could be estimated. For each adduct, approximately 2 mol of drug were bound per mol of calmodulin.

Effects of CPZ and the CPZ-CaM adduct on the activity of CaM-sensitive phosphodiesterase. Fig. 3 shows the effects of CPZ-CaM and CPZ on the basal and CaM-stimulated activity of phosphodiesterase. Although both agents inhibited the activation of phosphodiesterase by CaM, CPZ-CaM (IC_{50} = 0.2 μM) was about 200 times more potent than CPZ (IC_{50} = 42 μM). Neither CPZ nor CPZ-CaM affected the basal activity of phosphodiesterase.

Fig. 4 examines the effects of various drug-CaM adducts on the basal and CaM-stimulated activities of phosphodiesterase. CPZ-CaM completely inhibited the activation of phosphodiesterase by CaM, whereas FNM-CaM and PBZ-CaM, even at concentrations as high as 4 μM, had no inhibitory effect on the CaM-induced activation of the enzyme. None of the three adducts inhibited the basal activity of phosphodiesterase. The PBZ-CaM adduct slightly increased phosphodiesterase activity, indicating that it might have weak CaM agonist activity.

Effects of drug-CaM adducts on the activity of myosin light chain kinase. Fig. 5 shows the effects of the various drug-CaM adducts on the CaM-induced activation of myosin light chain kinase. CPZ-CaM inhibited the activation of the enzyme by CaM, with an IC_{50} value of about 3 μM. FNM-CaM and PBZ-CaM, even at concentrations as high as 6 μM, had

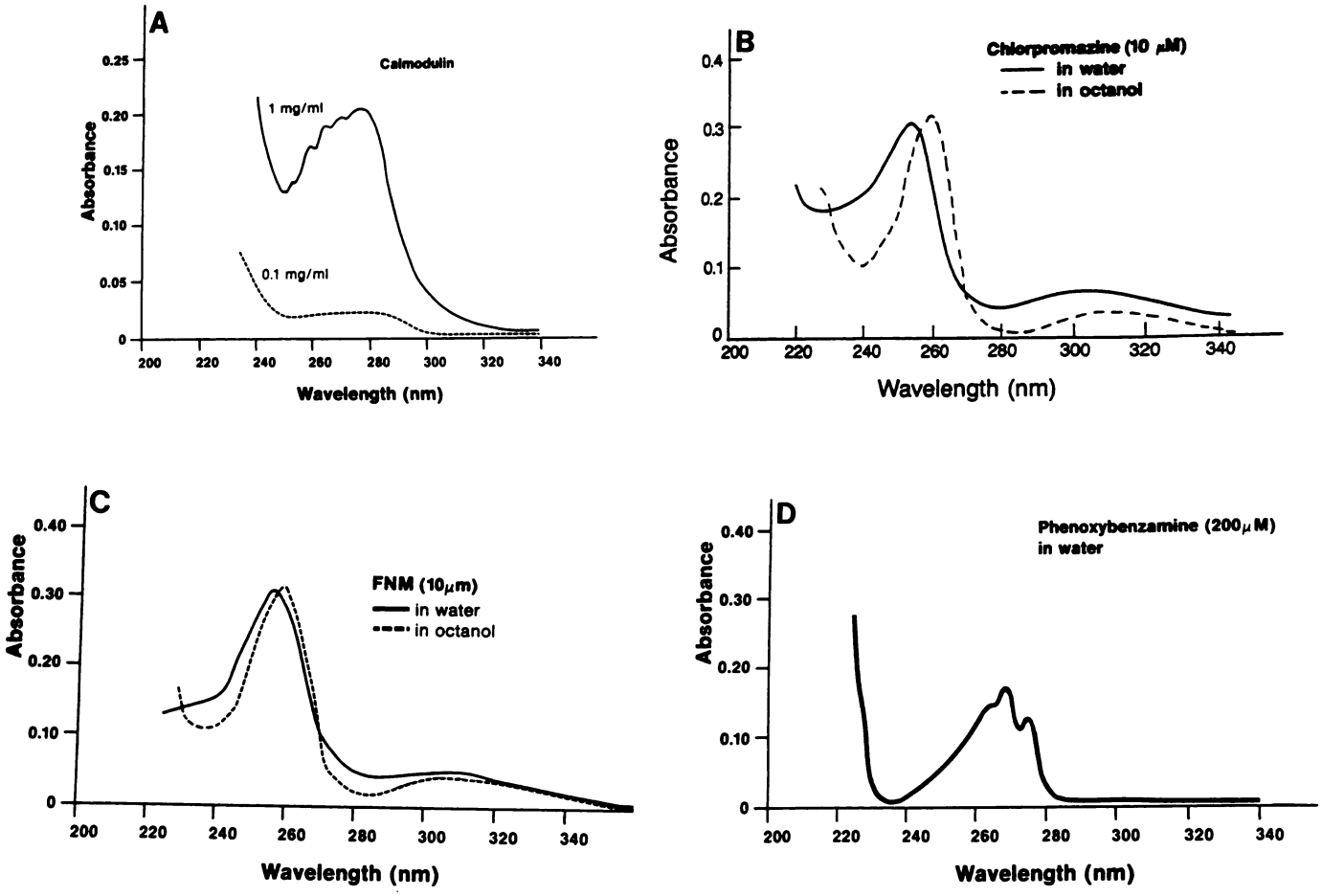

Fig. 1. UV absorption spectra of CaM and CaM antagonists. A, CaM; B, CPZ; C, FNM; D, PBZ.

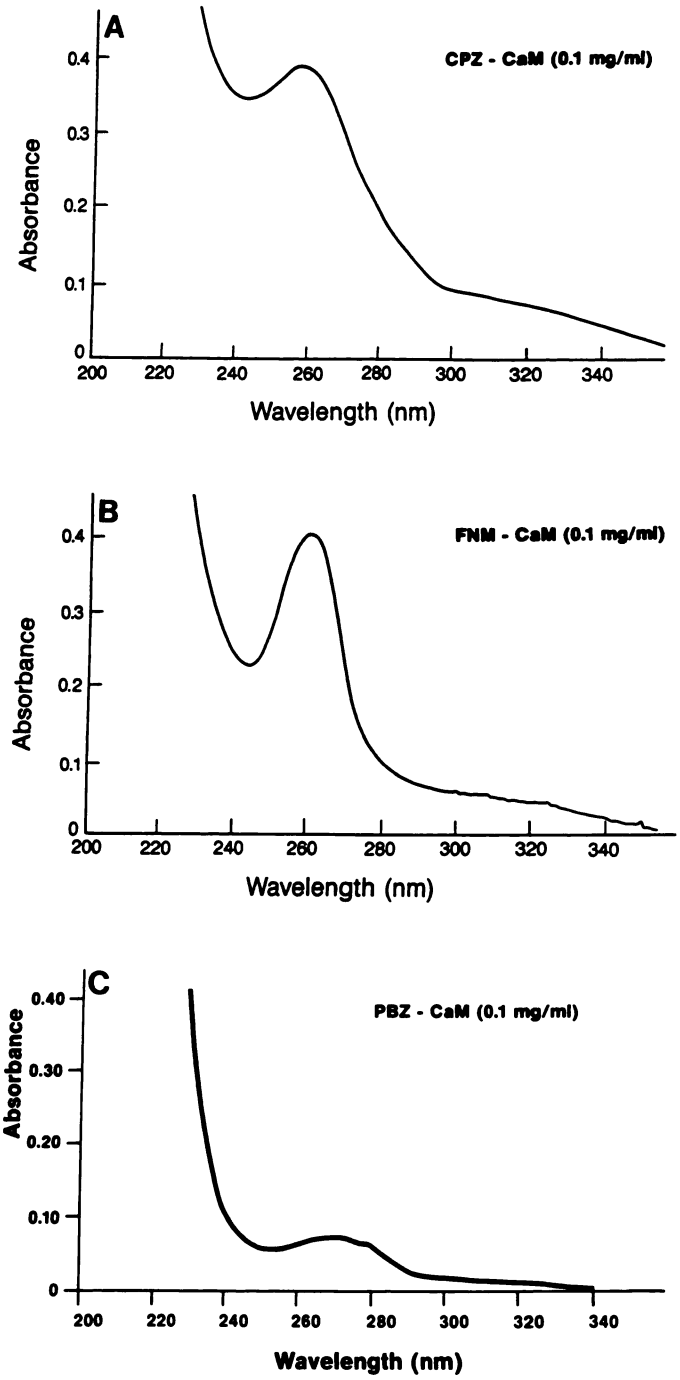

Fig. 2. UV absorption spectra of drug-CaM adducts. A, CPZ-CaM; B, FNM-CaM; C, PBZ-CaM.

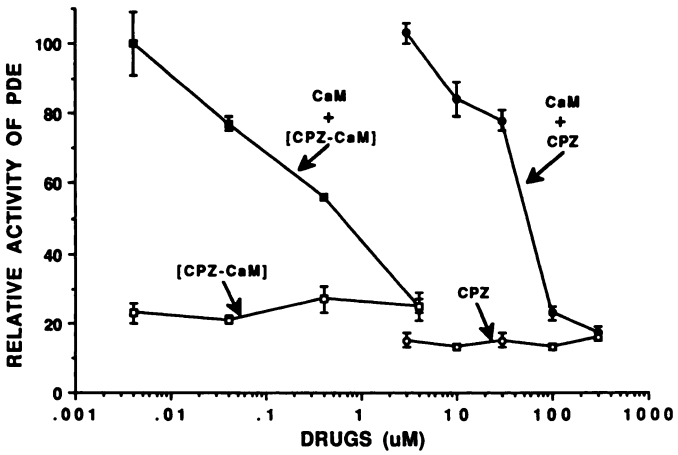

Fig. 3. Effects of CPZ-CaM and CPZ on the CaM-induced activation of phosphodiesterase. Phosphodiesterase activity was measured in the absence and presence of CaM (10 nM), with varied amounts of CPZ or CPZ-CaM. Each *point* represents the mean ± standard error of three samples.

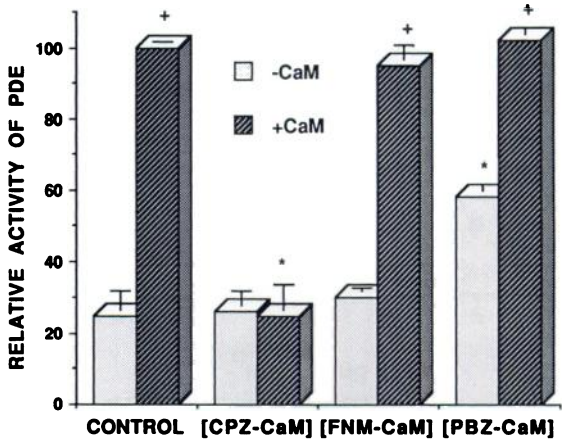

Fig. 4. Effects of CPZ-CaM, FNM-CaM, and PBZ-CaM on basal and CaM-stimulated phosphodiesterase activities. Phosphodiesterase activity was measured in the absence and presence of CaM (10 nM) and/or the various adducts (4 μM). Each *bar* represents the mean ± standard error of three samples. *, $p < 0.01$; +, $p < 0.01$.

little effect on the CaM-induced activation of myosin light chain kinase. None of the adducts had any significant effect on the basal activity of the enzyme.

Fig. 6 shows the concentration-effect curves for the activation of myosin light chain kinase by CaM in the absence and presence of CPZ-CaM. As may be seen, CPZ-CaM shifted the CaM activity curve to the right. However, an increase in the concentration of CaM could completely overcome the inhibitory effects of CPZ-CaM. CPZ-CaM did not alter the maximal activation of myosin light chain kinase but increased the EC_{50} value for the activation of the enzyme by CaM. In the absence of CPZ-CaM, the EC_{50} value for CaM was 0.12 μM; the addition of CPZ-CaM (4 or 6 μM) increased the EC_{50} value to 0.15 or 0.33 μM, respectively.

Effect of drug-CaM adducts on Ca^{2+}-ATPase activity. Studies of the effects of the drug-CaM adducts (CPZ-CaM, FNM-CaM, and PBZ-CaM) on the activity of Ca^{2+}-ATPase showed that none of them inhibited the CaM-induced activity of Ca^{2+}-ATPase, nor did they significantly affect the basal activity of the enzyme (data not shown).

Effect of TFP and drug-CaM adducts on CaM-dependent protein kinase activity. Table 1 shows that TFP inhibited CaM-dependent protein kinase activity, with an IC_{50} value of about 15 μM, but had no effect on the basal activity of the enzyme. However, none of the three drug-CaM adducts studied inhibited the activity of CaM-dependent protein kinase, even at concentrations as high as 6 μM. At these high concentrations, the FNM-CaM adduct slightly activated CaM-dependent protein kinase activity.

Comparative IC_{50} values of drugs and drug-CaM adducts for the activities of various enzymes. Table 1 shows the IC_{50} values of several CaM-binding drugs and drug-CaM

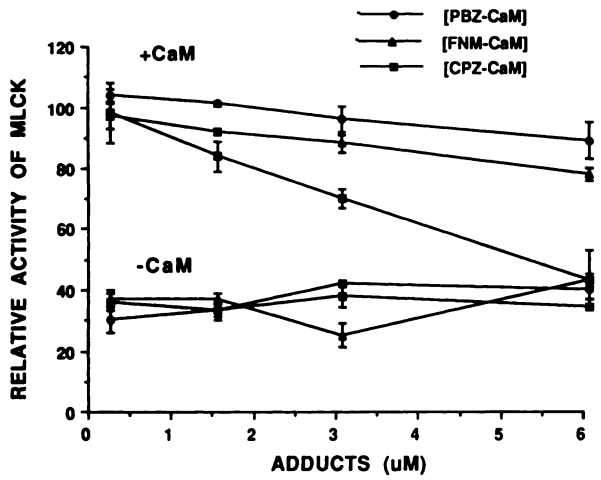

Fig. 5. Effects of drug-CaM adducts on the activity of CaM-stimulated myosin light chain kinase. Myosin light chain kinase (*MLCK*) activity was measured in the absence and presence of CaM (240 nM), with varied amounts of CPZ-CaM, FNM-CaM, or PBZ-CaM. Results represent the mean ± standard error of three samples.

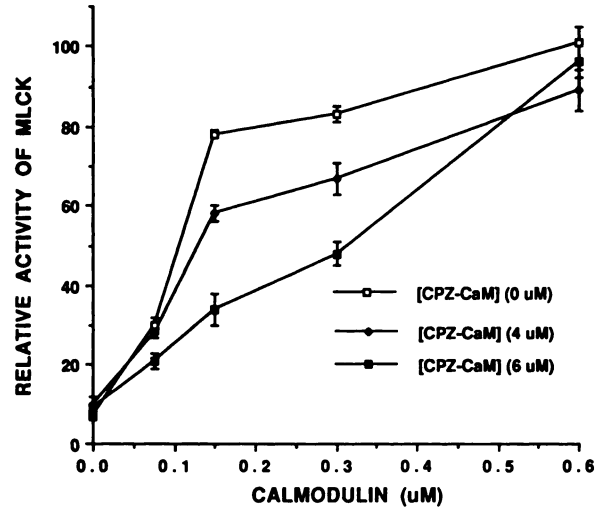

Fig. 6. Effects of CPZ-CaM on the activation of myosin light chain kinase by CaM. Myosin light chain kinase (*MLCK*) activity was measured in the absence and presence of varied amounts of CaM (0–0.6 μM) and/or the CPZ-CaM adduct (0, 4, or 6 μM). Each *point* represents the mean ± standard error of three samples.

adducts for the inhibition of the CaM-sensitive enzymes phosphodiesterase, Ca^{2+}-ATPase, myosin light chain kinase, and protein kinase and the CaM-insensitive enzyme protein kinase C. As may be seen, the CaM-binding drugs TFP, CPZ, FNM, and PBZ were relatively nonselective inhibitors of the enzymes. They inhibited the CaM-induced activation of all of the CaM-sensitive enzymes. The lack of specificity of these compounds was further evidenced by the fact that they displayed the same rank order of potency (PBZ < FNM ≅ TFP > CPZ) against all four of the CaM-sensitive enzymes. In addition, they all inhibited the CaM-insensitive enzyme protein kinase C. It should be noted that, although FNM was a very potent inhibitor of the CaM-sensitive enzymes (IC_{50} = 10–20 μM), it was a comparatively weak inhibitor of protein kinase C (IC_{50} = 160 μM).

Of the three drug-CaM adducts studied, only the CPZ-CaM adduct displayed significant anti-CaM activity. Of particular interest is the finding that CPZ-CaM differentially inhibited the various CaM-sensitive enzymes (Table 1 and Fig. 7). It was a potent inhibitor of CaM-sensitive phosphodiesterase (IC_{50} = 0.2 μM), was a moderate inhibitor of myosin light chain kinase (IC_{50} = 3 μM), and had little or no effect on CaM-sensitive Ca^{2+}-ATPase and protein kinase or the CaM-insensitive enzyme protein kinase C. The FNM-CaM and the PBZ-CaM adducts did not inhibit the effects of CaM on any of the enzymes studied, although they slightly activated two of the enzymes; FNM-CaM activated the CaM-sensitive protein kinase and the PBZ-CaM adduct slightly activated phosphodiesterase.

Discussion

CaM-sensitive enzymes are thought to consist of a hydrophobic regulatory domain, which binds the Ca^{2+}-CaM complex, and a catalytic region, which interacts with the substrate. The binding of the Ca^{2+}-CaM complex induces conformational changes in the enzyme, exposing additional catalytic sites and, thus, increasing activity (1–3, 28).

Several years ago (9, 10) we reported that CaM that had been irreversibly linked to CPZ by UV irradiation (CPZ-CaM) was able to inhibit the CaM-induced activation of phosphodiesterase by competing with CaM for regulatory sites on the enzyme. Results of the present studies show that the CPZ-CaM adduct, in addition to inhibiting the CaM-sensitive phosphodiesterase, competitively inhibited the CaM-induced activation of myosin light chain kinase but had little or no effect on the activity of other CaM-sensitive enzymes, including Ca^{2+}-ATPase and membrane-bound CaM-dependent protein kinase, or the CaM-insensitive enzyme protein kinase C. FNM-CaM and PBZ-CaM have no anti-CaM activity. The PBZ-CaM and the FNM-CaM adducts may actually act as selective, albeit weak, CaM agonists; PBZ-CaM partially activated CaM-sensitive phosphodiesterase and FNM-CaM partially activated CaM-dependent protein kinase. These results indicate that certain covalently linked drug-CaM adducts can differentially inhibit or activate various CaM-sensitive enzymes and they provide further evidence that it may be possible to develop a new class of CaM inhibitors that are directed against the CaM binding sites on CaM-sensitive enzymes.

There are two possible mechanisms that might explain how the CPZ-CaM adduct competitively inhibits the CaM-induced activation of phosphodiesterase and myosin light chain kinase. Either the adduct interacts with CaM itself or it competes with CaM for regulatory sites on the enzymes. Of these two possibilities, the second seems more likely to be correct. The reasons for this conclusion are as follows. First, the CPZ-CaM complex is unable to bind to a CaM-Sepharose affinity column (10), indicating that the adduct cannot interact directly with CaM. Furthermore, the fact that the adduct does not inhibit all CaM-sensitive enzymes strongly suggests that it does not bind to CaM. As was noted previously, all of the known CaM-binding drugs are relatively nonselective and inhibit all CaM-sensitive enzymes. If the comparatively large CPZ-CaM adduct did bind to CaM, one would expect that it too would be nonselective in its effects. The most likely explanation for the differential effects of CPZ-CaM is that it can bind to the CaM recognition sites on phosphodiesterase and myosin light chain kinase but not on the other enzymes.

It should be noted that, although these results indicate that the CPZ-CaM complex can interact with the CaM binding sites

TABLE 1

Comparative IC_{50} values of drugs and drug-CaM adducts on Ca^{2+}-sensitive enzymes

Drug	IC_{50} values								
	PDE[a]		Ca^{2+}-ATPase		MLCK		CaM-PK		PKC
	Basal	CaM	Basal	CaM	Basal	CaM	Basal	CaM	
					μM				
TFP	>300	10 ± 3	>40	11 ± 2	>40	10 ± 0.3	>50	15 ± 0.5	50 ± 5
CPZ	>500	42 ± 4	>100	36 ± 7	>100	36 ± 2	>100	46 ± 4	120 ± 16
FNM	>100	10 ± 2	>40	9 ± 3	>40	18 ± 2	>50	18 ± 3	160 ± 21
PBZ	>50	5 ± 0.3	>40	5 ± 1	>40	4 ± 0.3	>50	11 ± 3	
CPZ-CaM	>3	0.2 ± 1	>3	>3	>6	3 ± 0.3	>6	>6	>6
FNM-CaM	>3	>3	>3	>3	>6	>6	>6	>6	>6
PBZ-CaM	>3	>3	>3	>3	>6	>6	>6	>6	

[a] PDE, phosphodiesterase; MLCK, myosin light chain kinase; CaM-PK, CaM-dependent protein kinase; PKC, protein kinase C.

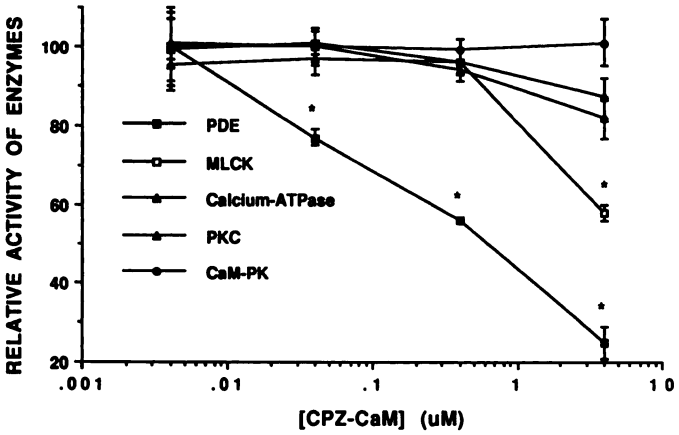

Fig. 7. Comparison of the effects of CPZ-CaM on various enzymes. The activities of the various enzymes were measured, as described in Materials and Methods, in the presence of varied amounts of CPZ-CaM. The results are expressed as the relative percentage of the activated enzyme activity. Each *point* represents the mean ± standard error of three samples. *, $p < 0.01$. *PDE*, phosphodiesterase; *MLCK*, myosin light chain kinase; *PKC*, protein kinase C; *CaM-PK*, CaM-dependent protein kinase.

on phosphodiesterase and myosin light chain kinase, it apparently does so with lower affinities than does native CaM. The EC_{50} value for the activation of phosphodiesterase by CaM was about 5 nM, whereas the K_i for the inhibition of the CaM-activated enzyme by CPZ-CaM was about 100 nM. Likewise, the EC_{50} value for the activation of myosin light chain kinase by CaM was 120 nM, whereas the K_i for the inhibition of the activated enzyme by CPZ-CaM was about 1 μM. These results are consistent with previous findings showing that the binding of phenothiazines and related drugs decreases the affinity of CaM for enzyme binding sites (28–30). However, our findings also indicate that drug binding does not necessarily prevent the binding of CaM to some of its target enzymes.

In contrast to the CPZ-CaM adduct, the FNM-CaM and the PBZ-CaM adducts do not inhibit any of the CaM-sensitive enzymes studied but act as weak CaM agonists for some of the enzymes. At high concentrations, the FNM-CaM adduct slightly activated the CaM-sensitive protein kinase from brain and the PBZ-CaM adduct slightly activated the CaM-sensitive phosphodiesterase. These agonist-like effects of the PBZ-CaM adduct are similar to those previously reported by Lukas *et al.* (17). These authors showed further that the potencies of the adducts depended upon the type of PBZ-CaM adduct formed. Although it is possible that the activation of these enzymes by FNM-CaM and PBZ-CaM could have resulted from the contamination of the adducts with native CaM (i.e., CaM that was not linked to FNM or PBZ), it seems more likely that the activation is due to the intrinsic CaM agonist activity of the adducts themselves. The reasons for this conclusion are as follows. First, analysis of the adducts by polyacrylamide gel electrophoresis, under conditions that separated the adducts from native CaM (0.1% SDS and 12% polyacrylamide), gave no evidence that unreacted CaM was present (data not shown). Furthermore, the fact that the adducts can activate some enzymes, but not others, strongly suggests that the activation did not result from the presence of native CaM. If native CaM were present, all of the enzymes would probably be activated. Finally, all of the adducts were formed under conditions in which there was a large (10-fold) molar excess of drug. Under these conditions, each of the drugs would saturate all of the available specific drug binding sites on CaM (8, 16, 18). It seems highly unlikely that any free CaM would remain under these conditions.

The conclusions described above are similar, in many respects, to those reported by Klee and co-workers (11–13), who studied a covalent adduct ($CAPP_1$-CaM) that was formed between CaM and norchlorpromazine isothiocyanate, an acylating phenothiazine. $CAPP_1$-CaM was unable to activate CaM-sensitive phosphodiesterase or myosin light chain kinase but did inhibit the activation of these enzymes by CaM. This same adduct acted as a partial agonist for the CaM-sensitive phosphoprotein phosphatase calcineurin and as a complete agonist for CaM-sensitive phosphorylase kinase (12, 14).

It should be noted that, although the CPZ-CaM adduct described here and the $CAPP_1$-CaM adduct described by Newton *et al.* (11, 12, 14) are both able to inhibit CaM-sensitive phosphodiesterase and myosin light chain kinase, they are not chemically identical. The $CAPP_1$-CaM adduct contains 1 mol of phenothiazine/mol of CaM, whereas the CPZ-CaM adduct contains 2 mol of drug/mol of CaM. Furthermore, the mechanism by which UV-irradiated CPZ binds to CaM is quite different from the mechanism by which norchlorpromazine isothiocyanate binds. The binding of norchlorpromazine isothiocyanate involves the specific acylation reaction of the drug with lysine-75 of CaM (31), whereas the binding of UV-irradiated CPZ probably involves a nucleophilic substitution reaction between electron-rich functional groups on CaM and a phenothiazine free radical that is formed during UV irradiation (8, 32). Interestingly, the covalent binding of PBZ and FNM to CaM probably involves a third mechanism, in which active

ethyleneimmonium intermediates of the drug molecules interact with methionine residues on CaM (17). The fact that the mechanisms for the binding of FNM and PBZ differ from the mechanism for the binding of CPZ may account for the different pharmacologic actions of the various adducts.

The findings described above further support the possibility of developing a new class of CaM antagonists that are directed against the CaM binding sites on CaM-sensitive enzymes. Such agents might exhibit a greater degree of selectivity than agents that act directly on CaM. Considerable evidence indicates that the various CaM-sensitive enzymes have different CaM binding sites (33–36). For example, various CaM-sensitive enzymes display different affinities for the Ca^{2+}-CaM complex (1) and slightly different sensitivities to inhibition by CaM-binding drugs (37, 38). Studies with proteolytic fragments of CaM indicate that different regions of CaM may be involved in its interaction with different target enzymes. Newton *et al.* (39) reported that CaM fragment 78–148 could interact with phosphodiesterase and prevent its activation by CaM, whereas this same fragment fully activated phosphorylase kinase. Guerini *et al.* (33) reported that CaM fragments 78–148 and 1–106 were both able to stimulate Ca^{2+}-ATPase but not phosphodiesterase.

Because of their ability to selectively influence the activity of certain CaM-sensitive enzymes, the covalently linked drug-CaM adducts described in this report could be useful in examining of the functions of CaM and its target enzymes. Because of their large molecular size, it is unlikely that these drug-CaM adducts or CaM fragments could penetrate cell membranes very well and, therefore, they probably would not inhibit CaM activity in most intact cells or tissues. However, such agents might be very useful for probing the effects of CaM in cell-free systems or in systems in which they can be introduced directly into cells.

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