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Functional *In Vitro* Test of Calmodulin Antagonism: Effect of Drugs on Interaction between Calmodulin and Glycolytic Enzymes.

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SUMMARY

A simple procedure has been elaborated to screen for the calmodulin antagonist effect of drugs. A covalently attached fluorescent probe was used to monitor the binding of enzymes known as target enzymes to calmodulin. Moreover, the probe made it possible to recognize a new target enzyme, aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13), for calmodulin among glycolytic enzymes. The calmodulin antagonist trifluoperazine prevented or eliminated the complex formation between calmodulin and enzymes studied in reconstituted systems; the Ca channel blockers had no effect. The functional consequences of the effect of drugs on calmodulin-phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) interaction were investigated as well. Whereas trifluoperazine suspended the calmodulin-mediated hysteretic inactivation of phosphofructokinase, Ca channel blockers (verapamil and nifedipine) were ineffective. Fendiline (regarded as a Ca channel blocker) seems to act as a functional calmodulin antagonist. Its binding to calmodulin does not prevent the complex formation of phosphofructokinase and calmodulin, but within this ternary complex phosphofructokinase preserves or recovers its original activity measured in the absence of calmodulin. The possible molecular effect of drugs on a calmodulin-enzyme complex is discussed.

The Ca^{2+}-dependent regulatory protein CaM has been shown to be involved in the regulation of numerous Ca^{2+}-mediated events (see Refs. 1–7 for review). Its role in signal transmission appears evident; whenever the free Ca^{2+} concentration in the cell changes, the concentration of the different Ca-CaM complexes varies, thus modulating the target enzymes that finally determine the cellular response. Although the effect of CaM on isolated enzymes is well established, its precise mode of action at the molecular level is not yet clearly defined. As a part of the activation mechanism, hydrophobic domains on CaM are exposed when Ca^{2+} binds, which are recognized by the target enzymes (8). CaM antagonists such as phenothiazines prevent the interaction of CaM with its target proteins by binding to CaM (9). However, it was also demonstrated that the CaM-norchlorpromazine adduct can interact with PDE but does not activate the enzyme and competitively inhibits its stimulation (10, 11).

CaM antagonist compounds are characterized by significant heterogeneity of chemical structure, which suggests multiple sites and mechanisms of action. Weiss *et al.* (9) proposed a model for CaM inhibitors. They ought to have a hydrophobic region formed by aromatic rings and a quaternary nitrogen atom at a distance of not less than three carbon atoms from the hydrophobic rings. A classic compound for this chemical structure is trifluoperazine (TFP).

There are some indications that CaM can mediate inhibition of enzymes. The action of PFK, being multimodulated by several effectors, is influenced by CaM (12, 13). From the dependence of catalytic activity on CaM concentration the apparent dissociation constant could be estimated (about 0.1 μM); however, this value very much depends on the modulated state of PFK. Recently, we[1] have found that CaM decreases the V_{max} value of aldolase activity without causing significant changes in the K_M value of fructose-1,6-bisphosphate. Moreover, CaM modulates the complex formation of PFK and aldolase that has been demonstrated (14).

In this study we investigated how the different drugs can modulate the interactions between CaM and soluble enzymes and elaborated simple and sensitive tests to study the binding of drugs to CaM-enzyme complexes, as well as the functional consequences of the binding.

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[1] F. Orosz, T. Y. Christova, and J. Ovádi, unpublished result

ABBREVIATIONS: CaM, calmodulin; TFP, trifluoperazine; EGTA, ethylene glycol bis(β-aminoethyleter)-*N,N'*tetraacetic acid; MLCK, myosin light chain kinase; PDE, phosphodiesterase; PFK, phosphofructokinase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials and Methods

ATP, NADH, and fructose-6-phosphate were purchased from Boehringer (Mannheim, FRG), EGTA from Sigma Chemical Co. (St. Louis, MO), and dansyl chloride from Calbiochem (Los Angeles, CA). The drugs TFP, fendiline, verapamil, and nifedipine were gifts from Chinoin Pharmaceutical Co. (Budapest, Hungary). All other chemicals were reagent-grade commercial preparations.

PFK (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11), glycerol-3-phosphate dehydrogenase, triosephosphate isomerase from rabbit skeletal muscle in $(NH_4)_2SO_4$ suspension, and CaM-deficient PDE (3′:5′ cyclic-nucleotide phosphodiesterase, EC 3.1.4.17) from beef heart, all in highest purity available, were from Boehringer. Aldolase (D-fructose -1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) was purified from rabbit skeletal muscle as described previously (15). Rabbit skeletal muscle MLCK (EC 3.6.1.3) was kindly provided by Dr. A. Jancsó (Eötvös Lorand Tudomanyegyetem, Budapest, Hungary). CaM from human erythrocytes was purchased from Reanal (Budapest, Hungary). The homogeneity of the proteins was checked by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

The enzyme suspensions were centrifuged at 14,000 × *g* for 10 min. The pellets were suspended in 50 mM HEPES, pH 7.0, containing 100 mM KCl, 5 mM $MgCl_2$, and 20% (w/v) glycerol, and dialyzed against the same buffer for 4 hr with several changes to remove the ammonium sulfate. In some cases PFK was filtered through a Sephadex G-50 column and eluted with 50 mM HEPES, pH 7.0, containing 100 mM KCl and 5mM $MgCl_2$. Enzyme concentrations were determined spectrophotometrically, by using absorption coefficients ($A_{280,\ 0.1\%}$) of 1.07 and 0.74 for PFK (16) and aldolase (17), respectively, and also by the method of Lowry *et al.* (18).

PFK assay. PFK activity was measured with fructose-6-phosphate as substrate in an assay mixture that contained 50 mM HEPES, pH 7.0, 100 mM KCl, 5mM $MgCl_2$, 50 μM MgATP, 1 mM KH_2PO_4, 0.6 mM NADH, 12 units/ml triosephosphate isomerase, 4 units/ml glycerol-3-phosphate dehydrogenase, and 0.6 units/ml aldolase in a total volume of 1 ml. The final concentration of PFK was 0.6 μM. The reaction was started by the addition of fructose-6-phosphate to a final concentration of 1 mM, and the decrease in absorbance at 340 nm was measured at 25°C. The velocity of the reaction was calculated from the linear steady state part of the progress curve. If CaM was present the assay mixtures contained also 300 μM $CaCl_2$ and 200 μM EGTA. The error of determination of enzymatic activities was less than ±5%.

Fluorescence measurements. Calmodulin was labeled according to Johnson and Wittenauer (19) with minor modifications as follows: 1 mg/ml protein was dansylated by reaction with a 5-fold molar excess of dansyl chloride in 10 mM 3-(*N*-morpholino)propanesulfonic acid, 90 mM KCl, 2 mM EGTA, and 3 mM $CaCl_2$, pH 7.0, for 24 hr. The excess free dye was removed by gel filtration through a Sephadex G-50 column equilibrated with 10 mM 3-(*N*-morpholino)propanesulfonic acid, 90 mM KCl, and 2 mM EGTA. The concentration of bound dye was determined on the basis of an absorption coefficient (A_{350}) of 3980 M^{-1} cm^{-1} (20). A total of 0.5 to 1 mol of dansyl group was incorporated per mol of CaM. Fluorescence intensity and polarization were measured at an excitation wavelength of 370 nm and an emission wavelength of 500 nm.

For each measurement, at least 10 determinations of the vertically and horizontally polarized components of the fluorescent emission were made with a standard deviation of less than ±5%. The anisotropy was calculated as described previously (21, 22).

The binding of enzymes and drugs to calmodulin was investigated after its saturation with Ca^{2+} (in the presence of 2 mM EGTA and 3 mM $CaCl_2$), which increased markedly (about 100%) the maximum fluorescence emission in parallel with a blue shift (from 525 to 500 nm).

Results and Discussion

Binding experiments. The usual systems for measuring anticalmodulin activity are the CaM-MLCK and CaM-PDE interactions. In smooth muscle, CaM is responsible for the activation of MLCK (23). The CaM antagonist TFP binds to CaM and disrupts the CaM-MLCK complex (24). The CaM-PDE interaction and the effect of TFP on it have been demonstrated in both *in vivo* and *in vitro* systems (5 and references therein).

We applied these systems to check the covalently attached fluorescent probe, as a simple tool for the investigation of the effect of drugs on the binding of enzymes to CaM.

CaM was labeled with dansyl chloride and MLCK or PDE was added to it in the absence and presence of TFP. As shown in Table 1, the anisotropy of labeled CaM increased on the addition of enzymes. Because the fluorescence intensity of dansyl-CaM is not changed by enzymes (not shown), the increase in anisotropy is probably related directly to the binding of MLCK or PDE to labeled CaM. The addition of 20 μM TFP to CaM-enzyme complexes reduced the anisotropy value to that of the free CaM, which essentially equals that of the CaM-TFP adduct (Table 1). This finding indicates that TFP prevents the binding of MLCK as well as PDE to CaM, probably because TFP, as expected, competes with the target enzymes for CaM binding. It should be added that labeled CaM activates MLCK similarly to native CaM (19); therefore, the modification of CaM by dye cannot be responsible for the observed phenomenon.

Fendiline (Sensit), verapamil, nifedipine, and others have been regarded as negative inotropic drugs acting in an apparently competitive manner against Ca^{2+} and have served as Ca channel blockers (25). These compounds are neither structurally nor pharmacologically similar. Whereas verapamil and nifedipine have a marked and selective effect on the Ca^{2+} component of the cardiac action potential, fendiline has a less selective effect (26). These drugs bind to CaM with different

TABLE 1

Effect of drugs on the labeled CaM-enzyme interactions as measured by fluorescence anisotropy

Concentrations were as follows: CaM, 4.5 μM; MLCK, 1.5 μM; PDE, 3 μM; aldolase, 3 μM; PFK, 0.6 μM; TFP, 20 μM; fendiline, 20 μM; and verapamil, 20 μM.

Enzymes	Drugs	Anisotropy[a]	Complex
		0.025	
	TFP	0.025	
	Fendiline	0.025	
	Verapamil	0.025	
MLCK		0.070	Yes
MLCK	TFP	0.030	No
MLCK	Fendiline	0.070	Yes
PDE		0.055	Yes
PDE	TFP	0.025	No
PDE	Verapamil	0.055	Yes
PDE	Fendiline	0.055	Yes
Aldolase		0.060	Yes
Aldolase	TFP	0.025	No
Aldolase	Verapamil	0.060	Yes
Aldolase	Fendiline	0.060	Yes
PFK[b]		0.068	Yes
PFK	TFP	0.025	No
PFK	Fendiline	0.093	Yes
PFK	Fendiline + TFP	0.050	Yes

[a] The anisotropy values are the average of at least five measurements. Error of determination is ±5%.

[b] PFK concentration in tetramers.

affinities (19). The primary mechanism of action of verapamil is the blockage of the Ca channel and not the interference with cytosolic CaM (27). However, fendiline, as a classical Ca channel blocker, may work in part by CaM antagonism (28–31). Therefore, we compared the effects of these chemically different drugs on CaM-enzyme interactions with TFP.

In contrast to TFP, fendiline or verapamil did not prevent the complex formation between CaM and target enzymes (Table 1). The anisotropy value of labeled CaM is increased by the addition of enzymes in the same manner even in the presence of these drugs. Because the covalently attached fluorescence probe provided a simple means of monitoring the effect of drugs on CaM-enzyme complexes expected to exist at physiological conditions, we extended our study to other CaM-enzyme complexes for the following two reasons: (i) to recognize new target enzymes for CaM interaction, and (ii) to compare the functional effects of drugs having different binding ability on CaM-enzyme complexes.

Two glycolytic enzymes were chosen that bind to the thin filament in muscle cell. The physiological concentration of aldolase is extremely high (32 μM) (32), and thus it can be expected that the cytosol contains significant amounts of unbound aldolase. The other enzyme is PFK, which plays a central role in the regulation of ATP production via glycolysis. In resting muscle the activity of this enzyme is several hundredfold lower than the maximal activity measured *in vitro* under optimal conditions, suggesting that some interacting mechanisms must exist (33); moreover, a CaM-mediated inactivation of PFK has been suggested (12, 13).

We have measured the fluorescence anisotropy of dansylated CaM at equilibrium in the presence of added aldolase or PFK. The quantitative results are presented in Table 1. It is clear that the anisotropy of labeled CaM is increased by the addition of aldolase, indicating complex formation between the two proteins. TFP (20 μM) prevents the binding of aldolase to labeled CaM. However, neither verapamil nor fendiline has any significant effect on the anisotropy of labeled CaM-aldolase complex.

Qualitatively the same result was obtained with PFK (cf. Table 1 and Fig. 1). As shown in Fig. 1A, the anisotropy value increases on the addition of PFK, due to its complex formation with dansyl-CaM. The experimental points could be fitted to a theoretical curve assuming an apparent dissociation constant of 0.3 μM for PFK - CaM complex. At a kinase concentration (2.4 μM) at which a significant fraction of CaM (about 50%) is complexed and the anisotropy is definitely higher than that of uncomplexed CaM, TFP was added to the samples. As shown in Fig. 1A, the anisotropy value decreased although the kinase concentration was unchanged. The reduction in the anisotropy is proportional to the concentration of TFP and about 30 μM TFP can suspend the effect of PFK on the anisotropy of dansyl-CaM, probably because TFP antagonizes the kinase for CaM binding.

In contrast to the TFP effect, if fendiline was added to the dansyl-CaM-PFK complex, the anisotropy is increased by increasing the concentration of fendiline (cf. Table 1 and Fig. 1B) without causing significant changes in the fluorescent intensity of labeled CaM at the concentrations used. This result suggests that fendiline can bind to the CaM-PFK complex. The increase of anisotropy value may be attributed to either conformational changes or an enhanced affinity of CaM-fendiline

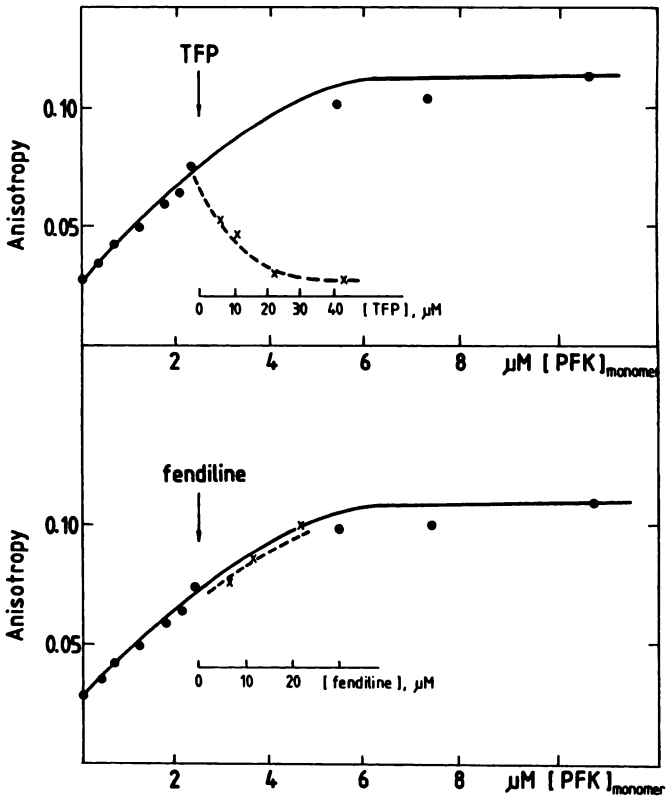


Fig. 1. Effect of drugs on the anisotropy of dansyl-CaM (4.5 μM) complexed with PFK. The *solid line* is computer fitting with an apparent dissociation constant of 0.3 μM for the PFK-CaM complex assuming that one protomer of PFK binds one CaM (12). *Arrows* indicate the constant concentration of enzyme at which TFP (A) or fendiline (B) is added to the PFK-CaM system. *Dashed lines* show the change of anisotropy as a function of drug concentrations.

adduct for PFK within the complex. When TFP and fendiline were added in equimolar concentration to CaM-PFK system, the anisotropy value was not reduced to that measured in the absence of fendiline (cf. Table 1). This finding indicates the partial dissociation of CaM-PFK complex induced by TFP in the presence of fendiline. TFP would have caused the complete dissociation of CaM-PFK complex if the drugs had acted independently.

Effect of drugs on the action of PFK complexed with CaM. PFK may exist in several conformational states having different catalytic properties. The effectors, metabolites, and macromolecules shift the equilibrium between the conformers of PFK by binding with different affinities to the different conformers (33). Because it was found (12, 13) that CaM can mediate the hysteretic inactivation of PFK, we used a sensitive enzyme kinetic probe to check the functional effect of drugs with different binding properties on the action of PFK complexed with CaM.

PFK at near-physiological concentration was preincubated with drugs or drugs plus CaM under the conditions described in Materials and Methods. The concentration of CaM was 3 μM, which is expected to be unbound *in vivo* (13). The presence of CaM decreased the catalytic activity of PFK to 40% (Table 2). The effect of drugs on CaM-induced inactivation of PFK was tested at protein concentrations at which PFK could be expected to exist predominantly in the complexed form (12). Control experiments indicated that drugs themselves had no

TABLE 2

Effect of drugs on calmodulin-induced inactivation of PFK

PFK and CaM concentrations were 0.6 and 3 μM, respectively.

Systems	Enzyme activity of PFK*
	%
PFK	100
PFK + calmodulin	40
PFK + 20 μM TFP	100
PFK + calmodulin + 4 μM TFP	55
PFK + calmodulin + 10 μM TFP	80
PFK + calmodulin + 20 μM TFP	100
PFK + 10 μM fendiline	100
PFK + calmodulin + 4 μM fendiline	70
PFK + calmodulin + 10 μM fendiline	90
PFK + calmodulin + 10 μM verapamil	50
PFK + calmodulin + 10 μM nifedipine	40

* The error of determination of enzymatic activities was less than ±5%.

significant effect on PFK activity (cf. Table 2). In accordance with the expectation, TFP suspended the inactivating effect of CaM and the effect was concentration dependent. Complete recovery of PFK activity was observed at 20 μM TFP (Table 2), which is in accord with the binding experiments.

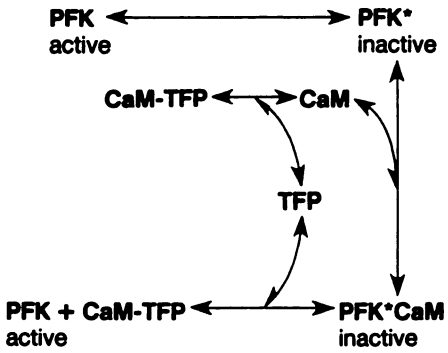
Surprisingly, fendiline had a functional effect similar to that of TFP, inasmuch as it could liberate PFK from CaM inhibition even in a PFK-CaM-fendiline complex form. It was effective in the same concentration range as TFP. Other Ca channel blockers like verapamil and nifedipine have practically no effect on the activity of PFK complexed with CaM in the same concentration range (Table 2), indicating that neither verapamil nor nifedipine can perturb the CaM-PFK interaction. Because the fendiline has a much higher affinity for the uncomplexed CaM than has TFP (34), however, their functional effects manifested themselves in the same concentration range; we suggest that the fendiline is bound to this protein complex with lower affinity than to the uncomplexed CaM. Moreover, CaM might have different sites for binding of the two different drugs, which mutually affect their binding properties.

Molecular models for effects of TFP and fendiline on CaM binding to PFK. As shown in Scheme 1, TFP effectively antagonizes CaM, by preventing the binding of CaM to the inactive form of PFK equilibrated with the active form of kinase. Alternatively, however, because CaM is a small protein, it cannot be excluded that inhibitors binding to sites different from those for the enzymes could sufficiently alter the conformation of CaM to prevent the binding of enzymes.

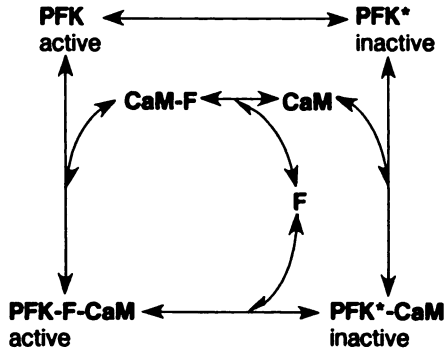
The effect of TFP on the CaM - PFK interaction can be explained as shown in Scheme 1.

Although fendiline binds to CaM complexed with PFK, it cannot prevent the binding of CaM to PFK (Scheme 2) as indicated by activity measurements. The possible explanation for this finding is that the fendiline (*F*) binding site on CaM differs from the TFP binding site at least in the CaM-fendiline-PFK (*CaM·F·PFK*) complex.

Inagaki and Hidaka (34) suggested that the binding site of prenylamine on CaM is different from that of TFP, which is known to be at the carboxy-terminal domain (35, 36). Because prenylamine differs only by one methylene group from fendiline, it is likely that the fendiline binding site is the same as that for prenylamine. Consequently, distinct binding sites for TFP and fendiline may exist. This possibility may be supported by results of our binding and kinetic experiments (cf. Schemes 1 and 2).



Scheme 1.



Scheme 2.

Johnson and Mills (6) showed that there are two hydrophobic drug binding domains on CaM, which exert allosteric control over each other. However, the precise relationship between these binding domains of CaM and the binding site(s) for the target enzymes has not been clarified. Our results suggest that the binding of fendiline to CaM does not compete directly with that of enzymes. However, the interaction of fendiline with CaM probably induces an alteration in the tertiary structure of CaM at least at the PFK binding surface. This may result in a complexed PFK species in which the catalytic properties of the uncomplexed form are preserved. This observation resembles and extends the finding of Klee and his co-workers (10, 11) whereby a phenothiazine-CaM adduct binds to PDE or MLCK but can no longer stimulate these enzymes. Moreover, recent evidence indicates that CaM substituted by gramicidin S, a cyclic decapeptide, activates PDE and displays the same activity as in the absence of gramicidin S (37).

The fluorimetric and kinetic approaches developed here offer a simple and suitable procedure to screen for CaM antagonists as well as to study the mechanism of action of CaM antagonists. The glycolytic enzymes used are suitable tools for this purpose because of their relatively low affinity for CaM. Dissociation constants are 4.6 and 0.3 μM (cf. Fig. 1) for aldolase-CaM[1] and PFK-CaM (cf. Fig. 1) complexes, respectively, which are commensurate with those of CaM-drug interactions. This system renders it possible to use relatively low drug concentrations, to eliminate their indirect and unspecific binding to the target enzymes (38–40). In this way our screening process may lead to the recognition of CaM antagonist compounds even if they are just "functional" antagonists.

The fact that CaM inhibitors can modulate the interactions between glycolytic enzymes and CaM cautions us that further effects *in vivo* of CaM antagonists must be taken into account.

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