0026-895X/90/060910-07$03.00/0

MOLECULAR PHARMACOLOGY, 38:910-916

Dissimilar Mechanisms of Action of Anticalmodulin Drugs: Quantitative Analysis

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Received February 26, 1990; Accepted September 18, 1990

SUMMARY

A novel molecule from the arylalkylamine family of drugs, KHL-8430, has been identified as a potent and specific inhibitor of calmodulin activity. The effect of this drug on calmodulin-mediated enzymatic actions has been analyzed to exemplify how to model the mechanism of action of a functional calmodulin antagonist. The approach used includes both binding and enzyme kinetic studies. In both types of experiments, the effects of drugs on calmodulin-phosphofructokinase [ATP:D[fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11] and calmodulin-phosphodiesterase (3′:5′ cyclic nucleotide phosphodiesterase, EC 3.6.1.3) interactions have been investigated. We have found that KHL-8430, in contrast to trifluoperazine, a classical anticalmodulin drug, competes with neither phosphofructokinase nor phosphodiesterase for calmodulin binding, yet it liberates phosphofructokinase from calmodulin inhibition and phosphodiesterase from calmodulin stimulation. The anticalmodulin activity occurs at lower KHL-8430 than trifluoperazine concentrations. These findings might establish the functional importance of these differences in the specificity of these drugs. The synthesis of the data suggests that (i) whereas trifluoperazine antagonizes both phosphofructokinase and phosphodiesterase binding to calmodulin, KHL-8430 interacts with calmodulin complexed with enzymes; (ii) KHL-8430 binds to the calmodulin-phosphofructokinase complex with an affinity constant of 0.8 μM, whereas the binding constant of trifluoperazine is 2.5 μM (iii) within the ternary complex the dimeric form of the kinase preserves activity that is otherwise inactive; and (iv) the binding of trifluoperazine and KHL-8430 to calmodulin exhibits negative cooperativity. The approach used in this study makes it possible to screen for the calmodulin antagonist effect of other drugs as well.

CaM, a Ca^{2+}-dependent regulatory protein, can modulate the activity of numerous enzymes. The effect of CaM usually manifests itself in an increase of the activity of enzymes, but there are some indications that CaM can inhibit the activity of some glycolytic enzymes (1, 2). CaM antagonists prevent the interaction of CaM with its target proteins by binding to CaM. The almost classical model for measuring anti-CaM activity is the CaM-PDE (3′:5′ cyclic nucleotide phosphodiesterase, EC 3.6.1.3) system, because of the competitive character of the antagonists. It has been suggested that, when PDE binds to one domain in the central helix region of CaM, this binding prevents, either sterically or allosterically, the stimulation of another target enzyme (Ref. 3 and references therein). The effectiveness of drugs in modifying CaM-stimulated events is characterized generally by IC_{50} values, half-maximum inhibition; however, in the case of drugs with competitive character, these values have no meaning unless the concentration of CaM is specified. Additionally, if the mechanisms of action of drugs are different, the IC_{50} values do not characterize the inhibitory potency of drugs.

In a previous paper (4), we reported a quantitative functional *in vitro* test of CaM antagonism, which involves a combination of enzyme kinetic and fluorescence measurements. In both types of experiments, the effects of drugs on the interactions of CaM and enzymes were investigated. The CaM-PFK (ATP:D-fructose-6-phosphate-1-phospho-transferase, EC 2.7.1.11) system has been preferentially selected to probe the effect of drugs, because (i) PFK is well known to exist in equilibria of distinct conformational and oligomeric states of different catalytic properties (5) and CaM shifts the equilibrium between these forms, resulting in an altered overall activity (1), and (ii) the affinity of PFK for CaM is commensurable with that of several anti-CaM drugs (3); therefore, the nonspecific binding of drugs to the enzymes can be minimized.

In this paper, both the binding and enzyme kinetic approaches were applied to characterize quantitatively the binding of drugs to CaM. We have investigated the effects of TFP and a new potent CaM antagonist on CaM-mediated inhibition of PFK and, in addition, on CaM-stimulated activity of PDE.

This paper is dedicated to the memory of Tamás Keleti.
This work was supported by Grant OTKA 315/86.

ABBREVIATIONS: CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyleter)-*N,N,N′,N′*-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; PDE, phosphodiesterase; PFK, phosphofructokinase; TFP, trifluoperazine; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

The synthesis of the results made it possible to develop molecular models for the mechanism of action of anti-CaM drugs and to compare their inhibitory potencies and specificities.

Experimental Procedures

Materials. 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). Fendiline, TFP, verapamil, diltiazem, nifedipine, and KHL-8430 were gifts from Chinoin Pharmaceutical Co. (Budapest, Hungary). [^{3}H] AMP (specific activity, 36.4 Ci/mmol) was the product of NEN. All other chemicals were reagent-grade commercial preparations. Stock solutions of drugs (4–20 mM) were made up fresh, at the start of each assay, in absolute ethanol or in water. The maximum final concentrations of ethanol in the kinetic and binding assay mixtures were 0.5% (v/v) and 2% (v/v), respectively; these concentrations had no effect on the activities and anisotropies measured.

Proteins. PDE from beef heart, PFK, glycerol-3-phosphate dehydrogenase, and triosephosphate isomerase from rabbit skeletal muscle in $(NH_4)_2SO_4$ suspension, all of the highest purity available, were from Boehringer. Aldolase (6) from rabbit skeletal muscle and CaM (7) from bovine brain were purified as described previously. The homogeneity of CaM was checked by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

The suspension of PFK was centrifuged at 14,000 × *g* for 10 min. The pellet was suspended in 50 mM HEPES, pH 7.0, containing 100 mM KCl and 5 mM $MgCl_2$. 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 5 mM $MgCl_2$. The PFK concentration was determined spectrophotometrically, using an absorption coefficient ($A_{276,0.1\%}$) of 1.07 (8), and also by the method of Lowry *et al.* (9).

PFK assay. The activity of PFK was assayed in a mixture that contained 50 mM Tris·HCl, pH 8.0, 50 μM MgATP, 1 mM KH_2PO_4, 230 μM NADH, 4 units of glycerol-3-phosphate dehydrogenase, 12 units of triosephosphate isomerase, and 0.6 units of aldolase, in a total volume of 1 ml. From the incubation assays (see below), the enzyme was diluted to 0.12 μM (10.5 μg/ml). The reaction was started by the addition of fructose-6-phosphate as substrate, at a final concentration of 2 mM, and the decrease in absorbance at 340 nm was monitored at 25°. The velocity of the reaction was calculated from the linear steady state part of the progress curve. The error of determination of enzymatic activities was less than ±5%.

Kinetic experiments. Concentrated (1.5 mg/ml) PFK was diluted to a protomer concentration of 0.48 μM (42 μg/ml), in the absence and presence of CaM (3 μM) and drugs, in a mixture containing 20 mM HEPES, pH 7.0, 40 mM KCl, 2 mM $MgCl_2$, 50 μM MgATP, 50 μM KH_2PO_4, 500 μM $CaCl_2$, and 4 mM dithiothreitol. The samples were incubated at 25° and aliquotes were withdrawn at appropriate intervals and diluted 4.5-fold into the assay mixture described above.

PDE assay. Cyclic AMP PDE activity was assayed according to the method of Solti *et al.* (10), in 40 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.5 mg/ml bovine serum albumin, pH 8.0, at 30°, with 0.1 mM cyclic AMP. The final concentrations of PDE and CaM were 1.66 and 0.2 μg/ml, respectively. Under our conditions, 20% of adenosine remained bound to the resin, which was taken into consideration when activities were calculated. The activities were calculated from the slopes of straight lines defined by the radioactivity increments after 10-, 20-, and 30-min reaction times. Care was taken that less than 25% of the initial substrate was consumed in the assays. The control sample did not contain CaM and its activity was taken to be 100%.

Binding experiments. CaM was labeled covalently with dansyl chloride as in Ref. 11, with slight modifications as follows: 1 mg/ml protein was dansylated with a 6-fold molar excess of dansyl chloride in 10 mM MOPS, 90 mM KCl, 2 mM EGTA, 3 mM $CaCl_2$, pH 7.0 (MOPS buffer), for 24 hr. The excess free dye was removed by gel filtration through a Sephadex G-25 column equilibrated with MOPS buffer without $CaCl_2$. The concentrations of bound dye and CaM were determined on the basis of their absorption, using absorption coefficients A_{350} of 3980 M^{-1} cm^{-1} (12) and A_{276} of 3240 M^{-1} cm^{-1} (13). About 0.5 mol of dansyl group was incorporated/mol of CaM.

The binding of enzymes and drugs to CaM was investigated after its saturation with Ca^{2+}. Fluorescence intensity was measured at an emission wavelength of 500 nm, with excitation at 370 nm. For each measurement, at least 10 determinations of 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 (14, 15).

Results

Effect of drugs on the CaM-promoted inactivation of PFK. As demonstrated previously (1, 2), the dilution of a concentrated PFK solution led to a significant decrease in the catalytic activity, due to partial dissociation of the active tetrameric form of the enzyme. In the presence of CaM, both the rate and extent of the inactivation were enhanced. The degree of the inactivation depended on the concentrations of both PFK and CaM (1). The inactivating effect of CaM on PFK activity has been interpreted as indicating that CaM is bound preferentially to the inactive dimeric form of PFK and shifts the equilibria towards the inactive forms of PFK, resulting in lower overall activity (1).

The effects of drugs on the CaM-induced inactivation were tested at protein concentrations at which both the uncomplexed form of PFK and the CaM-PFK complex existed (1). The data measured at the equilibrium state of the system are summarized in Table 1. TFP as well as fendiline can eliminate the inactivating effect of CaM, in a concentration-dependent manner. As expected, Ca^{2+} channel blockers of various chemical structures, like verapamil, nifedipine, and diltiazem, at concentrations of up to 20 μM, had practically no effect on the CaM-facilitated inactivation of PFK.

KHL-8430, an arylalkylamine derivative, liberates the kinase from the inhibition induced by CaM at a relatively low concentration of this drug (6 μM). Fig. 1 shows the dose-response curve of KHL-8430 in comparison with that of TFP. TFP at 20 μM or KHL-8430 at 6 μM was sufficient to suspend the CaM-induced inactivation of PFK. The IC_{50} values under our experimental conditions are 1.6 and 5.6 μM for KHL-8430 and TFP (see Fig. 1), respectively.

With an increase in the concentration of KHL-8430, the

TABLE 1

Effect of drugs of different chemical structures on CaM-induced inactivation of PFK

PFK activity was measured after a 15-min preincubation, as described in Experimental Procedures. Concentrations of PFK (in protomers) and CaM were 0.52 and 3 μM, respectively. The residual activity of PFK in the absence of CaM or drugs was 43% of the original activity. The error of determination of enzymatic activity was less than ±5%.

Addition to PFK + CaM	PFK activity
	%
None	23
TFP, 6 μM	33
TFP, 20 μM	43.5
Verapamil, 20 μM	23.5
Nifedipine, 20 μM	23
Diltiazem, 20 μM	22.5
Fendiline, 4 μM	33.5
Fendiline, 20 μM	42
KHL-8430, 6 μM	48

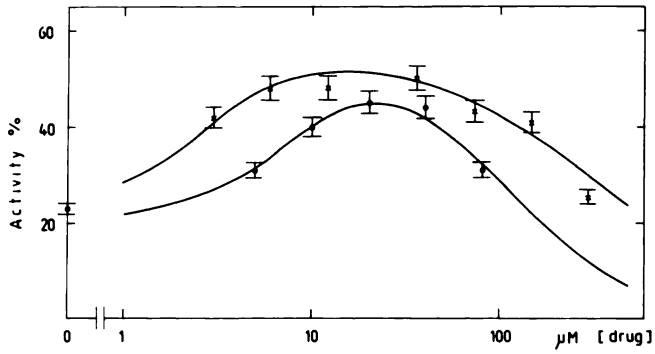


Fig. 1. Effect of TFP and KHL-8430 on the CaM-induced inactivation of PFK: dose-response curves. The mixtures contained 0.48 μM PFK, 3 μM CaM, and TFP (○) or KHL-8430 (×) at different concentrations. After 15-min preincubations, the activity of PFK was determined as described in Experimental Procedures. The activity of PFK without preincubation was taken as 100%. The residual activity of PFK was 43 ± 2% in the absence and 23 ± 1% in the presence of CaM. *Solid lines*, theoretical curves calculated on the basis of Schemes 1 (*lower curve*) and 2 (*upper curve*). The dissociation constants used for the calculation of the lower line were $K_1 = 0.1$ μM, $K_2 = 110$ μM, $K_3 = 110$ μM, $K_4 = 0.9$ μM, and $K_5 = 2.5$ μM and for the calculation of the upper line were $K_1 = 0.1$ μM, $K_2 = 130$ μM, $K_3 = 130$ μM, $K_4 = K_{14} = 0.9$ μM, and $K_5 = K_{15} = 0.8$ μM. The activity of PFK within the PFK-CaM-KHL-8430 ternary complex was 60%.

TABLE 2

Effect of TFP and KHL-8430 on CaM-induced activation of PDE

PDE activity was measured as described in Experimental Procedures. Concentrations of PDE and CaM were 1.66 and 0.2 μg/ml, respectively. The error of determination of enzymatic activity was less than ±5%.

Addition to PDE	PDE activity
	%
None	100
CaM	220
CaM + 10 μM TFP	147
CaM + 20 μM TFP	124
CaM + 50 μM TFP	79
CaM + 10 μM KHL-8430	105
CaM + 20 μM KHL-8430	95
CaM + 50 μM KHL-8430	87

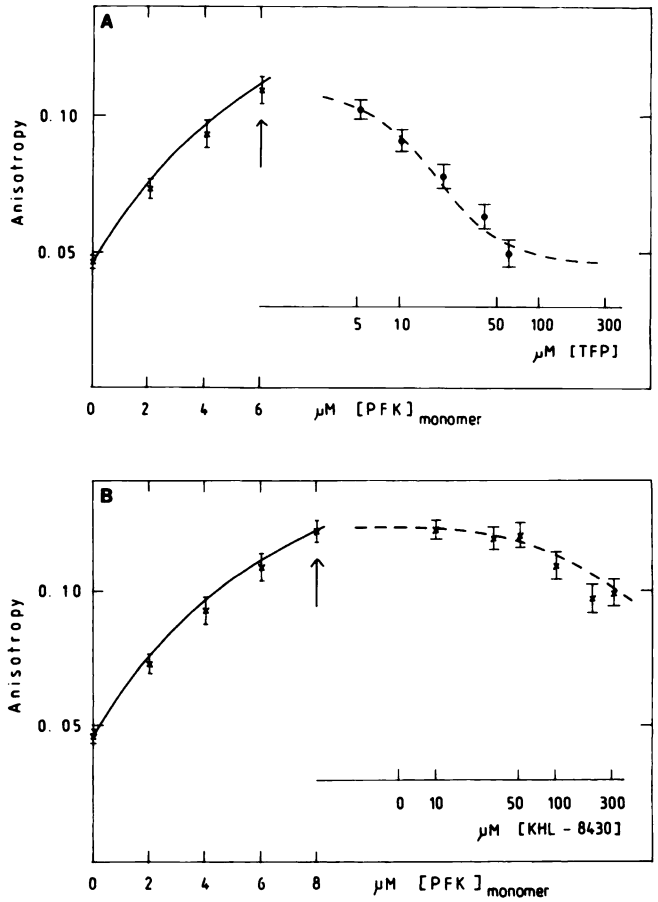


Fig. 2. Effect of TFP (A) and KHL-8430 (B) on the anisotropy of dansyl-CaM (10 μM) complexed with PFK. ——, Binding of PFK to CaM; – –, change of anisotropy as a function of drug concentration. The lines were calculated using Eqs. 1–16 and the same sets of dissociation constants as in Fig. 1. The values of specific anisotropies were: 0.046 for CaM, CaM-TFP, and CaM-KHL-8430 and 0.240 for CaM-PFK and CaM-PFK-KHL-8430. *Arrows*, the concentration of PFK at which TFP (○) or KHL 8430 (×) was added to the PFK-CaM system.

activity could surpass the value measured under the same condition in the absence of CaM (Fig. 1, Table 1). Further drastic elevation of the concentrations of drugs resulted in significant reductions of the enzyme activity (see Fig. 1). Control experiments showed that at extremely high drug concentrations (>200 μM) the enzyme was inactivated independently of the presence of CaM, suggesting the direct binding of drugs to PFK. This nonspecific effect manifests itself at about 3 times higher concentrations of KHL-8430 than of TFP (Fig. 1); therefore, the former can be considered as a more specific CaM antagonist.

The functional consequences of the binding of drugs to CaM were also assessed by study of their effects on the activation of PDE by CaM. Therefore, we have carried out a similar set of experiments using CaM-stimulated PDE. The concentration dependence of the effects of the two drugs is shown in Table 2. The inhibitory effect of TFP, as expected (16), occurs at low drug concentrations; however, KHL-8430 seems to be more potent.

Binding experiments. The influence of KHL-8430 on the binding of PFK to CaM was detected by using a covalently attached fluorescent probe. CaM was labeled with dansyl chloride (11) and the anisotropy was determined at various concentrations of PFK. As shown in Fig. 2. the value of the anisotropy increased due to the binding of PFK to the labeled CaM. In order to ensure that the observed changes were due to the binding of PFK to CaM, a number of positive and negative control experiments were done. As positive controls, well known target enzymes of CaM, myosin light chain kinase or PDE (4), were added to CaM instead of PFK. In these experiments, the anisotropy increased as a function of the concentration of unlabeled enzyme and reached saturation values (compare Ref. 4). The addition of TFP reduced the anisotropy values to that which is characteristic of the dansyl-CaM itself. These results are in accordance with the expectation that TFP can diminish the binding of the enzymes to CaM. Negative control experiments were performed, which utilized Ca channel blockers such as verapamil, nifedipine, and diltiazem. There was no change in the anisotropy of dansyl-CaM complexed with PFK or other target enzymes with up to 60 μM concentrations of the drugs. Changes in anisotropy could also have resulted from changes in the rotational freedom of the probe. However, we have found that (i) the addition of the tested drugs to dansyl-CaM itself had no effect on the anisotropy and (ii) the fluorescence emission spectrum of dansyl-CaM was not perturbed by addition of the enzymes.

As shown in Fig. 2A, the addition of TFP to the CaM-PFK system reduced the anisotropy value characteristic of the complex, in a concentration-dependent manner, down to that measured for the uncomplexed labeled CaM; 60 μM TFP could almost completely suspend the effect of PFK on the anisotropy of dansyl-CaM. Therefore, TFP, as expected (2, 4), prevents the binding of PFK to CaM.

In contrast to TFP, KHL-8430 at up to 60 μM did not change the anisotropy of the CaM-PFK complex (Fig. 2B), although KHL-8430 was more effective than TFP in bringing about complete reactivation of PFK. At high drug concentrations, some decrease of anisotropy could be observed; however, even 300 μM KHL-8430 was not enough to bring the anisotropy down to the level of free CaM. In another set of experiments, when PFK was added to the mixture of CaM and KHL-8430, ([CaM] = 10 μM; [KHL-8430] = 100 μM) the anisotropy increased in practically the same way as in the absence of this drug (data not shown). The data of the binding experiments could be interpreted in two different ways, (i) KHL-8430 cannot bind to CaM in the presence of PFK or (ii) the drug can bind to the CaM-PFK complex, forming a ternary complex. The results of the kinetic measurements allow us to come to the latter conclusion.

In order to check whether KHL-8430 can displace PDE from CaM or can bind to the CaM-PDE complex, a similar set of fluorescence anisotropy measurements has been carried out as with the PFK system. We have reported previously (4) that TFP abolishes the binding of PDE to fluoroscein isothiocyanate-labeled CaM. Now we have found that the anisotropy of dansyl-CaM measured in the presence of equimolar PDE was not influenced by KHL-8430 at up to 50 μM concentrations (data not shown). This observation suggests that KHL-8430 does not displace PDE from CaM but binds to CaM complexed with PDE.

To study whether the two drugs, TFP and KHL-8430, which exhibit similar functional effects on CaM-stimulated inactivation of PFK but via different molecular mechanisms, can mutually influence their binding to CaM, we added the two drugs simultaneously to the dansyl-CaM-PFK system. The PFK concentration was selected to be a subsaturating value. In one set of experiments, as shown in Table 3, the CaM-PFK system in the presence of 60 μM TFP was titrated with KHL-8430. The TFP concentration used was sufficient to prevent the binding of PFK to CaM in the absence of KHL-8430 (see Fig. 2A). With an increase in the concentration of KHL-8430, the anisotropy tended to reach the same limit value as measured in the absence of TFP. In another set of experiments, KHL-8430 in excess was first added to the CaM-PFK system, followed by the addition of 60 μM TFP. TFP at 60 μM could reduce the anisotropy but by a much lesser degree than in the absence of KHL-8430 (data not shown). It should be added that the steady state values of anisotropies were independent of the order of the addition of the two drugs. These findings suggest that the two drugs mutually affect their binding to CaM.

TABLE 3

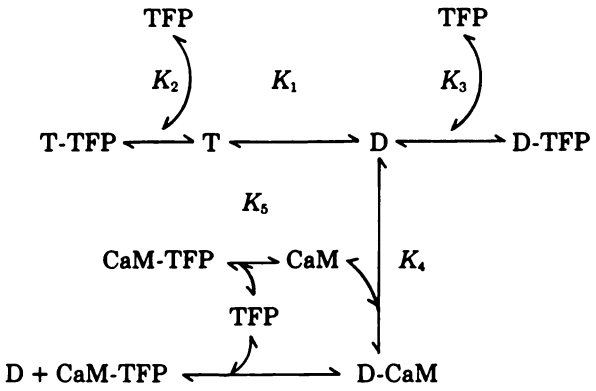
Joint effect of TFP and KHL-8430 on the anisotropy of dansyl-CaM complexed with PFK

Concentrations of both PFK and CaM were 10 μM. The anisotropy values are the average of at least five measurements. The error of determinations is ±5%.

System	Δ Anisotropy
PFK + CaM	0.100
PFK + CaM + 60 μM TFP	0
PFK + CaM + 60 μM TFP + 200 μM KHL-8430	0.030
PFK + CaM + 60 μM TFP + 400 μM KHL-8430	0.068
PFK + CaM + 60 μM TFP + 600 μM KHL-8430	0.076

Molecular models of drug action. The synthesis of the results allowed us to evaluate a quantitative model for the mechanism of action of KHL-8430, in comparison with that of TFP. The models rest upon the following observations and assumptions: (i) in solution, PFK exists as an equilibrium mixture of different forms (1, 5, 8, 17); in our calculations the dimer/tetramer equilibrium is considered; (ii) CaM preferentially binds to the dimeric form of PFK (1, 18); we have neglected the binding of CaM to the tetrameric form of PFK; (iii) the binding of TFP and PFK to CaM is alternative (4); (iv) KHL-8430 interacts with both free CaM and the CaM-PFK complex; (v) within the ternary complex, KHL-8430-CaM-PFK, the enzyme preserves some activity; and (vi) at relatively high concentrations of drugs both TFP and KHL-8430 can bind nonspecifically to PFK, resulting in inhibition of the enzyme.

Scheme 1 illustrates the equilibria in the PFK/CaM/TFP system; TFP prevents and eliminates the binding of CaM to the inactive, dimeric form of the kinase and, hence, liberates the PFK from CaM-induced inactivation. The nonspecific binding of TFP to both the tetrameric and dimeric forms of PFK is also taken into account.



Scheme 1

T and D stand for the tetrameric and dimeric forms of the enzyme, respectively.

We considered the following equilibra of the different species and defined the dissociation constants for binding sites:

$$K_1 = [D]^2/[T] \quad (1)$$

$$K_2 = [T][drug]/[T\text{-}drug] \quad (2)$$

$$K_3 = [D][drug]/[D\text{-}drug] \quad (3)$$

$$K_4 = [CaM][D]/[CaM\text{-}D] \quad (4)$$

$$K_5 = [CaM][drug]/[CaM\text{-}drug] \quad (5)$$

The values of apparent stoichiometry for the specific forms are taken from the literature, 1:2 for both D-CaM and CaM-TFP (1, 19). The apparent stoichiometry for the nonspecific binding of TFP to the T and D forms of PFK are assumed to be 2:1 and 1:1, respectively.

The following constraints for the concentrations were taken into account:

$$[\text{Enzyme}]_{\text{total, in dimers}} = 2[\text{T}] + [\text{D}] + 2[\text{T-TFP}] + [\text{D-TFP}] + [\text{D-CaM}] \quad (6)$$

$$[\text{CaM}]_{\text{total}} = [\text{CaM}] + 2[\text{D-CaM}] + [\text{CaM-TFP}] \quad (7)$$

$$[\text{TFP}]_{\text{total}} = [\text{TFP}] + 2[\text{T-TFP}] + [\text{D-TFP}] + 2[\text{CaM-TFP}] \quad (8)$$

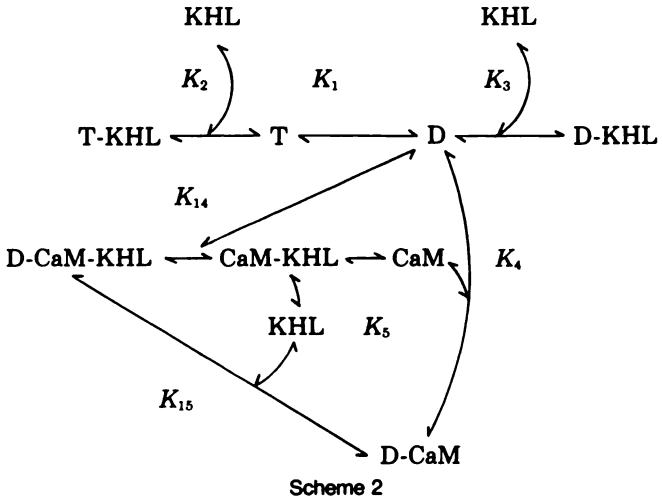
The overall activities of PFK measured at given concentrations of proteins and drugs are the sums of the specific activities of T,D,T-TFP, D-TFP and D-CaM weighed with the concentrations of corresponding enzyme species. Therefore, the overall activity can be deduced from Eq. 9,

$$\text{Activity}_{\text{overall}} = \frac{\sum a_i[\text{species}]}{[\text{PFK}]_{\text{total}}} = \frac{2a_T[\text{T}]}{[\text{PFK}]_{\text{total, in dimers}}} \quad (9)$$

where a_i values are the specific activities of the corresponding PFK species. Only the free tetrameric form has been assumed to be active.

The experimental points of the dose-response curve for TFP (see Fig. 1) could be fitted by the theoretical curve using dissociation constants, assuming 0.9 and 2.5 μM for the K_d values of PFK-CaM and TFP-CaM, respectively. The initial values of the dissociation constants for fitting were taken from the literature [0.6–0.8 μM (1) or 0.3–0.9 μM (2) for PFK-CaM and 1.5 μM (19) or 5 μM (20) for TFP-CaM] and they were varied slightly to get the best fit.

Experimental data showed that KHL-8430 could not prevent or eliminate the binding of CaM to PFK (see Fig. 2B); therefore, it is evident that KHL-8430 affects the CaM-PFK interaction with a different mechanism than does TFP. Consequently, the data gained from kinetic measurements were fitted to theoretical curves based on observations i to vi, with the exception of iii, and the assumed mechanism is illustrated in Scheme 2.



Scheme 2

KHL stands for KHL-8430.

For the calculations, we used Eqs. 1–5 and 10–15.

$$K_{14} = [\text{D}][\text{CaM-drug}]/[\text{D-CaM-drug}] \quad (10)$$

$$K_{15} = [\text{drug}][\text{CaM-D}]/[\text{D-CaM-drug}] \quad (11)$$

The apparent stoichiometries for CaM-KHL-8430 and D-CaM-KHL-8430 were 1:1 and 1:2:2, respectively.

The following total concentrations were taken into account:

$$[\text{Enzyme}]_{\text{total, in dimers}} = 2[\text{T}] + [\text{D}] + 2[\text{T-KHL}] + [\text{D-KHL}] + [\text{D-CaM}] + [\text{D-CaM-KHL}] \quad (12)$$

$$[\text{CaM}]_{\text{total}} = [\text{CaM}] + 2[\text{D-CaM}] + [\text{CaM-KHL}] + 2[\text{D-CaM-KHL}] \quad (13)$$

$$[\text{Drug}]_{\text{total}} = [\text{KHL}] + 2[\text{T-KHL}] + [\text{D-KHL}] + [\text{D-KHL}] + [\text{CaM-KHL}] + 2[\text{D-CaM-KHL}] \quad (14)$$

The overall activity of PFK in the PFK/CaM/KHL-8430 system was calculated from Eq. 15.

$$\text{Activity}_{\text{overall}} = \frac{2a_T[\text{T}] + a_{\text{D-CaM-KHL}}[\text{D-CaM-KHL}]}{[\text{PFK}]_{\text{total, in dimers}}} \quad (15)$$

According to this model KHL-8430 can bind not only to free CaM but also to the CaM-PFK complex. Because binding data showed that KHL-8430 does not influence significantly the binding of PFK to CaM (see Fig. 2B), that is, $K_4 = K_{14}$, it follows from the conservation of mass constraints that KHL-8430 binds with the same affinity to both free CaM and CaM complexed with PFK ($K_5 = K_{15}$). The same values of dissociation constants for tetrameric PFK ($K_1 = 0.1$ μM) and for the dimeric PFK-CaM complex ($K_4 = 0.9$ μM) were applied in the calculations as when the theoretical curve for the effect of TFP was fitted. Fig. 3 shows the theoretical dose-response curves of KHL-8430. In these calculations, only two parameters, the strength of the CaM-KHL-8430 bonds and the activity of PFK within the ternary complex, were varied (Fig. 3, *curves a, b,* and *c*). The best fit was obtained assuming a 0.8 μM binding constant for KHL-8430 and a $\approx$ 60% activity value for PFK within the ternary complex (Fig. 3, *curve b*). It should be emphasized that the fitting of curves was extremely sensitive to the activity of PFK within the ternary complex (Fig. 3, *curves a–c*). When no binding of KHL-8430 to the free CaM was assumed ($K_{15} \ll K_5 = \infty$), we failed to fit the experimental

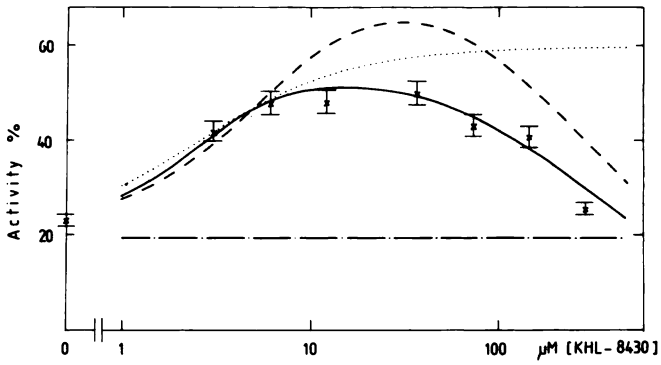


Fig. 3. Theoretical curves calculated on the basis of Scheme 2 using different parameters. The experimental points (×) measured in the presence of KHL-8430 are the same as in Fig. 1. Parameters for calculation of *curves a, b, c,* and *d* are, respectively (K_d values given in μM), for K_1, K_2, K_3, K_4, K_5, K_{14}, K_{15} and percentage of activity (PFK activity within the ternary complex), *curve a* (–·–·–), 0.1, ∞, ∞, 0.9, K_{15}, 0.9, K_5, and 0%; *curve b* (——), 0.1, 130, 130, 0.9, 0.8, 0.9, 0.8, and 60%; *curve c* (– – –), 0.1, 100, 100, 0.9, 4, 0.9, 4, and 100%; *curve d* (· · · · ·), 0.1, 100, 100, 0.9, ∞, 1/∞, 1, and 60%.

points (Fig. 3, *curve d*). The nonspecific binding of both drugs to PFK is much weaker (by 2 orders of magnitude) than their binding to CaM. Therefore, the fitting of curves, at least at low drug concentrations, were only slightly influenced by these values.

Eqs. 1–8 and 10–14 were used for the fitting of the anisotropy measurements, and the overall anisotropy was calculated on the basis of the following equation.

$$\text{Anisotropy}_{\text{overall}} = \frac{a_{\text{CaM}}[\text{CaM}] + 2a_{\text{D-CaM}}[\text{D-CaM}] + a_{\text{CaM-KHL}}[\text{CaM-KHL}] + 2a_{\text{D-CaM-KHL}}[\text{D-CaM-KHL}]}{[\text{CaM}]_{\text{total}}} \quad (16)$$

It has to be emphasized that the same values for dissociation constants applied for the fitting of the dose-response curves were appropriate for the simulation of the binding experiments data (see Fig. 2).

Discussion

We have combined fluorescence anisotropy measurements and enzyme kinetic studies to identify the mechanism of action of anti-CaM drugs of different chemical structures and to develop mathematical models for the mechanism of their actions. The compounds investigated from the phenothiazine and arylalkylamine families of CaM antagonists affect the binding of the target enzymes to CaM in different manners; however, they display similar functional effects on the CaM-mediated inhibition of PFK, namely, both can liberate PFK from CaM inhibition. A similar phenomenon has been observed when PDE was applied instead of PFK, that is, both drugs eliminated the CaM-stimulated PDE activity. At high drug concentrations, inhibition of the basal activity of PDE also occurs. On the basis of the activity measurements of both PFK and PDE systems, KHL-8430 seems to be more efficient and more specific than TFP. Binding experiments suggest that, in contrast to TFP, the binding of KHL-8430 to CaM does not antagonize that of the enzyme but KHL-8430 also binds to CaM complexed with PFK or with PDE. Therefore, the ability of KHL-8430 to share target enzymes with CaM does not seem to be characteristic for only the CaM-PFK system. Within the ternary complex, PFK in dimeric form does not lose its enzymatic activity; in the case of PDE, the CaM-stimulated activity is abolished. These observations are similar to those we found in the case of fendiline (4) and resemble and extend the finding of Newton *et al.* (21–23) that a phenothiazine-CaM adduct binds to the target enzymes, phosphodiesterase or myosin light chain kinase, but can no longer stimulate them. KHL-8430 probably induces an alteration in the tertiary structure of CaM, at least if it is complexed with enzymes, and abolishes the inhibitory/stimulatory effects of CaM. This effect is not specific for the CaM-PFK system but also occurs in the CaM-PDE complex. This surface within the CaM-PFK complex might relate to the contact surface of the dimeric PFK, because CaM preferentially interacts with the dissociated form of PFK. The hypothesis is supported by our previous result, namely, that dimeric PFK can preserve activity when it is complexed with aldolase (2, 24).

We propose two models to describe the different actions of TFP and KHL-8430. According to the models, we quantitatively characterized the interactions and determined the binding constant of KHL-8430 to the CaM-PFK complex. The K_d of KHL-8430-CaM binding is 0.8 μM, which shows that this drug has significantly higher affinity for CaM than does TFP (K_d = 2.5 μM). It does not compete with the enzyme for CaM but forms a ternary complex with dimeric PFK and CaM, in which PFK partially recovers its activity.

From the observation that KHL-8430 and TFP mutually influence each other in the binding to CaM (Table 2), one may infer that, although the KHL-8430 binding site on CaM differs from the TFP binding site, the drugs do not act independently but their binding to CaM exhibits negative cooperativity.

The combination of the fluorescence and kinetic approaches provides a simple and suitable procedure to screen for CaM antagonists, as well as to study the mechanism of their actions. PFK as the target enzyme is especially suitable for this purpose, because of its relatively low affinity for CaM ($K_d \approx 0.6$ μM), in comparison with those of the CaM-stimulated enzymes PDE and myosin light chain kinase, which are commensurate with those of CaM-drug interactions. This system made it possible to use relatively low drug concentrations, thus eliminating their nonspecific binding to the target enzyme (see Refs. 25–28). As can be seen from the dose-response curve (Fig. 1), not only the effectiveness but also the selectivity of the anti-CaM drugs from two different subclasses could be quantified.

In conclusion, data presented in this paper indicate the potency of our screening procedure and its theoretical and practical importance in drug research. Moreover, these results illustrate how specific drugs can be applied in the study to understand the ubiquitous character of CaM, which can mediate enzymatic events at the molecular level.

Acknowledgments

The authors are sincerely grateful to Dr. J. Gaál of Chinoin Pharmaceutical and Chemical Works Co., Ltd. (Budapest), for his constant and helpful interest in this work and for valuable discussions. Thanks are due to Mrs. M. Nuridsány for her skilful and conscientious technical assistance.

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