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EFFECT OF TRIFLUOPERAZINE, COMPOUND 48/80, TMB-8 AND VERAPAMIL ON THE RATE OF CALMODULIN BINDING TO ERYTHROCYTE Ca^{2+}-ATPase

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The erythrocyte Ca^{2+}-ATPase shifts reversibly between two states, the calmodulin-deficient A-state and the calmodulin-saturated B-state, dependent on calcium and calmodulin. The effects on this system of the four drugs, trifluoperazine, compound 48/80, TMB-8 and verapamil were studied. All four drugs inhibited the maximum activity of the B -state Ca^{2+}-ATPase and, in addition, trifluoperazine and compound 48/80 in higher doses inhibited the A-state. Furthermore, the four drugs decreased the calmodulin sensitivity of the Ca^{2+}-ATPase in the order of decreasing effect: trifluoperazine > compound 48/80 > TMB-8 > verapamil. In the same order of decreasing effect the drugs increased the time required for full calmodulin activation of the A-state of Ca^{2+}-ATPase, whereas the drugs had only small effects on the rate of deactivation of the B-state, caused by dissociation of calmodulin from the enzyme. It is discussed whether the effects on calmodulin activation were caused by a reduction of free calmodulin due to the formation of drug-calmodulin complexes or whether the drugs, especially trifluoperazine, compound 48/80 and TMB-8, by binding to the Ca^{2+}-ATPase, decreased the rate constants for association of calmodulin and enzyme.

Introduction

The Ca^{2+}-ATPase (EC 3.6.1.3) located in the plasma membrane in human red cells is part of the Ca^{2+} pump, and is regulated by calcium and calmodulin (for reviews see Refs. 1–5). The Ca^{2+}-dependent binding of calmodulin (Z) shifts reversibly the ATPase (E) from a ground state (A) to a more active state (B) with high Ca^{2+} affinity [4]:

$$E_A + Ca_iZ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_B(Ca_iZ) \; ; \; K_1 = k_2/k_1 \qquad (1)$$

The Ca^{2+}-ATPase binds almost exclusively Ca^{2+}-calmodulin complexes (Ca_iZ) with $i = 2$, 3 or 4 [6–7]. Rate constants for association (k_1) and dissociation (k_2) of calmodulin have been determined recently [8].

Inhibition of the Ca^{2+}-ATPase can occur by binding of drugs to the Ca^{2+}-calmodulin complex (Ca_iZ) or to the enzyme or to both.

We have compared the effects on the Ca^{2+}-ATPase of four drugs: trifluoperazine, compound 48/80, TMB-8 and verapamil. Trifluoperazine has been reported to inhibit several calmodulin-activated enzyme systems [9], including the calmodulin activation of the erythrocyte Ca^{2+}-ATPase [10–12]. Compound 48/80 is used as histamine releaser [13] and has been reported to be a more specific inhibitor of the calmodulin-dependent Ca^{2+}-ATPase activity than trifluoperazine as the calmodulin-independent Ca^{2+}-ATPase activity was unaffected by compound 48/80 [14]. The

Abbreviations: TFP, trifluoperazine dihydrochloride; TMB-8, 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride.

other two tested drugs have often been characterized as Ca^{2+} antagonists. Verapamil in micromolar concentrations inhibits slow calcium channels in cardiac cells [15] and the Ca^{2+}-ATPase in a cardiac sarcolemmal preparation [16], and in higher concentrations verapamil seems to bind calmodulin [17,18] and inhibits Ca^{2+}-ATPase in sarcoplasmic reticulum-enriched preparations [16]. TMB-8 inhibits various cellular reactions dependent on intracellular Ca^{2+} [19–21].

Methods

Drugs. Trifluoperazine dihydrochloride (TFP) was a gift from Rhone Poulenc (Pharma Norden A/S, Birkerød). Compound 48/80 is a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde from Sigma (product No. C4257). TMB-8 was from Niels Clauson-Kaas (Chemical Research Laboratory, Farum) and verapamil hydrochloride was a gift from Knoll AG (Meda A/S, Herlev).

Preparation of erythrocyte membranes. Two types of membrane-bound Ca^{2+}-ATPase were prepared from bank blood (stored 1–3 weeks) as described previously [6]: calmodulin-deficient ATPase (in the so-called A-state) and calmodulin-saturated ATPase (B-state). Before use the membranes were preincubated with saponin in order to secure full accessibility of the Ca^{2+}-ATPase, as described in Ref. 8.

Drug binding experiments. Erythrocyte membranes were incubated with drugs in the concentrations used in the inhibition experiments at room temperature in 70 mM Tris-HCl buffer, pH 7.6 for 20 min and centrifuged at $13\,000 \times g$ for 10 min. The absorbances of the supernatants were measured at 320 nm (TMB-8), 310 nm (TFP), 290 nm (verapamil) and 277 nm (compound 48/80) and the drug concentrations determined from standard curves. The supernatant from membranes incubated without drugs was used as blank. Drug binding to the Eppendorf centrifuge tubes was negligible.

ATPase assay. The ATPase activity was measured at 37°C as the production of ADP which was coupled to the oxidation of NADH and recorded continuously at 366 nm, as earlier [6,8]. The concentration of membrane protein during assay was about 0.5 g/l assay medium (10 nmol Ca^{2+}-ATPase per g membrane protein).

Ca^{2+}-ATPase activity is the difference between (Ca^{2+} + Mg^{2+})-dependent and Mg^{2+}-dependent ATPase, expressed as μmol/min per g membrane protein.

Values of maximum activity (V) of Ca^{2+}-ATPase were determined from double reciprocal plots, depicting activity (v) towards total or calculated (see Appendix) free calmodulin concentration (Z). Using free calmodulin concentrations, we found linear double reciprocal plots in the whole range of tested concentrations. The calmodulin concentration required for half maximum activation of Ca^{2+}-ATPase was determined from Hill plots, i.e. $\log(v/(V-v))$ vs. $\log Z$.

The rate constants for the binding of calmodulin to the Ca^{2+}-ATPase were calculated as described in Ref. 8.

Calmodulin was purified from bovine brain, and protein and ionized calcium were determined as described in Ref. 6.

Results

Fig. 1. shows, in agreement with the results of Hinds et al. [22], that the amount of TFP bound to the erythrocyte membranes was a linear function of the total TFP concentration. There was no difference between the TFP-binding to membranes deficient in calmodulin and the TFP-binding to membranes with calmodulin-saturated Ca^{2+}-ATPase. The membrane-bound fraction of the total TFP concentration during assay was dependent on membrane concentration and pH, but independent of Ca^{2+}-concentration (10^{-7}–10^{-3} M) and calmodulin concentration at the TFP concentrations used. During our assay conditions 70% of TFP and 35% of compound 48/80 was membrane-bound, whereas less than 5% of TMB-8 and verapamil was membrane-bound. In contrast to the binding, the inhibition of the membrane-bound Ca^{2+}-ATPase was a saturable function of the total TFP concentration. The different shapes of the two curves and the large difference between the Ca^{2+}-ATPase concentration (5 nmol/g dry membrane [6]) and the amount of TFP bound indicates that most of the TFP is 'non-specifically' bound. As a consequence of the results above, the

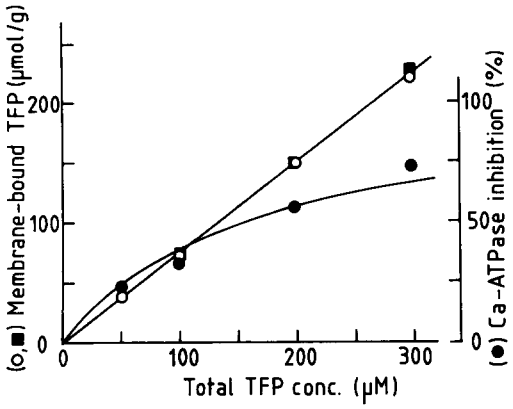

Fig. 1. Inhibition of Ca^{2+}-ATPase activity and binding of TFP to erythrocyte membranes vs. total TFP concentration. Inhibition of calmodulin-saturated Ca^{2+}-ATPase (●) was measured at 13 μM free Ca^{2+} and the activity without drug was 63.8 ± 2.0 $\mu mol \cdot min^{-1} \cdot g^{-1}$ (S.E., $n = 3$). The inhibition curve is calculated using a non-competitive model (% inhibition = $100 \cdot I/(I_{50} + I)$ where I is total TFP concentration and $I_{50} = 154$ μM (Table I)). TFP bound to membranes with calmodulin-deficient Ca^{2+}-ATPase (■) or calmodulin-saturated Ca^{2+}-ATPase (○) is given in μmol per g dry membranes (corresponding to μmol per 0.5 g membrane protein). Data points are the mean values of three determinations.

drug concentration in the buffer phase of the system was used in all calculations of kinetic models.

Fig. 2 shows dose-response curves, depicted as Dixon plots [23], of the four tested drugs. For the determination we used membranes in which the Ca^{2+}-ATPase was saturated with calmodulin at the start of assay. In addition, Dixon plots were obtained for calmodulin-deficient Ca^{2+}-ATPase (not shown). The Dixon plots were linear within the tested range of drug concentrations for all of the drugs, and the derived I_{50} values [24] are given in Table I.

This procedure deviates from those used in other investigations [10–12] in which drug inhibition was assayed by incubation of calmodulin and erythrocyte membranes which were deficient in calmodulin at the start of assay. The latter method led to lower I_{50} values than that used in the experiments shown in Fig. 2 and Table I and, furthermore, the Dixon plots were not linear. For instance, by assaying calmodulin-deficient membranes in the presence of 50–60 nM calmodulin we obtained the I_{50} values: TFP (88 μM), compound 48/80 (30 μg/ml), TMB-8 (2.13 mM), and verapamil (2.61 mM).

For further experiments we chose drug concentrations (see Fig. 3) corresponding to 35–40% inhibition of the calmodulin-saturated Ca^{2+}-ATPase. According to Fig. 3 the effect of the four drugs on maximum activity was not abolished even at high concentrations of calmodulin, i.e. every drug inhibited the calmodulin-associated state of Ca^{2+}-ATPase (B-state) in a non-competitive manner, probably by binding to the enzyme

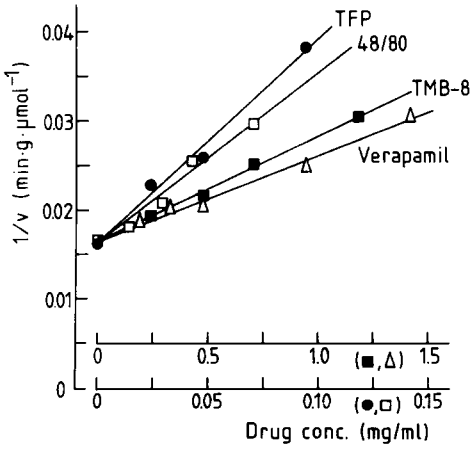

Fig. 2. Dixon plots for the four tested drugs. Reciprocal initial activity of calmodulin-associated Ca^{2+}-ATPase (B-state) vs. total drug concentration during assay. The concentration of free Ca^{2+} was 13.4 ± 1.8 μM (S.E., $n = 5$).

TABLE I

DRUG CONCENTRATION (I_{50}) REQUIRED FOR HALF MAXIMUM INHIBITION OF CALMODULIN-DEFICIENT Ca^{2+}-ATPase (A-STATE) AND CALMODULIN-SATURATED Ca^{2+}-ATPase (B-STATE)

The I_{50} values were obtained from Dixon plots as those in Fig. 2 and represent mean ± S.E. of three experiments. The I_{50}-values refer to total drug concentration. The concentrations of ionized calcium during assay were 130 μM (A-state) and 13 μM (B-state). The differences between A-state and B-state, tested by Student's t-test, were significant ($P < 0.05$).

Drug	I_{50}	
	A-state	B-state
TFP (mM)	0.271 ± 0.026	0.154 ± 0.013
48/80 (μg/ml)	258 ± 54	84 ± 1
TMB-8 (mM)	–	3.09 ± 0.34
Verapamil (mM)	23.1 ± 2.3	3.31 ± 0.28

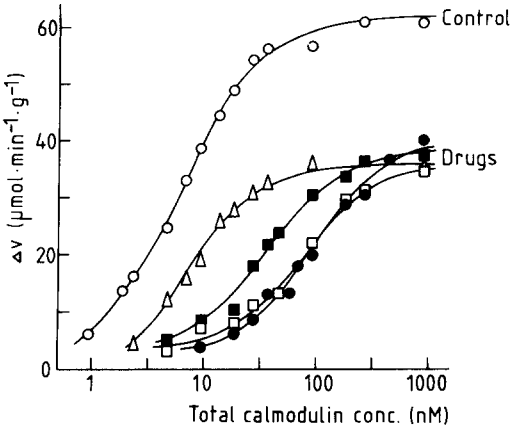

Fig. 3. Calmodulin-dependent Ca^{2+}-ATPase activity (Δv) vs. total calmodulin concentration during assay in the absence or presence of drugs. The drugs were (total concentrations): TFP (●, 0.10 mM), compound 48/80 (□, 57 μg/ml), TMB-8 (■, 1.65 mM), and verapamil (△, 0.97 mM). The Ca^{2+} concentration was as in Fig. 2. The Ca^{2+}-ATPase activities, determined without addition of calmodulin, were subtracted and they amounted to (μmol·min^{-1}·g^{-1}): 12.9 (control), 9.8 (TFP), 5.2 (compound 48/80), 9.7 (TMB-8), and 11.8 (verapamil).

molecule or its lipid environment, as suggested in the case of TFP [25–27].

In spite of the same magnitude of drug-effect on maximum activity (35–40% inhibition), the effects of the four drugs on the calmodulin sensitivity of the enzyme were different (Fig. 3). The drugs increased the calmodulin concentration ($K_{0.5}$) needed for half-maximum activation of the Ca^{2+}-ATPase. The $K_{0.5}$ values determined from three experiments like that in Fig. 3 were (nM, ± S.E.): control (6.0 ± 0.4), TFP (91 ± 13), compound 48/80 (50 ± 7), TMB-8 (25 ± 5), and verapamil (9.3 ± 0.5). All the $K_{0.5}$ values for the drugs were significantly different from the control value ($P < 0.01$).

The effect on $K_{0.5}$ could be due either (1) to a decreased calmodulin-affinity of the drug-inhibited enzyme or (2) to a reduction of free calmodulin due to binding of drug to calmodulin. Drug-binding to calmodulin has been reported in the case of TFP [28] and verapamil [17,18]. Of course, the effect can also be caused by a combination of these two modes of action, as discussed later (see Discussion).

Fig. 4 shows the time dependence of the activation of Ca^{2+}-ATPase in the presence of calmodulin and 13 μM free Ca^{2+}. In the absence of drugs (Control) the level of activation increased and the time needed to reach this level decreased when the calmodulin concentration was raised (from 6.5 to 56 nM in Fig. 4). In the presence of TFP, compound 48/80, or TMB-8, but not verapamil, we found an increase of the time required for reaching the constant level of activation, compared to Control at the same calmodulin concentration (shown for TFP and compound 48/80 in Fig. 4).

From time curves like those in Fig. 4, rate constants for association (k_1) and dissociation (k_2) of calmodulin and enzyme (see Eqn. 1) were calculated as previously [8]. Table II shows the rate constants calculated in two different ways (Mechanisms 1 and 2, see Discussion) from several experiments as those in Fig. 4.

At low Ca^{2+} concentrations calmodulin dissociated from the enzyme-calmodulin complex. This is exemplified in Fig. 5 which shows the time-dependent decrease of activity of calmodulin-associated Ca^{2+}-ATPase at 0.31 μM free Ca^{2+}. The reaction curves follow first order kinetics (in accordance with Eqn. 1), and from the slope of the regression line the rate constant (k_2) for dissociation of calmodulin and enzyme can be calculated, as described previously [8]. The rate of dissociation of calmodulin from the enzyme was not influenced by the presence of the drugs (Table III).

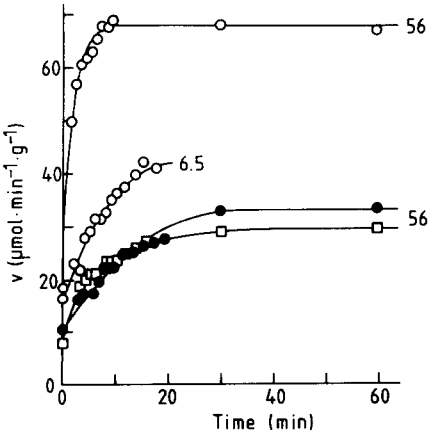

Fig. 4. Ca^{2+}-ATPase activity dependent on time in the presence or absence of TFP and Compound 48/80. Without drug (○), TFP (●, 0.10 mM), compound 48/80 (□, 57 μg/ml). Assayed in the presence of 13 μM free Ca^{2+} and different calmodulin concentrations (6.5 and 56 nM).

TABLE II

EFFECTS OF DRUGS ON RATE CONSTANTS FOR CALMODULIN BINDING (k_1) AND DISSOCIATION (k_2) AT HIGH Ca^{2+} CONCENTRATION

The concentrations of ionized calium was 13 μM and the drug concentrations were as in Fig. 3. The rate constants are mean ± S.E. of six experiments, tested by Student's t-test.

Drug	k_1 ($\mu M^{-1} \cdot min^{-1}$)		k_2 (min^{-1})	
	Mechanism 1	Mechanism 2	Mechanism 1	Mechanism 2
Control	13.8 ± 0.9	–	0.048 ± 0.003	–
TFP	0.8 ± 0.1 [a]	16.5 ± 1.0	0.066 ± 0.005 [b]	0.058 ± 0.004
48/80	1.3 ± 0.1 [a]	13.3 ± 1.0	0.061 ± 0.003 [b]	0.047 ± 0.004
TMB-8	2.9 ± 0.4 [a]	13.9 ± 1.3	0.070 ± 0.010 [b]	0.049 ± 0.005
Verapamil	10.3 ± 0.8	18.9 ± 1.4 [b]	0.068 ± 0.005 [b]	0.066 ± 0.005 [b]

[a] $P < 0.001$.
[b] $P < 0.01$.

The apparent increase of the rate constants determined at 0.18 μM free Ca^{2+} in the presence of TMB-8 or verapamil (Table III) may be due to a decrease of the free Ca^{2+} concentration, exerted by the rather high concentrations of these two drugs (1.7 and 1.0 mM, respectively) during assay. At this low level of Ca^{2+} the rate constant (k_2) is strongly sensitive to even small changes in the concentration of free Ca^{2+} (see Ref. 8). Unfortunately, this possibility could not be verified by measurements of the Ca^{2+} concentrations, since

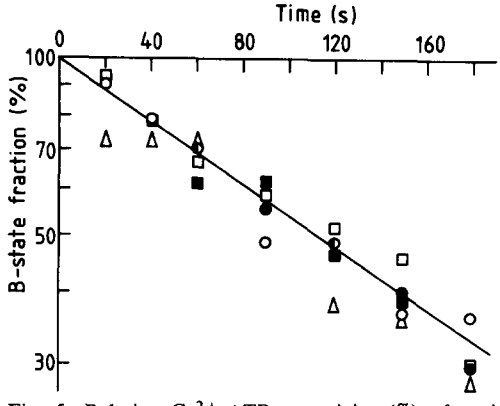

Fig. 5. Relative Ca^{2+}-ATPase activity (%) of preincubated membranes vs. time of preincubation, in the presence or absence of drugs. Without drugs (○), TFP (●, 0.10 mM), compound 48.80 (□, 57 μg/ml), TMB-8 (■, 1.65 mM), and verapamil (△, 0.97 mM). The membranes were calmodulin-saturated at the start of preincubation. During the preincubation, in which the free Ca^{2+} concentration was 0.31 μM, calmodulin dissociated from the membranes, leading to a shift of the Ca^{2+}-ATPase from B-state to A-state. At the end of preincubation the initial Ca^{2+}-ATPase activity (before re-binding of calmodulin) was assayed at 1 μM free Ca^{2+} and related to the activity of non-preincubated Ca^{2+}-ATPase in the B-state (see also Ref. 8). The activities ($\mu mol \cdot min^{-1} \cdot g^{-1}$) of the non-preincubated membranes (100% values) were 33.0 (control), 25.2 (TFP), 21.0 (compound 48/80), 21.6 (TMB-8), and 24.4 (verapamil).

TABLE III

EFFECT OF DRUGS ON RATE CONSTANT FOR CALMODULIN DISSOCIATION (k_2) AT LOW Ca^{2+} CONCENTRATIONS

The concentrations of drugs were as in Fig. 3. The rate constants (min^{-1}) are mean ± S.E. of three experiments (controls six experiments), tested by Student's t-test.

Drug	0.18 μM Ca^{2+}	0.31 μM Ca^{2+}
Control	0.74 ± 0.06	0.34 ± 0.04
TFP	0.81 ± 0.08	0.42 ± 0.02
48/80	0.86 ± 0.13	0.41 ± 0.04
TMB-8	1.83 ± 0.15 [b]	0.35 ± 0.06
Verapamil	1.21 ± 0.08 [b]	0.46 ± 0.02

[b] $P < 0.01$.

TABLE IV

DISSOCIATION CONSTANTS (K_6) FOR DRUG-CALMODULIN COMPLEXES

The dissociation constants (K_6) for the reaction shown in Eqn. 6 were obtained by fitting mechanism 2 (see Discussion) to calmodulin activation experiments like those in Fig. 3.

Drug	K_6
TFP	1.3 μM
48/80	2.9 μg/ml
TMB-8	300 μM
Verapamil	1030 μM

TMB-8 and verapamil in these high concentrations interacted with the Ca^{2+}-selective electrode. TFP and compound 48/80 did not interact with the electrode at the concentrations used.

Discussion

The results suggest that the four drugs inhibit the Ca^{2+}-ATPase by competitive and non-competitive reaction mechanisms. The inhibition of the calmodulin-saturated Ca^{2+}-ATPase, the so-called B-state (Fig. 2 and Table I), and the inability of high calmodulin concentrations to abolish the drug-inhibition of the enzyme (Fig. 3) suggest a 'non-competitive reaction mechanism' (drug indicated by I, K_2–K_6 are dissociation constants, for other symbols see Eqn. 1):

$$E_B(Ca_iZ) + I \overset{K_2}{\rightleftharpoons} I \cdot E_B(Ca_iZ) \quad (2)$$

On the other hand, the drugs increased the calmodulin concentration required for half-maximum activation of the Ca^{2+}-ATPase ($K_{0.5}$) (Fig. 3), which suggest a competitive reaction mechanism. Three different 'competitive mechanisms' are possible:

(a) The drug combines with the calmodulin-binding site on the enzyme:

$$E_A + I \overset{K_3}{\rightleftharpoons} E_A(I) \quad (3)$$

(b) The drug reduces the calmodulin-affinity, i.e. $K_5 > K_1$ (cf. Eqn. 1), by binding to another site on the enzyme:

$$E_A + I \overset{K_4}{\rightleftharpoons} I \cdot E_A \quad (4)$$

$$I \cdot E_A + Ca_iZ \overset{K_5}{\rightleftharpoons} I \cdot E_B(Ca_iZ) \quad (5)$$

(c) The drug binds to calmodulin, thereby reducing the concentration of free calmodulin, without affecting the calmodulin-binding of the enzyme (i.e. $K_1 = K_5$):

$$I + Ca_iZ \overset{K_6}{\rightleftharpoons} I(Ca_iZ) \quad (6)$$

Generally, the equilibrium constants of the reactions in Eqns. 1, 2, 4 and 5 are mutually interdependent, since $K_1K_2 = K_4K_5$.

According to results obtained by Al-Jobore and Roufogalis (see Ref. 29), the competitive mechanism (a) (Eqn. 3) seems unlikely, at least in the case of drug-inhibition by the use of chlorpromazine. In our experiments mechanism (a) cannot explain the found non-competitive reaction mechanism (cf. Eqn. 2). Mechanism (c) implies a calcium-dependent binding of drug to calmodulin, and this has been demonstrated for a variety of drugs [18,28,30,31].

Therefore, in the following we only consider two possibilities: mechanism 1, which is the non-competitive reaction mechanism combined with the competitive mechanism (b) (Eqns. 2, 4 and 5), and mechanism 2, which is the non-competitive mechanism combined with the competitive mechanism (c) (Eqns. 2 and 4–6).

In mechanism 1 the binding of drug to a site on the enzyme different from the calmodulin-binding site (Eqns. 2 and 4) has a dual effect: the reduction of both maximum activity and calmodulin-affinity ($K_5 > K_1$). The latter effect implies that the drug must affect the rate of association or dissociation of calmodulin and enzyme, by decreasing k_1 and/or by increasing k_2 (see Eqn. 1). According to Table II (mechanism 1) and Table III, the effect of drug on k_1 exceeded that on k_2, i.e. the main effect of drug would be to decrease the rate of association of Ca^{2+}-ATPase and calmodulin.

It is to be noticed that, due to the general relationship between the equilibrium constants (cf. above), the inequality $K_5 > K_1$ leads to $K_2 > K_4$, i.e. that in the case of mechanism 1 the inhibitor should bind to the A-state of Ca^{2+}-ATPase (Eqn. 4) with higher affinity than to the B-state (Eqn. 2).

In mechanism 2 the binding of drug to the enzyme only inhibits in a non-competitive way (Eqns. 2 and 4), and the apparent decrease of calmodulin affinity is due to the drug-binding of calmodulin (see Eqn. 6). The mechanism means that $K_1 = K_5$ which imply that $K_2 = K_4$ (cf. above), i.e. that the A-state (Eqn. 4) and the B-state of Ca^{2+}-ATPase (Eqn. 2) bind the inhibitor with the same affinity.

In order to test the consequences of using mechanism 2 we attempted to fit this model to the results from the calmodulin activation experiments

with any of the four drugs (Fig. 3). In this model we chose: $K_1 = K_5 = 3.5$ nM, $K_2 = K_4$ was equal to the I_{50} values (cf. below) for the B-state in Table I, and the K_6 values were those shown in Table IV (see also Appendix). The K values were corrected for the non-specific binding of drugs to the membranes, as described in Results. Table II (Mechanism 2) shows that the rate constants for association (k_1) and dissociation (k_2) of calmodulin and enzyme were virtually unaffected by the drugs when the rate constants were calculated by the aid of mechanism 2. According to this model the delayed calmodulin activation of the Ca^{2+}-ATPase in the presence of drug (see Fig. 4) was due to the low concentration of free calmodulin caused by the formation of drug-calmodulin complex.

In the attempts to evaluate whether mechanism 1 or 2 best describes the mode of inhibition exerted by the four drugs, the I_{50} values in Table I should be included. The I_{50} values depend partly on the dissociation constants for the drug-enzyme complex (K_2 or K_4), partly on the ratio (α): activity of drug-enzyme complex/activity of enzyme (without drug-binding). In case of purely non-competitive enzyme inhibition (mechanism 2), the I_{50} value is equal to the equilibrium constant (K_2 or K_4) when $\alpha = 0$. If $\alpha > 0$, the I_{50} value will be higher than the corresponding equilibrium constant, and the I_{50} value increases towards infinity as α approaches 0.5.

The effects of the four drugs on the calmodulin/Ca^{2+}-ATPase system differ. Studying the calcium-dependent binding of TFP to calmodulin, Levin and Weiss [28] found that the apparent dissociation constant for the drug-calmodulin complex was 1–1.5 μM, in agreement with our value, $K_6 = 1.3$ μM (Table IV), obtained from the kinetic data (for simplicity we ignored the multi-site model for TFP-binding, suggested by Levin and Weiss). Therefore, the results obtained with TFP can be explained satisfactorily by mechanism 2. Then TFP should bind to the A-state and the B-state of Ca^{2+}-ATPase with the same affinity, and the difference between the I_{50} values of the two enzyme states (Table I) indicates that the B-state was inhibited relatively more (α low) by the drug-binding than the A-state (α higher than for the B-state). However, we cannot entirely exclude that TFP, in addition, decreases the rate constant for association of enzyme and calmodulin, and thereby decreases the calmodulin-affinity of the Ca^{2+}-ATPase.

The effects of compound 48/80 were similar to those of TFP. It is not known whether compound 48/80 binds to calmodulin. If so, the K_6 value in Table IV predicts the minimum dissociation constant. Using calmodulin-deficient erythrocyte membranes in the presence of 30 nM calmodulin, Gietzen et al. [14] found a much lower I_{50} value for compound 48/80 (0.85 μg/ml) than the corresponding value found by us (30 μg/ml), and in contrast to Gietzen et al. we found an inhibition in the absence of added calmodulin. We cannot explain these differences. Possibly, the used lots of compound 48/80 differed in composition, since this drug is not a well defined single substance.

The effects of TMB-8 and verapamil clearly deviated from those of TFP and compound 48/80. The B-state Ca^{2+}-ATPase was much less sensitive to TMB-8 and verapamil and the A-state was not (TMB-8) or only little (verapamil) affected (Table I). However, TMB-8 had a pronounced inhibitory effect on the calmodulin-sensitivity of the Ca^{2+}-ATPase (Fig. 3), and this could be due either to a decreased rate constant for association of enzyme and calmodulin in the presence of TMB-8 (Table II, Mechanism 1) or to the formation of a complex of calmodulin and TMB-8 with an apparent dissociation constant of minimum 300 μM (Table IV, Mechanism 2).

Verapamil had only a small effect on the calmodulin sensitivity of the Ca^{2+}-ATPase (Fig. 3), and this effect could be ascribed to a low-affinity binding of verapamil to calmodulin (Table IV). Recently, Andersson et al. [31] determined a dissociation constant of 100-1000 μM for the binding of D600 (a methoxy derivative of verapamil) to calmodulin, in reasonable agreement with the value of K_6 shown in Table IV, whereas Johnson and Wittenauer [18] found lower K_d values for verapamil and D600 (30 μM for both). The effects of verapamil on the rate constants for calmodulin binding (Table II) are too small to allow a conclusion. Obviously, the micromolar concentrations of verapamil that are effective in therapeutic doses do not affect the calmodulin/Ca^{2+}-ATPase system in red cells.

Appendix

In mechanism 1 the concentration of free calmodulin was calculated from

$$[Ca_iZ] = Z_t/(1 + E_t/(K + [Ca_iZ]))$$

where Z_t and E_t are the total concentrations of calmodulin and enzyme, respectively, and where K, calculated as $K = K_{0.5} - 0.5 \cdot E_t$ [6], represents a mean dissociation constant for the reactions between calmodulin and enzyme, the latter with or without drug-binding (see Eqns. 1 and 5).

In mechanism 2 the concentrations of free calmodulin (Ca_iZ) and free drug (I) were calculated by iteration of the expressions:

$$[Ca_iZ] = Z_t/(1 + [I]/K_6 + E_t/(K_1 + [Ca_iZ])) \quad (7)$$

$$[I] = I_t/(1 + [Ca_iZ]/K_6 + E_t/(K_2 + [I])) \quad (8)$$

which are derived from the expressions for the total concentrations of enzyme, calmodulin, and drug (I_t):

$$E_t = [E_A] + [E_B(Ca_iZ)] + [I \cdot E_A] + [I \cdot E_B(Ca_iZ)]$$

$$I_t = [I] + [I(Ca_iZ)] + [I \cdot E_A] + [I \cdot E_B(Ca_iZ)]$$

$$Z_t = [Ca_iZ] + [I(Ca_iZ)] + [E_B(CA_iZ)] + [I \cdot E_B(Ca_iZ)]$$

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