Calmodulin antagonist action in smooth-muscle myosin phosphorylation

Different mechanisms for trifluoperazine and calmidazolium inhibition

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The mechanism of inhibition of myosin phosphorylation by calmodulin antagonists [trifluoperazine (TFP) and calmidazolium (CAL)] was investigated in two enzyme–substrate systems: (1) mixtures of isolated myosin phosphorylatable light-chain (L_{20}), myosin light-chain kinase (MLCKase) and calmodulin (CM); (2) synthetic self-assembled myosin filaments containing tightly bound endogenous MLCKase and CM. Double-reciprocal plots obtained with the first system were non-linear, indicating that the antagonists did not act exclusively on CM to inhibit MLCKase. First-order phosphorylation progress curves obtained at different CM antagonist concentrations for the more native filamentous myosin system indicated that the antagonists could also inhibit phosphorylation by interaction with the myosin phosphorylation site. Further analysis of these data in accordance with Reiner [(1969) *Behavior of Enzyme Systems*, 2nd edn., pp. 185–201, Van Nostrand–Reinhold, New York] showed that over a range of concentrations required to inhibit phosphorylation TFP interacted with free CM as well as with the myosin phosphorylation site: accordingly inhibition was of an activator- and substrate-depletion type. CAL inhibition was more CM-specific and operated via an activator-combination mechanism, inhibiting free CM as well as the CM–MLCKase complex. Both CM and the isolated L_{20} light chain antagonized the inhibitory effects of CAL and TFP, consistent with the above analysis.

INTRODUCTION

Since the discovery of calmodulin (CM) as a Ca^{2+} -dependent regulator of phosphodiesterase (Cheung, 1970; Kakiuchi *et al.*, 1970) a multitude of enzymes have been found to be similarly regulated by this ubiquitous protein [for reviews see Klee & Vanaman (1982) and Mannhold (1984)]. It is now generally accepted that CM responds to intracellular Ca^{2+} and thereby mediates its cellular actions.

Attempts to elucidate the mechanism of CM action have led to the widespread use of CM antagonists (see Roufogalis, 1985). The first of these, trifluoperazine (TFP), a phenothiazine-type compound, was described by Weiss *et al.* (1974). Among others is a more recently described antagonist, an antimycotic miconazole derivative, calminazolium (CAL; Van Belle, 1981). Although these and other CM antagonists share common hydrophobic and cationic features, it has become apparent that many of those commonly employed may have additional biological effects at concentrations similar to those that block CM. For this reason it has become important to establish the exact mechanism of inhibition of the different antagonists in defined cellular systems. Rather few studies of this type have, however, been performed, and none has hitherto been made at all for the CM-dependent smooth-muscle regulatory system. In smooth muscle, calmodulin not only regulates myosin light-chain kinase (MLCKase) and thereby the phosphorylation of myosin (Small & Sobieszek, 1980;

Marston, 1982; Kamm & Stull, 1985) but also modulates the actin–myosin interaction by acting on caldesmon (Smith *et al.*, 1987). In the present study the inhibitory mechanisms of TFP and CAL were elucidated in relation to the MLCKase system. These two CM antagonists are commonly used in studies on smooth muscle and, as shown in the present paper, inhibit myosin phosphorylation by different mechanisms.

MATERIALS AND METHODS

Protein preparations

Turkey gizzard myosin was used throughout. The myosin was purified from freshly extracted actomyosin as described previously (Sobieszek, 1985), with the use of a myofibril-like preparation as starting material (Sobieszek & Bremel, 1975).

The myosin light chain fraction was isolated from purified myosin or tropomyosin-free actomyosin by the methods of Perrie & Perry (1970) with some modifications (Sobieszek, 1988). The homogeneous and CM-free L_{20} preparation so obtained was stored as a 1 mM stock solution at -30°C .

MLCKase was extracted from gizzard myofibrils and purified as described previously (Sobieszek & Barylko, 1984). MLCKase was subjected to an additional purification/concentration step on a CM–Sephacrose affinity column. After dialysis, it was stored in small portions at -70°C . CM was obtained as a by-product

Abbreviations used: CAL, calmidazolium {1-[bis-(*p*-chlorophenyl)methyl]-3-[2,4-dichloro-(2,4-dichlorobenzyloxy)phenyl]imidazolium chloride}; TFP, trifluoperazine {10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10*H*-phenothiazine}; CM, calmodulin; MLCKase, myosin light-chain kinase; L_{20} , 20 kDa myosin light chain.

of the MLCKase purification (Sobieszek & Barylko, 1984) and additionally purified by gel filtration and ion-exchange chromatography.

Inhibition assays

TFP and CAL were purchased from Serva (Heidelberg, Germany) and Janssen Pharmaceutica (Beerse, Belgium) respectively. A 0.8 mM stock solution of CAL [dissolved in 75% (v/v) dimethyl sulphoxide in AA buffer (see below)] was kept at -30°C . It was normally diluted to the required concentration with the assay AA buffer of the following composition: 60 mM-KCl/2 mM-MgCl₂/0.5 mM-dithiothreitol/10 mM-imidazole/HCl buffer, pH 7.1 at 25°C . A 5 mM-TFP stock solution (also in AA buffer) was similarly stored at -30°C . Unless otherwise stated, the concentration of filamentous myosin suspensions in the assay mixture was between 15 and 20 mg/ml (30–40 μM).

Incorporation of ^{32}P into myosin (or its L_{20} light chain) was measured as described previously (Sobieszek, 1985). For assay times shorter than 20–30 s termination was achieved by the addition of 50 μl of saturated guanidinium chloride solution (final assay volume 175 μl), except for samples analysed, in addition, by urea/glycerol/polyacrylamide-gel electrophoresis, in which case 102 mg of solid urea was added (Sobieszek & Jertschin, 1986).

Inhibition of the initial phosphorylation rates, at different antagonist concentrations, was measured in two

different ways. In the first method, the rates were obtained directly from ^{32}P incorporation in short assays (10–30 s). In the second and more accurate assay, the rates were calculated from phosphorylation progress curves obtained for a number of inhibitor concentrations. A best asympto-exponential fit to the experimental points was then computed and this yielded the effective initial myosin concentration ($[M]_0$) as well as the apparent first-order rate constant (k_1), taking the initial rate at a given antagonist concentration as $v_i = k_1 \cdot [M]_0$.

RESULTS

Double-reciprocal analysis

The phosphorylatable 20 kDa myosin light chain (L_{20}) is the substrate most commonly used for analysis of myosin phosphorylation. In contrast with myosin, the L_{20} preparations are not contaminated by MLCKase or myosin light chain phosphatase and are readily soluble in purified form (Sobieszek, 1988). I have attempted to elucidate the inhibitory mechanism of TFP and CAL with the use of this convenient substrate. In a conventional approach, the initial phosphorylation rates were measured at different concentrations of L_{20} and for a series of concentrations of antagonist at a fixed concentration of CM-MLCKase complex. Double-reciprocal plots obtained from the collected data (Figs. 1a and 1b) showed that, for a relatively wide range of light

Fig. 1. Double-reciprocal plots of TFP and CAL inhibition of phosphorylation of isolated L_{20} light chains

TFP inhibition (a) was non-competitive at lower L_{20} concentrations (straight lines with broken extensions intersecting abscissa). At higher L_{20} concentrations the inhibition became competitive (intercept on ordinate). A similar change from non-competitive to competitive inhibition was also observed for CAL (b) except that the lines remained linear over the whole range of L_{20} concentrations. MLCKase concentration was 60 nM and 90 nM respectively for (a) and (b) and a 2-fold molar excess of CM was added in all assays.

Fig. 2. Relationship between initial phosphorylation rate and the L_{20} light chain concentration for increasing TFP concentrations

The hyperbolic relationship seen in the absence of TFP ($0 \mu\text{M}$) changed to a sigmoidal type at high TFP concentrations ($50 \mu\text{M}$ and $150 \mu\text{M}$), consistent with a substrate-depletion mechanism of inhibition. MLCKase concentration was 15 nM .

chain concentrations, the inhibition patterns were complex and did not show a simple type of inhibition.

As is apparent from Fig. 1, both antagonists exhibited a combination of uncompetitive and competitive types of inhibition. There was, however, an interesting difference in the character of their transition from uncompetitive to competitive inhibition. For CAL (Fig. 1b) the plots remained linear over a wide concentration range, whereas for TFP (Fig. 1a) the corresponding traces were bent and remained 'uncompetitive' at low light chain concentrations.

The reason for the observed non-linearity in the double-reciprocal plots for TFP could be seen more clearly in normal v -versus- $[S]$ curves obtained at different TFP concentrations (Fig. 2). At low TFP concentrations ($25 \mu\text{M}$) the curve was hyperbolic, as it was in the absence of inhibitor ($0 \mu\text{M}$). At higher TFP concentrations (50 and $150 \mu\text{M}$) the relationship changed to a sigmoidal type, and could indicate inhibition via a substrate-depletion mechanism (Segel, 1975).

Filamentous myosin: first-order approach

As established earlier (Sobieszek, 1985), in the filamentous myosin system the phosphorylation reaction appears to be first-order, irrespective of the substrate concentration. By measuring the progress of myosin phosphorylation with time, at different antagonist concentrations, it was therefore possible to see whether inhibition was related to CM and/or to the myosin

phosphorylation site. If inhibition operates via CM this should be revealed by a decrease in the apparent first-order rate constant of the phosphorylation reaction. Alternatively, any inhibition at the myosin phosphorylation site should diminish the final extent of myosin phosphorylation since less myosin would be available for phosphorylation.

Smooth-muscle myosin purified via filament assembly has the additional advantage that it retains endogenous MLCKase and CM. Therefore it provides an ideal system for testing the inhibitory action of CM antagonists under conditions that resemble the situation *in vivo* more closely than the isolated light chain system. The amounts of MLCKase and CM relative to myosin in such preparations were established from measurements of the phosphorylation rates before and after addition of known amounts of CM or MLCKase. In fresh preparations there were approximately stoichiometric amounts of endogenous CM and MLCKase but always with some excess of CM. The molar ratio of myosin to CM was rather variable and depended on the type of myosin preparation. The established ratio ranged from 30 to 200 molecules of myosin per molecule of CM.

Figs. 3(a) and 3(b) demonstrate the inhibitory effects of CAL and TFP analysed in the above manner. The consecutive panels in Figs. 3(a) and 3(b) represent phosphorylation progress curves obtained at increasing concentrations of antagonists. The data are presented in the form of semi-logarithmic plots, which give directly

Fig. 3. Inhibitory effects of TFP (a) and CAL (b) on the phosphorylation of filamentous myosin containing endogenous MLCKase and CM

The successive plots of $\ln([M]_0 - [M])$ versus time were derived from the phosphorylation progress curves for increasing TFP or CAL concentrations given at the top of each panel. $([M]_0 - [M])$ is the concentration of phosphorylatable myosin (in c.p.m.) remaining at the given time. Note that, with increasing antagonist concentrations, not only k (slope) but also $[M]_0$ become progressively smaller. The total myosin concentration was constant and equal to $34 \mu\text{M}$.

the apparent first-order rate constant ($k_1 = \text{slope}$) and the effective initial concentration of the substrate ($[M]_0 = \text{intercept}$). The slope reflects the initial rate and the initial concentration equals that of 'uninhibited' myosin.

As is clear from Fig. 3, neither antagonist acted exclusively via calmodulin, since they not only inhibited the phosphorylation rate but also decreased the effective myosin concentration. This is seen as a gradual decrease in the intercept value. Such a decrease in the number of myosin phosphorylatable sites could not be detected from simple initial-rate measurements and took place for TFP approximately within or above the range, and for CAL a little below the range, of the myosin head molar concentration.

The interaction with CM, reflected by a decrease in the slopes, indicated for CAL (Fig. 3b) that rate inhibition occurred before a significant decrease in the extent of phosphorylation. In contrast, TFP both inhibited the rate and decreased the extent of phosphorylation simultaneously (Fig. 3a).

Removal of inhibition

More illustrative data demonstrating the specificity of the antagonist used came from experiments in which the effectiveness of CM and purified L_{20} light chain on the abolition of CAL and TFP inhibition was tested. For filamentous myosin CAL inhibition was readily removed by CM (Fig. 4), when added in amounts severalfold higher than those already present in myosin (see above). In the same system, removal of TFP inhibition required higher concentrations of excess CM. The additional CM

acted in the L_{20} light chain system in a manner similar to that observed for myosin (Fig. 5). It was rather ineffective in removal of TFP inhibition (Fig. 5c), whereas for CAL an increase in CM resulted in an increase of the initial slope of the inhibition curve and removal of inhibition was almost complete (Fig. 5b).

The ineffectiveness of CM in the removal of TFP inhibition was consistent with the sigmoidal velocity relationship shown in Fig. 2. This suggested that, for the higher range of concentrations used, TFP interacted with the substrate (myosin phosphorylation sites) to inhibit the phosphorylation rate. This conclusion was reinforced by a similar experiment with addition of the isolated L_{20} light chain. Thus TFP inhibition could be readily blocked by adding L_{20} light chain in a 2–3-fold molar excess (Fig. 6). Inhibition by CAL in the same system was unaffected by excess L_{20} light chain (Fig. 5a). Since endogenous MLCKase present in myosin could readily phosphorylate added L_{20} light chain, the latter type of experiments were only practicable for the L_{20} system (there the extents of ^{32}P incorporation due to myosin were severalfold lower than those of added L_{20} light chain). In addition, the V_{max} conditions necessary for this type of experiment were also readily obtained only for the L_{20} system.

Ca^{2+} -independent inhibition

Limited proteolysis of myosin, containing endogenous MLCKase and CM, with chymotrypsin produced heavy meromyosin that retained MLCKase after ion-exchange chromatography. Heavy meromyosin prepared in this way could be phosphorylated in the absence of Ca^{2+} (2 mM-EGTA) at rates about 10 times lower than for the

Fig. 4. Removal of CAL inhibition by addition of increasing amounts of CM

Note that, for filamentous myosin at a concentration of $32 \mu\text{M}$, the presence of $4.5 \mu\text{M}$ -CM restored the phosphorylation rates to approximately normal in the presence of CAL.

Fig. 5. Removal of inhibition of CAL and TFP by added L_{20} light chain and calmodulin in the L_{20} system

A 2.5-fold increase in the L_{20} concentration (Δ in *a*) did not affect the phosphorylation rate ($\bullet$ in *a*). CM was a more effective antagonist of CAL inhibition (*b*), but was essentially ineffective with TFP (*c*). MLCKase concentration was 115 nM.

Fig. 6. Removal of TFP inhibition by the L_{20} light chain

Note that the TFP inhibition was practically abolished by a 2.5-fold increase in the light chain concentration (given at the top of each panel). MLCKase concentration was 115 nM.

corresponding parent myosin. Such heavy meromyosin preparations were used to assess CM-independent effects of the inhibitors under study. (Since the inhibitors react with CM only in the presence of Ca^{2+} , the possible

presence of any residual CM in the heavy meromyosin preparations could be disregarded.) Consistent with the above proposed mechanisms, TFP inhibited the Ca^{2+} -independent phosphorylation of heavy meromyosin

Fig. 7. CM-independent inhibition of heavy meromyosin phosphorylation by TFP (●) and CAL (○)

Note that at their normally effective concentrations only TFP inhibited phosphorylation in the absence of Ca^{2+} . Heavy meromyosin concentration was $42 \mu\text{M}$; for further details see the text.

equally effectively as that of myosin, a comparable inhibition requiring only about a 2-fold higher concentration of TFP (Fig. 7). From this Figure it is also evident that CAL was equally effective in the absence of Ca^{2+} in this heavy meromyosin system, although it was ineffective at concentrations that inhibited filamentous myosin (compare Figs. 7 and 10). The sigmoidal form of the inhibition curves suggests that neither TFP nor CAL acted at the level of CM-independent MLCKase.

Alternative analysis of kinetic data in accordance with Reiner (1969)

A simpler but less conventional analysis of inhibition data has been offered by Reiner (1969), who suggests the use of the inverse function of inhibition $\{[I]_t = f(u)\}$, where $[I]_t$ is the total inhibitor concentration added and u is a simple parameter of fractional inhibition ($u = v_0/v_i - 1$). Experimentally, the approach requires measurement of initial phosphorylation rates as a function of antagonist concentration as for ' v_i -versus- $[I]_t$ ' inhibition curves commonly used to evaluate I_{50} , an ambiguous parameter characterizing the inhibitor in a defined assay system. As demonstrated below, Reiner's replots of $[I]_t$ versus u yield more information.

As is apparent from Figs. 8–10, the simpler (v_i -versus- $[I]_t$) TFP and CAL inhibition curves appeared rather similar, disregarding the different ranges of antagonist required to produce a comparable inhibition. The difference between the inhibition by CAL and TFP became more obvious when these data were replotted as

Fig. 8. Simple inhibition curves (●) and corresponding Reiner $[I]_t$ -versus- u replots (○) for TFP (a) and CAL (b) inhibition of phosphorylation of L_{20} light chains (L_{20} system)

Note that the asymptote (broken line) of the Reiner's replot in (a) crossed the $[I]_t$ axis at a value corresponding to the substrate concentration, indicating a substrate-depletion type of inhibition. For CAL the replot was inflected downwards so that its asymptote crossed the $[I]_t$ axis at a negative value, indicating that the substrate (L_{20}) could also effectively displace the inhibitor from the CM-MLCKase complex. (For convenience Reiner's replots were drawn here using the same $[I]_t$ axis as for the inhibitor curves. Thus the x and y co-ordinates are inverted in comparison to the original Reiner form.) The MLCKase and L_{20} concentrations in (a) and (b) were respectively 110 nM and 190 nM .

Fig. 9. TFP inhibition curves for phosphorylation of filamentous myosin

In (a) and (b) the inhibition curves were obtained from single-point rate measurements whereas for (c) the rates were calculated from the phosphorylation progress curves. The substrate concentration was $11 \mu M$ in (a), $24 \mu M$ in (b) and $32 \mu M$ in (c). The asymptote intercept of the Reiner's replots [drawn for (a) and (b)] indicated that between one and two TFP molecules were bound per myosin head.

$[I]_i$ versus u . For the L_{20} system, the TFP inhibition points fitted a hyperbola-like curve with an asymptote crossing the $[I]_i$ axis at a value equal to the substrate concentration (Fig. 8a). According to Reiner this indicates that TFP acts by depleting substrate with a 1:1 binding stoichiometry.

The $[I]_i$ -versus- u plots obtained for CAL were more simple and could normally be approximated to a straight line crossing the co-ordinate origin. This line was, however, often inflected downwards, so that its asymptote crossed the CAL concentration axis below zero (Fig. 8b, broken line). Therefore the amount of bound CAL in

these experiments was negligible in relation to the total L_{20} concentration, and the observed plots were indicative of inhibition of MLCKase by CM-CAL or CM-CAL- L_{20} complexes. In this case the activator-antagonist complex (CAL-CM) and not the antagonist alone was the actual inhibitor. The observed downward inflexion suggests, in addition, that at high L_{20} concentration the L_{20} displaced the inhibitor (CM-CAL or CM-CAL- L_{20}) from MLCKase.

$[I]_i$ -versus- u plots were also obtained for the inhibition of phosphorylation of filamentous myosin. In the case of TFP (Fig. 9) they were of the same sigmoidal shape as

Fig. 10. CAL inhibition of phosphorylation of the filamentous myosin

The Reiner's replots (●) yield an overestimate of the CM content (broken-line asymptote on $[I]_i$ axis) of myosin as a result of some direct binding of CAL to myosin (see the text). In (b) the initial rates were calculated from the phosphorylation progress curves, whereas for (a) they were obtained from single-point rate measurements. Myosin concentration was $45 \mu\text{M}$ in (a) and $32 \mu\text{M}$ in (b).

those for the isolated light chain, with the asymptote intercept occurring in the range of myosin head concentration. In contrast, the same plots for the CAL inhibition of myosin filaments were different from those obtained with the L_{20} system. As shown in Fig. 10, the plots for myosin filaments more closely resembled the TFP curves, with the difference that the asymptote crossed the $[I]_i$ axis at concentration values about 2–4 times higher than that of CM present in myosin. Since each CM molecule binds at least two CAL molecules (Mills *et al.*, 1985), one can suggest, in agreement with Reiner (1969), that CAL inhibited myosin phosphorylation by depleting free CM. A direct inhibition of the CM–MLCKase complex, as suggested by the experiments with the isolated L_{20} light chain, could represent an independent inhibitory pathway.

DISCUSSION

CM antagonists are employed not only as tools for the study of CM-regulated enzymes (see Roufogalis, 1985) but also in studies of CM-dependent systems *in vivo*, including smooth-muscle tissue (Sheterline, 1980; Silver *et al.*, 1986; Tanaka *et al.*, 1986). Their further applications in pharmacology have, however, been restricted owing to a lack of understanding of the kinetic mechanisms of their action. Only recently was an attempt made to classify these drugs according to their specificity for two CM-dependent enzymes, namely MLCKase and phosphodiesterase, both from bovine heart (Zimmer & Hofmann, 1987). These authors proposed, contrary to an earlier conclusion (Zimmer & Hoffman, 1984), that this class of drugs acts exclusively on CM. Other reports claim, however, that the inhibition may not exclusively occur via interaction with CM but may involve interaction with the respective enzyme (Mannhold, 1984).

The results presented here are a first attempt to elucidate the kinetic mechanism of inhibition by CM antagonists. Smooth-muscle MLCKase was chosen as a test system for three reasons. First, this enzyme can be readily purified. Secondly, its activity is totally dependent on the presence of CM and Ca^{2+} . Thirdly, it is tightly associated with filamentous myosin, so that kinetic experiments can be carried out in two systems *in vitro*, one composed of the isolated myosin light chain and purified MLCKase and the other comprising filamentous myosin and endogenous MLCKase and CM. The latter system may be considered to resemble the situation *in vivo*.

It is concluded that TFP inhibits MLCKase-dependent phosphorylation by depleting activator (CM) as well as substrate (phosphorylatable light chain or intact myosin). Thus TFP appears to interact with free CM, preventing formation of the active CM–MLCKase complexes and independently with the myosin (or L_{20}) phosphorylation site, inhibiting its phosphorylation. The relative contributions of these two independent inhibitory pathways could not be evaluated, since this would require independent determinations of the respective dissociation constants.

A similar CM-independent interaction of CAL with the phosphorylation site was also observed, but at CAL concentrations required for inhibition this interaction did not inhibit myosin phosphorylation. CAL inhibited the phosphorylation more effectively by depleting CM and inhibiting the CM–MLCKase complex (activator-combination-type mechanism). As shown also by Van Belle (1981), this is consistent with a much greater affinity of CM for CAL than for TFP. In the MLCKase system CAL produced an inhibition at 10–20 fold lower concentrations than required for TFP, implying a similar difference in the respective CM-related dissociation constants for these drugs. This difference is, however, lower

than the 1000-fold value indicated by fluorescence measurements by Johnson & Wittenauer (1983), which gave dissociation constants of 3 nM (CAL) and 5 μ M (TFP). The reason for this difference is unclear at present and can only be settled by more direct binding studies.

An interaction of CM antagonists with the myosin phosphorylation site implies that they interact directly with the L₂₀ light chain, consistent with the fluorescence measurements made by Zimmer & Hofmann (1984). From the present study it appears that this interaction does not lead to a complete CM-independent inhibition of MLCKase activity, as concluded by these authors. A contribution of this inhibitory pathway appeared to be significant only in the case of TFP. Furthermore, in the L₂₀ system the antagonist–light chain interaction was not always of the same kinetic type. For TFP, the L₂₀–TFP complex was apparently not bound to MLCKase, whereas for CAL binding did occur, resulting in different inhibition curves (compare Figs. 8a and 8b).

The modes of interaction of CAL and TFP with CM also appear to differ. For example their binding to dansylated CM results in opposite changes of the fluorescence (Johnson & Wittenauer, 1983). More significantly, binding of CAL to one drug-binding domain allosterically potentiates the affinity of the other (Mills *et al.*, 1985). It is therefore not surprising that CAL inhibited MLCKase via interaction with the CM–MLCKase complex as well as by depleting free CM. In contrast with the action of TFP, this interaction appeared not to lead to dissociation of the enzyme–activator complex, since then interaction with dissociation would be indistinguishable from a simple depletion. I suggest, in accordance with Mills *et al.* (1985), that at least one drug-binding site of CM is available for CAL and that its binding stabilizes the inactive CM–MLCKase complex. This type of inhibition, characterized by a simple hyperbolic inhibition curve, was shown to predominate in the isolated L₂₀ light chain system (Fig. 8b). The CAL inhibition curves obtained for filamentous myosin were sigmoidal, demonstrating a significant contribution of the depletion mechanism. However, Reiner plots obtained for the latter system (Fig. 10), taken together with the estimations of CM content of myosin, indicated that more than two molecules of CAL were bound per molecule of CM. This is inconsistent with the existence of only two hydrophobic binding sites (see Johnson & Mills, 1986), but may result from some binding of CAL by myosin, usually present in a large molar excess over CM.

The significance of the present study hinges on the demonstration of different inhibitory mechanisms for TFP and CAL in filamentous myosin. At the same time I have documented the ranges of antagonist concentrations at which these mechanisms predominate. This type of data should prove useful for clarifying the molecular aspects of Ca²⁺-dependent regulation of MLCKase and other CM-dependent enzymes.

I thank Dr. J. V. Small for revision of the manuscript and Dr. R. A. Cross for comments. The technical assistance of Ms. U. Mueller and Mrs. A. Jankela as well as the general assistance of Ms. E. Hofer are also acknowledged. These studies were supported by a grant from the Muscular Dystrophy Association Inc.

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