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Mechanism for the Inhibition of Acto-Heavy Meromyosin ATPase by the Actin/Calmodulin Binding Domain of Caldesmon†

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ABSTRACT: Caldesmon, an actin/calmodulin binding protein, inhibits acto-heavy meromyosin (HMM) ATPase, while it increases the binding of HMM to actin, presumably mediated through an interaction between the myosin subfragment 2 region of HMM and caldesmon, which is bound to actin. In order to study the mechanism for the inhibition of acto-HMM ATPase, we utilized the chymotryptic fragment of caldesmon (38-kDa fragment), which possesses the actin/calmodulin binding region but lacks the myosin binding portion. The 38-kDa fragment inhibits the actin-activated HMM ATPase to the same extent as does the intact caldesmon molecule. In the absence of tropomyosin, the 38-kDa fragment decreased the K_{ATPase} and $K_{binding}$ without any effect on the V_{max}. However, when the actin filament contained bound tropomyosin, the caldesmon fragment caused a 2-3-fold decrease in the V_{max}, in addition to lowering the K_{ATPase} and the $K_{binding}$. The 38-kDa fragment-induced inhibition is partially reversed by calmodulin at a 10:1 molar ratio to caldesmon fragment; the reversal was more remarkable in 100 mM ionic strength at 37 °C than in 20 or 50 mM at 25 °C. Results from these experiments demonstrate that the 38-kDa domain of caldesmon inhibits the binding of myosin head to actin; however, when the actin filament contains bound tropomyosin, caldesmon fragment affects not only the binding of HMM to actin but also the catalytic step in the ATPase cycle. The interaction between the 38-kDa domain of caldesmon and tropomyosin-actin is likely to play a role in the regulation of actomyosin ATPase and contraction in smooth muscle.

Regulation of actomyosin ATPase and force development in smooth muscle are myosin-mediated, through the Ca^{2+}-calmodulin-dependent myosin light chain phosphorylation [Gorecka et al., 1976; Sobieszek & Small, 1977; Chacko et al., 1977; Dillon et al., 1981; Butler & Siegman, 1982; for a review, see Kamm and Stull (1985)]. There is also evidence for the existence of a thin filament mediated regulation through the interaction of proteins associated with the thin filament [for reviews, see Marston et al. (1985) and Chacko et al.

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(1987)]. Tropomyosin, a component of the thin filament in smooth muscle, causes a 2–3-fold increase in actin-activated ATP hydrolysis by phosphorylated smooth muscle myosin (Chacko et al., 1977; Chacko, 1981; Small & Sobieszek, 1977). The potentiation of actin-activated ATP hydrolysis by tropomyosin is caused by a rise in the V_{max} with slight increases in $K_{binding}$ and K_{ATPase} (Chacko & Eisenberg, 1990).

Caldesmon, another component of the thin filament in smooth muscle cell (Kakiuchi et al., 1983; Bretscher & Lynch, 1985; Lehman et al., 1989), inhibits actin-activated ATP hydrolysis by myosin; this inhibition is released by Ca^{2+}–calmodulin (Marston et al., 1985; Sobue et al., 1985; Dabrowska et al., 1985; Horiuchi et al., 1986; Ngai & Walsh, 1984). The caldesmon-induced inhibition of actin-activated ATPase is amplified by tropomyosin (Marston et al., 1985; Sobue et al., 1985; Dabrowska et al., 1985; Horiuchi et al., 1986). It has also been shown that caldesmon and tropomyosin, which are bound to the actin filament, interact with each other causing conformational changes in both tropomyosin and caldesmon (Horiuchi & Chacko, 1988; Dobrowolski et al., 1988). Despite the inhibition of the actin-activated ATPase associated with the interaction between tropomyosin and caldesmon, the exact mechanism for the inhibition of ATPase is not known.

The region of the caldesmon responsible for the inhibition of actin-activated ATPase of myosin is near the C-terminus (Szpacenko & Dabrowska, 1986; Fujii et al., 1987; Yazawa et al., 1987; Ball & Kovala, 1988; Mornet et al., 1988; Bryan et al., 1989; Katayama et al., 1989). However, the portion of the caldesmon molecule close to the N-terminal region interacts with smooth muscle myosin, specifically with the subfragment 2 region (Katayama et al., 1989; Ikebe & Reardon, 1988; Sutherland & Walsh, 1989). This interaction increases the binding of heavy meromyosin (HMM)[1] to actin; however, this binding is "non-productive" with respect to ATP hydrolysis (Hemric & Chalovich, 1988). Therefore, using the intact caldesmon, it is difficult to distinguish the binding of myosin heads to actin, which is associated with the ATPase cycle, from the "non-productive" binding of HMM to actin, mediated through the myosin binding domain of the caldesmon.

Utilizing a 38-kDa chymotryptic fragment of caldesmon which possesses the actin/calmodulin binding domain but is devoid of the subfragment 2 binding region, the "non-productive" binding of HMM can be distinguished from the binding associated with the ATP hydrolysis (Horiuchi & Chacko, 1989). Moreover, unlike the intact molecule, the 38-kDa fragment inhibits both the acto–HMM ATPase and the binding of HMM to actin (Horiuchi & Chacko, 1989; Velaz et al., 1990). In the absence of tropomyosin, the 38-kDa fragment-induced inhibition of ATPase is correlated with an inhibition of the binding of HMM to actin, while in its presence, the ATPase decreases more rapidly than does the binding. These data suggest that, in the presence of tropomyosin, the 38-kDa fragment modulates not only the binding of myosin heads to actin but also the rate of ATP hydrolysis. Velaz et al. (1989) report that the mechanism for the inhibition of actin-activated HMM ATPase by intact caldesmon in the presence of tropomyosin is different from that in its absence; however, the level of binding of myosin heads to actin has not been determined under this condition.

In this study, we utilize the 38-kDa proteolytic fragment to study the exact mechanism for the inhibition of acto–HMM ATPase by caldesmon. We demonstrate that this fragment decreases the K_{ATPase} (the apparent binding constant of HMM to actin, obtained from the double-reciprocal plot of ATPase versus actin concentration) and the $K_{binding}$ (the binding constant of HMM to actin in the presence of ATP) without any effect on the V_{max}. However, in the presence of tropomyosin, the caldesmon fragment causes a 2–3-fold decrease in the V_{max} as well as decreases in the K_{ATPase} and the $K_{binding}$. Results from these experiments show that the binding of myosin heads to actin and the rate of actin-activated MgATPase of HMM are modulated by both tropomyosin and caldesmon bound to the actin filament.

Materials and Methods

Chicken gizzard myosin, actin, and tropomyosin were prepared as described (Chacko, 1981; Heaslip & Chacko, 1985). The HMM was made from phosphorylated chicken gizzard myosin according to Kaminski and Chacko (1984), and was free of phosphatase and kinase activities. The level of phosphorylation was found to be 98–100% on urea gel electrophoresis (Perrie & Perry, 1970). Chicken gizzard caldesmon prepared from heated muscle extract (Horiuchi & Chacko, 1988) was subjected to chymotryptic digestion, and the 38-kDa fragment from the digest was purified as described (Horiuchi & Chacko, 1989). Bovine brain calmodulin was prepared as described (Dedman & Kaetzel, 1983) or purchased from Sigma.

Conditions for the ATPase assay are given in the figure legends. Actin or actin containing tropomyosin (molar ratio of actin to tropomyosin, 6:1) was mixed with the 38-kDa fragment in reaction tubes, in either the presence or the absence of calmodulin. The level of binding of the 38-kDa fragment to actin or tropomyosin–actin, under the experimental condition used for HMM binding and for the ATPase assay, was determined by SDS gel electrophoresis of the pellet after centrifugation. More than 90% of the added fragment bound to actin and tropomyosin–actin at a 1:20 molar ratio of 38-kDa to actin. After incubation of the reaction mixture for ATPase assay for 15 min at 25 or 37 °C, aliquots of 0.15 mL were transferred into airfuge tubes and used for the binding assay. The remaining portion (0.5 mL) was utilized for the ATPase assay. Samples for the ATPase assay were mixed with Mg[γ-^{32}P]ATP, and the assay was initiated by adding the HMM. Aliquots of 0.1 mL were removed at 0 time, 3 min, and 6 min, and the inorganic phosphate (^{32}P) liberated from [γ-^{32}P]ATP was determined according to Martin and Doty (1949) with a slight modification (Chacko & Eisenberg, 1990).

For the binding experiments, HMM made from chicken gizzard myosin phosphorylated with [γ-^{32}P]ATP was utilized. Prior to use, the phosphorylated HMM (containing covalently bound ^{32}P) was centrifuged (130000g for 20 min), and the pellet (less than 10% of the HMM) was discarded. The supernatant containing soluble HMM was used for the binding experiments. Aliquots (0.15 mL) taken from the ATPase assay mixture, containing all the other proteins except for the HMM, were gently mixed with 4 mM MgATP followed by ^{32}P-HMM. The ATP concentration was adjusted to ensure a sufficient amount of unhydrolyzed nucleotide in the assay mixture for 30 min. After being mixed, the samples were immediately centrifuged (130000g) for 20 min either in an Airfuge (Beckman) at 25 °C or in a Beckman TL-100 at 37 °C. The supernatant and the pellet were transferred separately into vials and counted in a liquid scintillation counter. The free HMM and bound HMM were estimated from radioac-

[1] Abbreviations: HMM, heavy meromyosin; S1, myosin subfragment 1; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

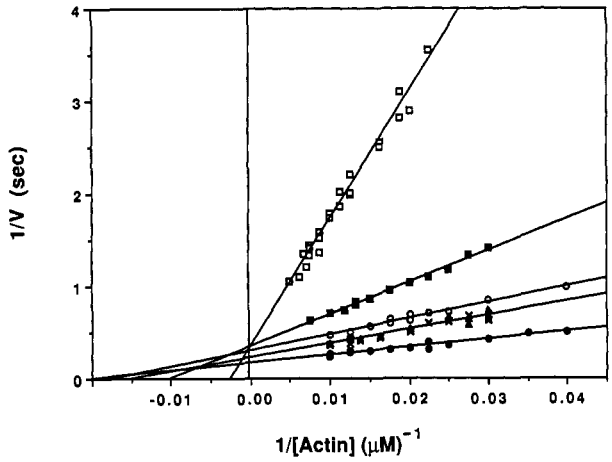

FIGURE 1: Double-reciprocal plots of the actin-activated ATPase of phosphorylated HMM versus actin concentration. Conditions for the assay are 20 mM ionic strength (adjusted by KCl), 3 mM free Mg^{2+}, 0.1 mM $CaCl_2$, 2.3 mM Mg[γ-^{32}P]ATP, 10 mM imidazole (pH 7.0), and 2.5 mM DTT at 25 °C. The HMM concentration is 0.33 μM, and actin concentrations are increased as indicated. The molar ratios of tropomyosin or calmodulin to actin are 1:6 or 1:2 (38-kDa:calmodulin ratio = 1:10), respectively. (●) Tropomyosin–actin; (▲) tropomyosin–actin + 38-kDa (20:1 = actin:38-kDa ratio); (×) tropomyosin–actin + 38-kDa + calmodulin (20:1:10); (■) tropomyosin–actin + 38-kDa (10:1); (○) actin; (□) actin + 38-kDa (10:1).

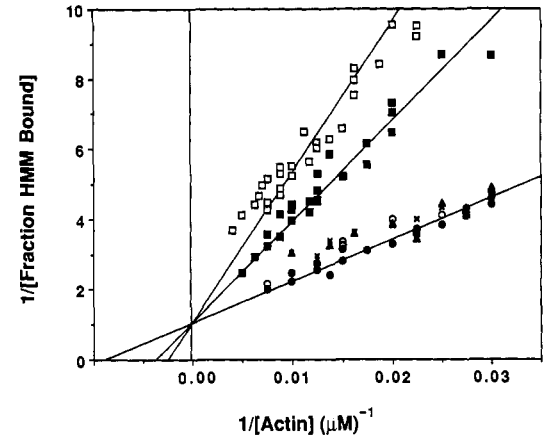

FIGURE 2: Double-reciprocal plots of HMM binding to actin. Conditions and symbols are the same as in Figure 1 except that the concentration of MgATP is 4 mM. The solid lines drawn through data points were obtained by fitting the data to a linear regression in which the ordinate intercept is 1 (100% binding).

tivity in the supernatant and pellet, respectively. The HMM alone in the binding buffer served as a control.

The concentration of protein in 38-kDa fragment preparations was determined by the method of Lowry et al. (1951). Concentrations of other proteins were determined spectrophotometrically using E_{280} = 0.647 (mg/mL)$^{-1}$ cm^{-1} for gizzard HMM, E_{290} = 0.638 (mg/mL)$^{-1}$ cm^{-1} for gizzard actin, and E_{277} = 0.19 (mg/mL)$^{-1}$ cm^{-1} for gizzard tropomyosin. The molecular weight of the caldesmon fragment was assumed to be 38 000, although the real molecular weight may be lower (Graceffa et al., 1988; Leszyk et al., 1989).

Results

The effect of the 38-kDa caldesmon fragment on the actin-activated ATP hydrolysis by phosphorylated HMM in the presence or absence of bound tropomyosin is depicted in Figure 1. The steady-state ATPase rates versus actin concentrations in 20 mM ionic strength and at 25 °C are plotted as double reciprocals. In the absence of the 38-kDa caldesmon fragment, the V_{max} and the K_{ATPase} were 3.3 s^{-1} and 1.72 × 10^4 M^{-1} for actin, and 5.8 s^{-1} and 2.07 × 10^4 M^{-1} for tropomyosin–actin, respectively. These values are in agreement with previous data (Chacko & Eisenberg, 1990). In order to study the effect of the 38-kDa fragment on ATP hydrolysis, this fragment was added to actin or tropomyosin–actin at a fragment to actin ratio of 1:10; at this ratio, the inhibition by caldesmon is close to maximum (Horiuchi & Chacko, 1989). In the absence of tropomyosin, the K_{ATPase} was decreased 6-fold by the 38-kDa fragment, but there was almost no effect on the V_{max}. When tropomyosin was bound to actin, the addition of 38-kDa fragment decreased both the V_{max} and the K_{ATPase} 2-fold.

Since the stoichiometry of caldesmon on the actin filament in the intact cell is thought to be between 1:28 to 1:14 (Marston & Lehman, 1985; Graceffa et al., 1988), experiments were also carried out at a 38-kDa fragment to actin molar ratio of 1:20. At this ratio, the inhibition of ATPase is half-maximal (Horiuchi & Chacko, 1989), and it is likely that the actin or the tropomyosin–actin filaments are saturated partially with 38-kDa fragment. Results from these experiments are also shown in Figure 1. At the 1:20 ratio of 38-kDa fragment to tropomyosin–actin, the 38-kDa fragment decreased the K_{ATPase} about 30%. The V_{max} was also reduced by about 30%. Under this condition, the reversal of the caldesmon-induced inhibition by calmodulin was less than 10% (molar ratio of 38-kDa fragment to actin to calmodulin, 1:20:10).

The binding of HMM to actin was determined parallel to the ATPase assay to see if the effects of 38-kDa fragment on the kinetic steps of ATP hydrolysis are associated with a difference in the binding constant for HMM to actin. Double-reciprocal plots of the HMM binding to actin or to tropomyosin–actin versus the actin concentration, under the same conditions as in Figure 1, are shown in Figure 2. At a 1:10 molar ratio of 38-kDa fragment to actin, the $K_{binding}$ was lowered from 0.83 × 10^4 to 0.23 × 10^4 and to 0.33 × 10^4 M^{-1} in the absence and the presence of tropomyosin, respectively. When the molar ratio of 38-kDa fragment to actin was lowered to 1:20 in 20 mM ionic strength, the $K_{binding}$ of HMM to tropomyosin–actin–38-kDa fragment obtained from the linear regression curve of data was very close to the binding constant of HMM to actin and tropomyosin–actin (around 0.83 × 10^4 M^{-1}), although the 38-kDa fragment slightly inhibited the binding of HMM to actin in some experiments.

It is likely that the lack of a notable inhibition as shown by the binding constants in a low ratio of caldesmon fragment to actin (1:20 molar ratio), despite an effect on the K_{ATPase} and the V_{max}, is due to the binding of both heads of the HMM to actin and tropomyosin–actin in low ionic strength (Greene & Eisenberg, 1980; Chacko & Eisenberg, 1990). The HMM binding study using the sedimentation technique would not distinguish the double-head binding from the single-head binding since the binding of one head to actin would bring both heads to sediment with actin. Even a 50% inhibition of binding would not be evident from the sedimentation studies. In order to see if this phenomenon contributes to the lack of an effect on the $K_{binding}$ at a low ratio of caldesmon fragment, the ionic strength was raised from 20 to 50 mM to minimize the double-head binding, and the experiments in Figures 1 and 2 were repeated. At a 1:20 molar ratio of 38-kDa fragment to actin, the $K_{binding}$ decreased from 0.74 × 10^4 to 0.38 × 10^4 M^{-1} for tropomyosin–actin and from 0.46 × 10^4 to 0.32 × 10^4 M^{-1} for actin. At a 1:10 molar ratio of 38-kDa fragment to actin, the $K_{binding}$ decreased from 0.74 × 10^4 to 0.19 × 10^4 M^{-1} for tropomyosin–actin and from 0.46 × 10^4 to 0.095 × 10^4 M^{-1} for actin (Table I).

Table I: Binding and Kinetic Constants Obtained from Figures[a]

	$K_{binding}$[b] × 10^{-4} (M^{-1})			K_{ATPase}[c] × 10^{-4} (M^{-1})			V_{max}[c] (s^{-1})		
IS (mM)	20	50	100	20	50	100	20	50	100
temp (°C)	25	25	37	25	25	37	25	25	37
Tm-A[d]	0.83	0.74	0.75	2.07	0.87	1.00	5.83	5.38	8.73
Tm-A + 38K (20:1)	0.83	0.38	0.44	1.51	0.73	0.73	4.36	3.23	3.73
Tm-A + 38K + CaM(20:1:10)	0.83	0.41	0.54	1.55	0.81	0.98	4.92	3.20	3.83
Tm-A + 38K (10:1)	0.33	0.19		1.02	0.43		2.86	2.25	
Tm-A + 38K (6:1)				0.72			0.40		
A	0.83	0.46	0.42	1.72	0.47	0.65	3.32	3.15	3.53
A + 38K (20:1)		0.32	0.29		0.32	0.34		3.20	3.83
A + 38K (10:1)	0.23	0.095		0.29	0.12		3.04	3.30	
A + 38K (6:1)				0.04			3.00		

[a] Each constant was estimated from the intercepts of double-reciprocal plots as determined by linear regression analysis of 15–30 data points for each condition. Data were obtained from a total of 10 preparations of HMM. [b] Conditions: same as in Figure 2 except ionic strength and temperature as indicated. [c] Conditions: same as in Figure 1 except ionic strength and temperature as indicated. [d] Abbreviations: Tm-A, tropomyosin-actin; A, actin; 38K, 38-kDa fragment; CaM, calmodulin.

In general, the effect of 38-kDa fragment on ATPase activity at 50 mM ionic strength was the same as that at 20 mM, although the K_{ATPase} was lower. At a 1:10 ratio of 38-kDa fragment to actin in the absence of tropomyosin, the 38-kDa fragment decreased the K_{ATPase} 4-fold without any effect on V_{max}. At the same ratio in the presence of tropomyosin, the V_{max} was decreased 2.5-fold in addition to a 2-fold decrease in K_{ATPase}. At a 1:20 ratio, the effects of the 38-kDa fragment are comparable, although weaker than those observed at a 1:10 ratio (Table I).

Addition of calmodulin (molar ratios of calmodulin to actin to 38-kDa fragment, 10:20:1) did not cause a significant reversal of the caldesmon-induced inhibition of the binding and ATPase in 20 mM ionic strength. The reversal was slightly higher (about 15%) in 50 mM ionic strength. In view of the finding that the reversal of caldesmon-induced inhibition of ATPase by calmodulin is more evident at 37 °C in 70–100 mM KCl than at 25 °C in lower ionic strengths (Pritchard & Marston, 1989), the experiments shown in Figure 1 (ATPase) and Figure 2 (binding) were repeated at 37 °C and in 100 mM ionic strength using a 1:20 ratio of 38-kDa fragment to actin in order to see if the reversal of inhibition by calmodulin was increased. The viscosity of the protein mixture, especially in the presence of the 38-kDa fragment, increased on raising the temperature under these conditions. It was difficult to get reliable data from experiments at high concentration of actin mixed with 38-kDa fragment at a 1:10 ratio due to high viscosity of the mixture. At a 1:20 ratio, the 38-kDa fragment caused a 2-fold decrease in the K_{ATPase} and $K_{binding}$ without any change in V_{max} when actin alone was used (data shown in Table I). In the presence of tropomyosin, the V_{max} decreased 2.5-fold, and $K_{binding}$ decreased 40% with a 30% decrease in K_{ATPase}. The reversal of the inhibition by calmodulin was about 25%, slightly greater than that in 20 or 50 mM ionic strength.

The binding and kinetic constants obtained from double-reciprocal plots are summarized in Table I. In the absence of tropomyosin, $K_{binding}$ and K_{ATPase} are decreased without any effect on V_{max}. In contrast, in the presence of tropomyosin, V_{max} was decreased in addition to the inhibition of both $K_{binding}$ and K_{ATPase}. The ratio of the 38-kDa fragment to actin was increased to 1:6 to minimize the possibility for the existence of a small population of actin filament not saturated with 38-kDa fragment, and the ATPase was measured in 20 mM ionic strength at 25 °C (data shown in Table I). At a 1:6 ratio, caldesmon fragment inhibited the ATPase in the same manner as it did at the 1:10 ratio, although the effect was more pronounced. The $K_{binding}$ was too low to measure (less than 400 M^{-1}) in both cases. Further increase in the fragment ratio causes extremely high turbidity in the protein mixture, presumably due to the formation of the bundle of actin filament (Dabrowska et al., 1985).

Discussion

In this study, we utilized the actin/calmodulin binding fragment of caldesmon to eliminate the caldesmon-induced increase in the binding of HMM to actin. This enabled us to determine, without any interference by the "non-productive" binding of HMM to actin, if the actin binding domain of caldesmon has any effect either on the binding of HMM heads to actin or on the acto-HMM ATPase. Furthermore, all the proteins utilized for this study were isolated from smooth muscle tissue to rule out the possibility that proteins from different types of muscle may have different levels of affinity to each other, and thus diffrent levels of inhibition. Utilization of HMM enabled us to elucidate the effect of caldesmon on the interaction between actin and a double-headed, soluble fragment of the myosin molecule. Unlike the single-headed subfragment 1 (S1), the HMM is regulated by light-chain phosphorylation and is subjected to conformational changes in response to different ionic conditions and phosphorylation level (Suzuki et al., 1985; Ikebe & Hartshorne, 1985). In this study, the HMM was fully phosphorylated for maximal actin activation.

The effect of caldesmon on the acto-HMM ATPase in the presence of tropomyosin is different from that in its absence. In the absence of tropomyosin, the 38-kDa fragment does not alter the V_{max}, but it decreases the K_{ATPase} and $K_{binding}$. In 20 mM ionic strength, $K_{binding}$ was decreased 3.5-fold at a 1:10 ratio of fragment to actin, while K_{ATPase} was decreased 6-fold, almost 2-fold higher than the decrease in $K_{binding}$ (Table I). The $K_{binding}$ accounts for the physical binding of HMM to actin. The 2-fold difference between K_{ATPase} and $K_{binding}$ may be attributed to a change from two-head binding to single-head binding in the presence of caldesmon fragment, caused by a decrease in the affinity of HMM heads to actin. Since the binding of one head to actin causes HMM to sediment with actin, the binding experiment does not estimate the double-head binding. On increasing the ionic strength to 50 or 100 mM, the inhibition of $K_{binding}$ correlates with that of K_{ATPase}. Since the decrease in K_{ATPase} is associated with a decrease in the binding of HMM to actin ($K_{binding}$), the inhibition of ATPase in the absence of tropomyosin is likely to be caused by a reduction in the interaction of myosin heads with actin; this is presumably due to a change in conformation of the actin filament, induced by the 38-kDa fragment. In contrast, in the presence of tropomyosin, the 38-kDa fragment decreased the V_{max} by a maximum of 2–3-fold, in addition to lowering the

$K_{binding}$ and K_{ATPase}. The decreased V_{max} is close to that seen when the actin filament devoid of tropomyosin was used. Hence, it appears that the caldesmon fragment abolishes the 2–3-fold potentiation of the V_{max} produced by tropomyosin (Chacko & Eisenberg, 1990). However, when tropomyosin–actin was used to activate the HMM ATPase, the augmentation of the ATPase occurred due to an increase in the V_{max} with only a slight effect in the K_{ATPase} and $K_{binding}$ (Chacko & Eisenberg, 1990). In contrast, with tropomyosin–actin–caldesmon, not only the V_{max} but also the K_{ATPase} and $K_{binding}$ are affected. At a 1:10 ratio of fragment to tropomyosin–actin, diminutions in $K_{binding}$ and K_{ATPase} are 2–3-fold in both 20 and 50 mM ionic strengths. Raising the 38-kDa fragment to actin molar ratio from 1:10 to 1:6 shows the same effect. However, the affinity of HMM to actin is too low at the 1:6 ratio, making the measurements unreliable. Results in the presence of tropomyosin indicate that the caldesmon fragment affects not only the formation of cross-bridges but also a certain kinetic step altered by tropomyosin, presumably the P_i release step.

Interestingly, Bartegi et al. (1990) show that caldesmon inhibits acto–S1 ATPase; however, when S1 is cross-linked to actin in the absence of tropomyosin, the ATPase is not inhibited. This result indicates that the dissociation of S1 from actin by caldesmon is essential for inhibition of the acto–S1 ATPase. However, in the presence of tropomyosin, the ATPase of cross-linked acto–S1 is inhibited; this suggests that tropomyosin and caldesmon together can regulate acto–S1 ATPase, independent of the dissociation of the myosin head from actin.

The kinetic data from experiments using a 1:20 molar ratio of 38-kDa fragment to actin are interesting in view of the reports by Marston and Lehman (1985) and Graceffa et al. (1988) that the stoichiometry of caldesmon to actin in intact smooth muscle cells is between 1:28 and 1:14. The finding that the inhibition in V_{max} with tropomyosin–actin–38-kDa fragment increases on raising the ionic strength and temperature to approach the physiological conditions even at a low ratio of fragment to actin (Table I) implies that a substantial level of inhibition can be obtained with a low stoichiometry of caldesmon to actin under the physiological condition.

The reversal of caldesmon-induced inhibition by calmodulin was only 20–30% in this study, although calmodulin was added in a 10-fold molar excess of the caldesmon fragment. The mechanism of reversal by calmodulin seems to be caused by a decrease in the affinity of caldesmon to actin when calmodulin binds to caldesmon (Wang, 1988). Affinities to actin and to calmodulin are in the range of 10^{-7} and 10^{-6} M, respectively (Smith et al., 1987; Shirinsky et al., 1988; Velaz et al., 1989). Considering the difference in the affinity of caldesmon to actin and to calmodulin, a 6–10 times higher molar ratio of calmodulin to actin (not to caldesmon) needs to be implemented to obtain a complete reversal. In our experiments, the actin concentration is increased up to 250 μM, and it is not practical to add the sufficient amount of calmodulin for complete reversal of the inhibition. Despite the low level of reversal by calmodulin, the effect of calmodulin on caldesmon-induced inhibition appears to be a reduction of the binding of caldesmon fragment to actin rather than a change in the kinetic steps.

In the reconstituted system containing actin and HMM, phosphorylation of the myosin light chain causes a 25-fold rise in the V_{max} for actin-activated MgATPase activity (Sellers et al., 1982). Compared to the effect of dephosphorylation of myosin light chain, the caldesmon-induced effect is low, but it is still about 3-fold at close to physiological ionic strength. Although this is not a major factor in the regulation, it cannot be ignored. Data from this study demonstrate that the C-terminal region of caldesmon can modulate the ATP hydrolysis by affecting a catalytic step in the ATPase cycle and cause a decrease in the binding of myosin heads to actin. Further kinetic study is needed to elucidate the mechanism for caldesmon inhibition and thin filament regulation. In any case, the different effects of caldesmon on actin and tropomyosin–actin indicate that the tropomyosin plays an important role in the regulation of the thin filament by caldesmon.

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Stereochemistry of the Microsomal Glutathione S-Transferase Catalyzed Addition of Glutathione to Chlorotrifluoroethene†

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ABSTRACT: The stereochemistry of *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione formation was studied in rat liver cytosol, microsomes, *N*-ethylmaleimide-treated microsomes, 9000*g* supernatant fractions, purified rat liver microsomal glutathione S-transferase, and isolated rat hepatocytes. The absolute configuration of the chiral center generated by the addition of glutathione to chlorotrifluoroethene was determined by degradation of *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione to chlorofluoroacetic acid, followed by derivatization to form the diastereomeric amides *N*-(*S*)-α-methylbenzyl-(*S*)-chlorofluoroacetamide and *N*-(*S*)-α-methylbenzyl-(*R*)-chlorofluoroacetamide, which were separated by gas chromatography. Native and *N*-ethylmaleimide-treated rat liver microsomes, purified rat liver microsomal glutathione S-transferase, rat liver 9000*g* supernatant, and isolated rat hepatocytes catalyzed the formation of 75-81% (2*S*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione; rat liver cytosol catalyzed the formation of equal amounts of (2*R*)- and (2*S*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione. In rat hepatocytes, microsomal glutathione S-transferase catalyzed the formation of 83% of the total *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione formed. These observations show that the microsomal glutathione S-transferase catalyzes the first step in the intracellular, glutathione-dependent bioactivation of the nephrotoxin chlorotrifluoroethene.

Glutathione S-transferases (EC 2.5.1.18) catalyze the addition of glutathione to electrophilic substrates. Glutathione S-conjugate formation in the liver, followed by conversion to mercapturates, serves to detoxify potentially harmful xenobiotics. Alternatively, glutathione S-conjugate formation is an important bioactivation mechanism for several classes of compounds (Anders, 1990; Vamvakas & Anders, 1990). The nephrotoxicity of several haloalkenes is attributable to hepatic glutathione S-conjugate formation, metabolism of the gluta-

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