Caldesmon Inhibits the Cooperative Turning-On of the Smooth Muscle Heavy Meromyosin by Tropomyosin–Actin†

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ABSTRACT: The 38-kDa chymotryptic fragment of caldesmon, which possesses the actin/calmodulin binding domain, was purified and utilized to study the mechanism for the inhibition of acto–myosin ATPase by caldesmon. The intact caldesmon inhibited the acto–HMM ATPase although it caused an increase in the binding of HMM to actin, presumably due to the interaction between the S-2 region of HMM and the caldesmon located on the actin filament. The 38-kDa fragment, which lacks the S-2 binding domain, inhibited both the acto–HMM ATPase and the HMM binding to actin. The ATPase and the HMM binding to actin decreased in parallel on increasing the 38-kDa fragment bound to actin. In the presence of tropomyosin, the ATPase activity fell more rapidly than did the HMM binding to actin. Binding of intact caldesmon or 38-kDa fragment to actin inhibited the cooperative turning-on of tropomyosin–actin by NEM·S-1, which forms rigor complexes in the presence of ATP. The absence of cooperative turning-on of the acto–HMM ATPase by rigor complexes in the presence of 38-kDa fragment was associated with an inhibition of the binding of HMM to tropomyosin–actin. Addition of NEM·S-1 to tropomyosin–actin–caldesmon caused a gradual decrease in the caldesmon-induced binding of HMM to actin. The calmodulin restored the caldesmon-induced binding of HMM to tropomyosin–actin, but it had only a slight effect on the acto–HMM ATPase. These data suggest that the cooperative turning-on of the smooth muscle tropomyosin–actin by rigor bonds is modulated by the interaction of caldesmon, tropomyosin, and calmodulin on the thin filament.

Thin filaments in the smooth muscle and microfilaments in nonmuscle cells contain tropomyosin, a known component of the regulatory system in the striated muscle (Bailey, 1948; Ebashi, 1980), and caldesmon, an actin/calmodulin binding protein (Sobue et al., 1981; Bretscher & Lynch, 1985; Lehman et al., 1989). Caldesmon is a highly asymmetric molecule with a molecular weight of 93 000 (Graceffa et al., 1988; Bryan, 1989). The binding of caldesmon to actin inhibits actin–activated ATP hydrolysis by myosin, and this phenomenon is partially reversed by calmodulin in the presence of Ca^{2+} (Ngai & Walsh, 1984; Marston et al., 1985; Dabrowska et al., 1985; Sobue et al., 1985; Horiuchi et al., 1986). The domain of caldesmon responsible for the inhibition of the ATPase is the 38-kDa actin/calmodulin binding region derived from the C-terminus (Szpacenko & Dabrowska, 1986; Yazawa et al., 1987; Fujii et al., 1987; Ball & Kovala, 1988; Mornet et al., 1988; Katayama et al., 1989).

Caldesmon also interacts with smooth muscle myosin, specifically with the S-2 region (Ikebe & Reardon, 1988). Although structural evidence for this interaction is lacking, the binding studies indicate that this interaction causes an increase in the binding of myosin or heavy meromyosin to actin filaments which contain bound caldesmon (Lash et al., 1986; Hemric & Chalovich, 1988; Ikebe & Reardon, 1988). The portion of the caldesmon molecule which interacts with the myosin is believed to be close to the N-terminal region, and the molecular weight of this fragment is around 80K–110K (Katayama et al., 1989; Sutherland & Walsh, 1989; Bryan et al., 1989). The linking of myosin to actin, mediated through caldesmon, is nonproductive with respect to ATP hydrolysis (Hemric & Chalovich, 1988), but it is thought to play a role in the maintenance of force in smooth muscle (Lash et al., 1986; Hemric & Chalovich, 1988; Sutherland & Walsh, 1989) during the latch state (Dillon & Murphy, 1982).

Although the phosphorylation of the smooth muscle myosin light chain is a prerequisite for actin activation of the Mg-ATPase of myosin (Gorecka et al., 1976; Chacko et al., 1977; Sobieszek & Small, 1977; Sellers et al., 1981) and force development (Kerrick et al., 1980; Dillon et al., 1981; Barany & Barany, 1981; Butler & Siegman, 1982; Paul et al., 1983; Kamm & Stull, 1985), tropomyosin potentiates the actin activation about 3-fold (Chacko et al., 1977; Small & Sobieszek, 1977). Furthermore, as in the skeletal muscle (Bremel et al., 1972; Lehrer & Morris, 1982; Murray et al., 1982; Williams et al., 1988; Greene & Eisenberg, 1988), the smooth muscle actin filament is turned on by rigor complexes in the presence of tropomyosin (Chacko & Eisenberg, 1988). Tropomyosin also amplifies the inhibition of actomyosin ATPase by caldesmon (Marston et al., 1985; Dabrowska et al., 1985; Sobue et al., 1985; Horiuchi et al., 1986). It also interacts with caldesmon, and this interaction causes conformational changes in both tropomyosin and caldesmon. These changes are evident even when these proteins are bound to the actin filament (Horiuchi & Chacko, 1988; Galazkiewicz et al., 1987; Dobrowolski et al., 1988). These data raise the possibility that the interaction between caldesmon and tropomyosin may function similar to the troponin–tropomyosin system in skeletal muscle (Ebashi, 1980).

In this study, we utilized the 38-kDa chymotryptic fragment of caldesmon to distinguish the caldesmon-induced "nonproductive" binding of heavy meromyosin (HMM)[1] to actin

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[1] Abbreviations: HMM, heavy meromyosin; NEM, *N*-ethylmaleimide; S-1, myosin subfragment 1; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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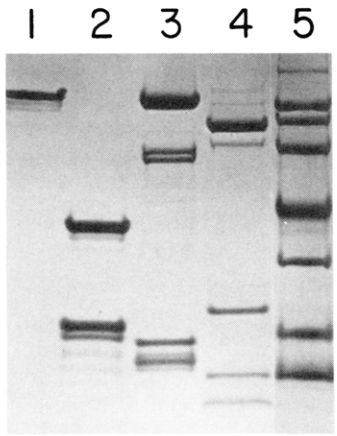

FIGURE 1: SDS–polyacrylamide gels of the proteins used in experiments. (1) Intact caldesmon; (2) 38-kDa fragment; (3) smooth muscle HMM; (4) skeletal S-1; (5) molecular weight standards (Bio-Rad) are 200K, 116.25K, 92.5K, 66.2K, 45K, 31K, 21.5K, and 14.4K from top to bottom.

from the HMM binding to actin associated with the acto–myosin ATPase cycle. While the intact caldesmon inhibited acto–HMM ATPase with an increase in the binding of HMM to actin, 38-kDa actin/calmodulin binding fragment inhibited both the acto–HMM ATPase and the binding of HMM to actin. Utilizing this fragment, we also studied the effect of caldesmon on the cooperative turning-on of smooth muscle HMM ATPase activity by tropomyosin–actin in the presence of rigor bonds.

Materials and Methods

Chicken gizzard HMM was prepared from phosphorylated myosin according to Kaminski and Chacko (1984). The phosphorylation level was found to be 98–100% on urea gel electrophoresis (Perrie & Perry, 1970). NEM·S-1 was prepared from rabbit skeletal muscle by the method of Nagashima and Asakura (1982). Chicken gizzard myosin, actin, and tropomyosin were prepared as described (Chacko, 1981; Heaslip & Chacko, 1985). Chicken gizzard caldesmon was prepared from heated muscle extract as described (Horiuchi & Chacko, 1988).

Chymotryptic Fragments of Caldesmon. Chymotryptic digestion of caldesmon (6 mg/mL) was performed at 25 °C for 7 min in 50 mM KCl, 15 mM imidazole (pH 7.0), 1 mM DTT, and 1.45 μg/mL α-chymotrypsin. Digestion was stopped by the addition of PMSF to obtain a final concentration of 1 mM. The fragments were purified on a DEAE-Sephacel column equilibrated with 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 1 mM DTT. The flow-through fraction contained the 38-kDa fragment and its breakdown products, whereas the bound fraction contained the intact caldesmon, the high molecular weight fragments (110K, 80K, and 60K), and an approximately 20-kDa fragment based on SDS–PAGE. The flow-through fraction was purified further by a calmodulin–agarose column (Sigma) equilibrated with 100 mM NaCl, 0.1 mM $CaCl_2$, 20 mM Tris-HCl (pH 7.5), and 1 mM DTT. The bound proteins were eluted with the same buffer containing 1 mM EDTA. The bound fraction contained predominantly the 38-kDa fragment and small amounts of 23- and 21-kDa fragments which were derived from further breakdown of the 38-kDa fragment. The SDS–PAGE patterns of the purified caldesmon and the 38-kDa fragment are shown in Figure 1.

Binding Assays. The binding experiments utilized chicken gizzard heavy meromyosin which was made from myosin phosphorylated with [γ-^{32}P]ATP. Prior to use, the HMM was centrifuged, and the pellet containing sedimented HMM (less than 10%) was discarded. The supernatant containing soluble HMM was utilized for the binding assay. Actin (60 μM) or actin containing bound tropomyosin (A:Tm ratio of 6:1) was first mixed with caldesmon or 38-kDa fragment. It was then transferred to airfuge tubes and mixed with ^{32}P-HMM (1 μM) in 30 mM KCl, 2 mM free Mg^{2+}, 10 mM imidazole hydrochloride (pH 7.0), and 2.5 mM DTT. The final volume of the assay was 0.15 mL. Assays were initiated by adding 4 mM Mg-ATP. Following mixing, the samples were immediately sedimented at 25 °C (130000*g*) for 20 min using a Beckman airfuge. After centrifugation, the supernatant containing unbound HMM was transferred to the scintillation vial. The airfuge tubes with the pellets containing actin and the bound HMM were also dropped into scintillation vials and counted. The HMM alone in the binding buffer served as a control.

The binding experiments in the presence of NEM·S-1 were performed as described above except for the protein concentrations. Actin was mixed with varying concentrations of NEM·S-1. The free actin concentration was maintained at 20 μM by raising the total actin concentration on increasing the NEM·S-1 concentration. The HMM concentration was kept constant at 0.3 μM. Sufficient caldesmon or 38-kDa fragment was added to obtain a caldesmon or 38-kDa fragment to actin (total) molar ratio of 1:6.7. This ratio corresponds to the 1:10 ratio used in a previous paper (Horiuchi & Chacko, 1988) in which the molecular weight of caldesmon was considered to be 140K.

Other Assays. The actin-activated ATPase activity of gizzard HMM was determined under the conditions of the binding assays except that 2 mM Mg-ATP mixed with [γ-^{32}P]ATP was utilized as described (Martin & Doty, 1949).

The protein concentrations were determined by the method of Lowry et al. (1951), and all the calculations were performed considering the molecular weights of intact caldesmon and the actin binding fragment to be 93K (Graceffa et al., 1988; Imai et al., 1988) and 38K, respectively.

Results

The effect of intact caldesmon and the 38-kDa caldesmon fragment on the actin-activated ATP hydrolysis by phosphorylated HMM is depicted in Figure 2A. Addition of either the intact caldesmon or the 38-kDa fragment inhibited the activation of the Mg-ATPase of phosphorylated HMM by smooth muscle actin, and the inhibition was completed at a caldesmon to actin molar ratio of around 1:5. The ATPase activity was potentiated 3–4-fold by tropomyosin bound to actin. Raising the caldesmon or the 38-kDa fragment concentration lowered the ATPase activity both in the presence and in the absence of tropomyosin; the activity decreased to the same level for both actin and tropomyosin–actin when the ratio of caldesmon or 38 kDa to actin reached 1:5.

The binding of the HMM to actin or tropomyosin–actin increased on raising the ratio of intact caldesmon to actin (Figure 2B). On the other hand, the addition of 38-kDa fragment caused a diminution of the binding of HMM to the actin and tropomyosin–actin. At a 38-kDa fragment to actin molar ratio of 1:4, the inhibition of HMM binding to actin devoid of tropomyosin was complete. The binding of HMM to actin containing tropomyosin was not completely inhibited under these conditions (Figure 2B).

In order to correlate the 38-kDa fragment-induced inhibition of the ATPase and the binding of HMM to actin, the percentages of the ATPase and the binding were plotted as a function of the ratios of 38 kDa to actin (Figure 3). HMM binding to actin and the ATPase (data from Figure 2) in the

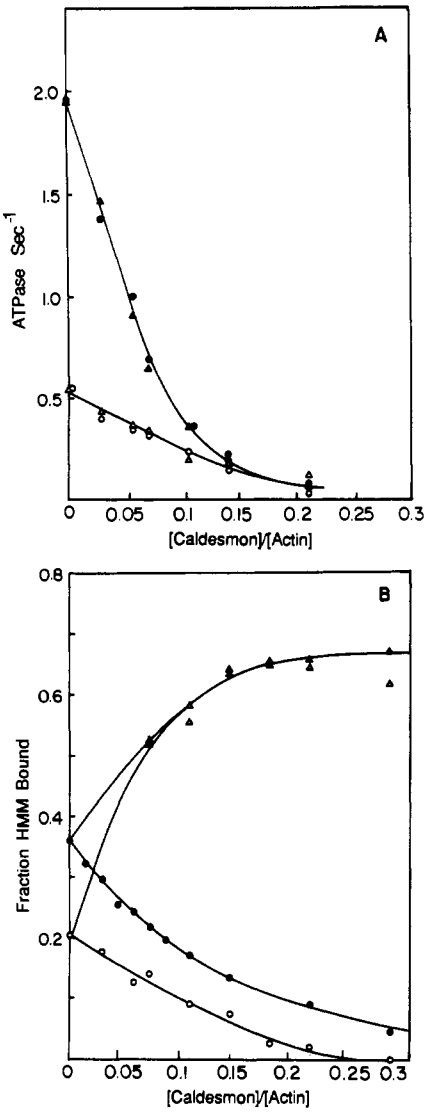

FIGURE 2: (A) Effects of intact caldesmon or the 38-kDa fragment on the actin-activated ATPase of phosphorylated HMM. Intact caldesmon (Δ) or 38-kDa fragment (O) was mixed with 60 μM actin with (closed symbols) or without (open symbols) 10 μM tropomyosin. Conditions of the assays were 30 mM KCl, 2 mM $MgCl_2$, 10 mM imidazole hydrochloride (pH 7.0), and 2.5 mM DTT at 25 °C; 1 μM HMM was mixed with the actin, and the ATPase was initiated by the addition of 2 mM Mg-ATP. (B) Effects of intact caldesmon or 38-kDa fragment on the binding of HMM to the actin filament. Symbols are the same as in (A). The binding experiments were performed under the same conditions as the ATPase assay except for the Mg-ATP concentration which was raised to 4 mM.

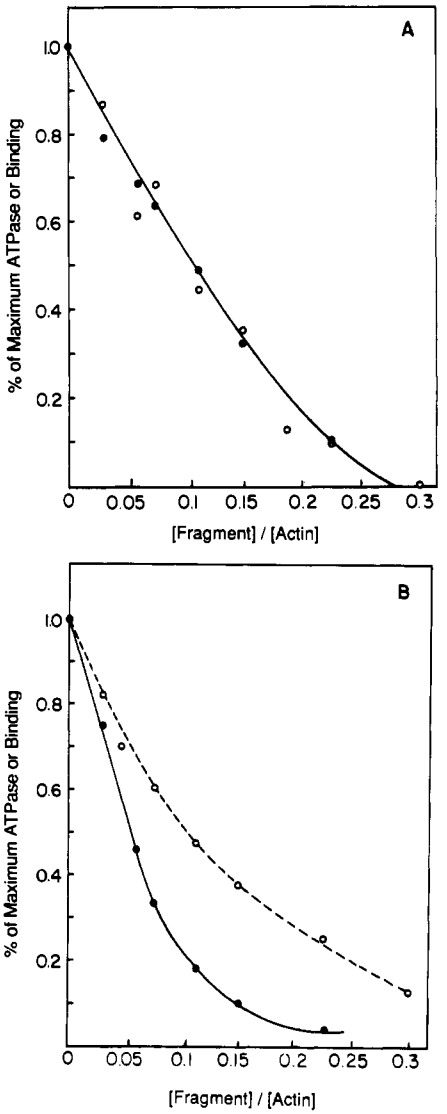

FIGURE 3: Relationship between the 38-kDa fragment-induced inhibitions of the ATPase and the binding of HMM to the actin filament in the absence (A) or presence (B) of tropomyosin. Data from Figure 2A,B were plotted as the percentage of HMM binding and ATPase. Values without caldesmon were considered as 100%. Data from ATPase, closed circles; binding, open circles.

absence of tropomyosin showed a parallel decrease on raising the 38-kDa fragment concentration (Figure 3A). In the presence of tropomyosin, the ATPase was 90% inhibited at a 38-kDa fragment to actin molar ratio of around 1:7, but the binding was inhibited only 60% (Figure 3B). Hence, in the presence of tropomyosin, the inhibition of the ATPase by 38-kDa fragment fell more rapidly than did the inhibition of HMM binding to actin.

The effects of the intact caldesmon and the 38-kDa fragment on the ATPase and the binding of HMM to actin were also examined in the presence of rigor complexes which cooperatively turn on the tropomyosin–actin (Chacko & Eisenberg, 1988). The effect of increasing the rigor complexes, produced by the binding of NEM-S-1 to the actin, on the actin-activated ATPase of HMM is shown in Figure 4A. In these experiments, the free actin concentration was kept constant by raising the total actin concentration as the NEM-S-1 concentration was increased. The addition of NEM-S-1 to the actin devoid of tropomyosin had no effect on the ATPase. However, the activation of the Mg-ATPase of HMM by tropomyosin–actin was enhanced about 6-fold at a 25% saturation of the actin monomer with NEM-S-1. The intact caldesmon or the 38-kDa fragment abolished the turning-on of the tropomyosin–actin by rigor bonds; the ATPase activity remained at the same level as with actin alone. Calmodulin had only a slight effect on the reversal of the inhibition of the ATPase.

The binding of HMM to actin on increasing the rigor bonds is shown in Figure 4B. Rigor bonds had no effect on the binding of HMM to actin devoid of tropomyosin. On the other hand, the HMM binding to tropomyosin–actin increased on raising the ratio of NEM-S-1 to actin monomer. The binding of HMM to tropomyosin–actin increased 3-fold when the ratio of NEM-S-1 to actin reached 1:4 (Figure 4B). This increase in the binding of HMM to tropomyosin–actin in the presence of rigor bonds was not evident when the 38-kDa fragment was added to tropomyosin–actin (38-kDa fragment to actin molar ratio around 1:7). In the presence of intact caldesmon, the binding of HMM was increased irrespective of the presence

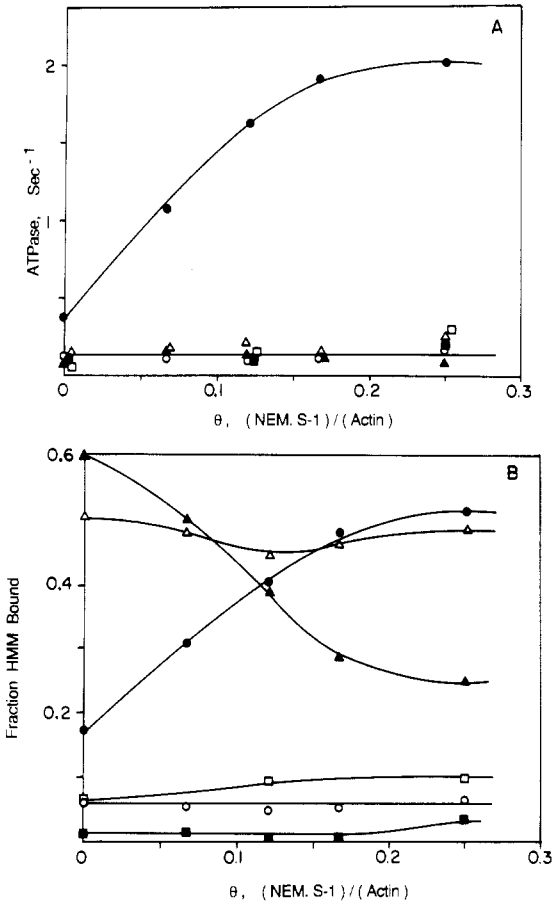

FIGURE 4: (A) Actin-activated ATPase activity of HMM in the presence of rigor bonds. Free actin concentration was kept constant at 20 μM by raising the total actin concentration when the NEM·S-1 concentration was increased. Tropomyosin to actin and caldesmon to actin (total) molar ratios were 1:6 and 1:6.7, respectively. Calmodulin was added to obtain a caldesmon to calmodulin molar ratio of 1:5.3. The HMM concentration was 0.3 μM. Conditions were the same as in Figure 2A except for the concentration of Mg-ATP (2.3 mM) and the presence of $CaCl_2$ (0.1 mM). (○) Actin alone; (●) actin containing tropomyosin; (▲) actin + tropomyosin + intact caldesmon; (■) actin + tropomyosin + 38-kDa fragment; (△) actin + tropomyosin + intact caldesmon + calmodulin; (□) actin + tropomyosin + 38-kDa fragment + calmodulin. (B) Binding of HMM to actin filament in the presence of rigor bonds. Conditions and symbols are the same as in (A).

or the absence of tropomyosin (as shown in Figure 2B). Introduction of rigor bonds to tropomyosin–actin–caldesmon lowered the HMM binding to actin. There was a 33% decrease in the binding when the tropomyosin–actin–caldesmon was saturated with NEM·S-1 to around 10%. Increasing the saturation of actin with NEM·S-1 up to 25% caused a 60% decrease in the binding.

Calmodulin modulated the binding of HMM to tropomyosin–actin–caldesmon in the presence of rigor bonds. While the rigor complexes decreased the binding of HMM to tropomyosin–actin–caldesmon, it had no effect in the presence of calmodulin; the HMM binding remained high (Figure 4B). This phenomenon was independent of the Ca^{2+} concentration (data not shown). The addition of calmodulin to tropomyosin–actin–caldesmon in the presence of rigor bonds had only a slight effect on the ATPase (Figure 4A). Calmodulin caused only a slight increase in the binding of HMM to tropomyosin–actin–38-kDa fragment in the presence of rigor complexes, but the ATPase remained inhibited. Calmodulin added to tropomyosin–actin for a control experiment had no effect either on the ATPase or on the HMM binding (data not shown).

Discussion

Caldesmon modulates the interaction between actin and myosin or HMM. This results in a decrease in the actin-activated ATP hydrolysis; however, the binding of HMM to actin, mediated through the linking of the S-2 region of the HMM and the caldesmon located on the actin filament, is increased (Lash et al., 1986; Hemric & Chalovich, 1988; Ikebe & Reardon, 1988). In a previous study (Chalovich et al., 1987), this "non-productive" binding is eliminated by using smooth or skeletal muscle S-1. Unlike the smooth or skeletal muscle S-1, smooth muscle HMM is regulated (Seidel, 1978; Sellers et al., 1981; Kaminski & Chacko, 1984), and it is double headed like myosin. Utilization of the 38-kDa fragment which is devoid of the S-2 binding domain of the caldesmon enables us to use the acto–HMM system to study the effect of caldesmon on the regulation of ATPase and the binding of the myosin heads to actin without any interference by the nonproductive linking of HMM to actin through caldesmon.

The 38-kDa fragment inhibits the ATPase at the same level as does the intact caldesmon (Figure 2A), indicating that the inhibition of the actin-activated ATP hydrolysis requires only the actin/calmodulin binding domain. This inhibition of ATP hydrolysis in the presence of 38 kDa is caused by an inhibition of the binding of HMM heads to actin since the 38-kDa fragment is devoid of the S-2 binding domain of the caldesmon. Hence, the HMM binding in this case is associated with the ATPase cycle. On the other hand, the increased binding observed in the presence of the intact caldesmon is presumably due to a nonproductive binding between the S-2 region of the HMM and the caldesmon.

The 38-kDa fragment-induced inhibition of acto–HMM ATPase parallels a decrease in the binding of HMM to actin in the absence of tropomyosin (Figure 3A). On the other hand, the ATPase falls more rapidly than does the HMM binding in the presence of tropomyosin (Figure 3B). The finding that the inhibition of the ATPase was not well correlated with the inhibition of the HMM binding to tropomyosin–actin suggests that the tropomyosin modulates the effect of the actin/calmodulin binding domain of the caldesmon on the HMM binding and ATPase. It has been reported that the caldesmon inhibits the potentiation of smooth muscle acto–myosin ATPase by tropomyosin (Sobue et al., 1985; Horiuchi et al., 1986); the ATPase remains similar to that in the presence of actin alone. However, this is not evident when acto–HMM is used instead of acto–myosin (Figure 2A). Caldesmon inhibits the acto–HMM ATPase both in the presence and in the absence of tropomyosin. The reason for this difference is not clear. Interestingly, in the presence of caldesmon, the potentiation of the actin-activated ATPase of skeletal muscle myosin by smooth muscle tropomyosin is inhibited to a level lower than the ATPase with actin alone (Marston et al., 1985; Dabrowska et al., 1985).

In the present study, the effect of caldesmon on the cooperative turning-on of smooth muscle tropomyosin–actin by rigor bonds is investigated to explore the possibility that the actin–tropomyosin–caldesmon may function as a regulated thin filament in smooth muscle. The increased binding of the HMM to tropomyosin–actin in the presence of rigor complexes is associated with a rise in the acto–HMM ATPase activity. Although the binding of HMM to tropomyosin–actin is high in the presence of caldesmon, it decreases gradually on adding the NEM·S-1 (Figure 4B). However, the binding of HMM in this case is nonproductive since the ATPase activity is low. The decrease in the binding on raising the NEM·S-1 bound to actin filament is presumably caused by the detachment of

caldesmon from actin filament. The SDS gel of the pelleted actin filament shows that caldesmon is partially released from the actin filament by rigor complexes (data not shown). This is in agreement with a previous report which shows that the caldesmon is displaced by HMM or S-1 under rigor conditions, and this effect was more pronounced when skeletal HMM or S-1 was used (Hemric & Chalovich, 1988). The amount of caldesmon that still remains bound to actin filament is sufficient to cause the inhibition of the ATPase. Both intact caldesmon and the 38-kDa fragment prevent the turning-on of the tropomyosin–actin by rigor complexes as evidenced by a lack of increase in the ATPase activity. The inhibition of the cooperativity by 38 kDa is associated with an inhibition of the binding of HMM heads to tropomyosin–actin and a decrease in ATP hydrolysis.

The caldesmon-induced inhibition of the turning-on of the acto–HMM ATPase by rigor complexes is slightly reversed by Ca^{2+}–calmodulin. Rigor bonds decrease the caldesmon-induced enhancement of the binding of HMM to tropomyosin–actin; however, they have no effect in the presence of calmodulin. Hence, in the presence of rigor bonds, calmodulin restores the caldesmon-induced binding of HMM to tropomyosin–actin. Since it is not associated with a concomitant increase in the acto–HMM ATPase, the binding is nonproductive with respect to ATP hydrolysis. Therefore, calmodulin is not sufficient to reverse the binding of HMM heads to actin, that is responsible for the ATP hydrolysis although it is capable of facilitating the interaction between the caldesmon and the S-2 region of the HMM.

The possibility that tropomyosin–actin–caldesmon–calmodulin (or another Ca^{2+} binding protein) functions as a thin filament mediated regulatory system in smooth muscle is considered in previous reports (Ngai & Walsh, 1984; Sobue et al., 1985; Horiuchi et al., 1986; Lash et al., 1986; Hemric & Chalovich, 1988; Horiuchi & Chacko, 1988; Dobrowolski et al., 1988; Pritchard & Marston, 1989). The data presented in this paper clearly demonstrate that the different domains of the caldesmon have different effects on the interaction between myosin and the thin filament. The caldesmon-induced inhibition of the cooperative turning-on of the smooth muscle tropomyosin–actin indicates that the activation of the smooth muscle myosin ATPase by tropomyosin–actin is modulated by caldesmon. Interestingly, calmodulin maintains the nonproductive binding of the HMM to tropomyosin–actin–caldesmon in the presence of rigor complexes though it has only a slight effect on the ATPase. This phenomenon is independent of Ca^{2+} concentration (data not shown). The modulation of the effect of rigor complexes on the smooth muscle tropomyosin–actin by caldesmon–calmodulin may play an important role in the development and maintenance of contractile force in smooth muscle. This mode of regulation coexists with the regulation by the Ca^{2+}–calmodulin-dependent myosin light chain phosphorylation.

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Disulfide Formation within the Regulatory Light Chain of Skeletal Muscle Myosin†

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ABSTRACT: Thiol–disulfide exchange reactions between myosin and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) lead to the formation of 5-thio-2-nitrobenzoic acid (TNB)-mixed disulfides as well as to protein disulfide bonds. After incubation with DTNB, myosin was treated with an excess of *N*-ethylmaleimide (NEM) before electrophoretic analysis of the protein subunits in sodium dodecyl sulfate (SDS) without prior reduction by dithiothreitol (DTT). Without NEM treatment, thiol–disulfide rearrangement reactions occurred in the presence of SDS between the residual free thiols and DTNB. In the absence of divalent metal ions at 25 °C, DTNB was shown to induce an intrachain disulfide bond between Cys-127 and Cys-156 of the RLC. This intrachain cross-link restricts partially the unfolding of the RLC in SDS and can be followed as a faster migrating species, RLC′. Densitometric evaluation of the electrophoretic gel patterns indicated that the stoichiometric relation of the light chains (including RLC and RLC′) remained unchanged. The two cysteine residues of the fast migrating RLC′ were no more available for reaction with [^{14}C]NEM, but upon reduction with DTT, the electrophoretic mobility of the RLC′ reverted to that of unmodified RLC and of the RLC modified with two TNB groups. Ca^{2+} or Mg^{2+} was able to prevent this disulfide formation in the RLC of myosin by 50% at a free ion concentration of 1.1×10^{-8} and 4.0×10^{-7} M, respectively, at 25 °C and pH 7.6. Intrachain disulfide formation of RLC never occurred in myosin at 0 °C. Incubation of isolated RLC with DTNB always resulted in intrachain disulfide formation, irrespective of temperature or the presence or absence of divalent metal ions.

All double-headed myosin species seem to contain two types of light chains (LC),[1] one of each associated noncovalently with the heavy chain (HC) in each head portion. Not much is known about the function of the alkali-type LC (LC-1 and LC-3) in fast skeletal muscle myosin. The second type of LC in the 19 000-Da molecular mass region is often referred to as DTNB-LC, as P-LC, or as regulatory light chain (RLC). These different trivial names derive from some of its properties: (i) part of this LC may be removed by treatment of myosin with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of EDTA at elevated pH; (ii) this LC can be reversibly phosphorylated at its Ser-15 in vitro and in vivo (Perrie et al., 1973); and (iii) homologous protein subunits serve as

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[1] Abbreviations: Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HC, myosin heavy chain; LC-1, myosin light chain 1; LC-3, myosin light chain 3; NEM, *N*-ethylmaleimide; [^{14}C]-NEM, *N*-ethyl[^{14}C]maleimide; [^{14}C]NES, *N*-ethyl[1-^{14}C]succinimide; PAGE, polyacrylamide gel electrophoresis; RLC, myosin regulatory light chain; SDS, sodium dodecyl sulfate; TNB, 5-thio-2-nitrobenzoic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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