Inhibition of Smooth Muscle Actomyosin ATPase by Caldesmon Is Associated with Caldesmon-Induced Conformational Changes in Tropomyosin Bound to Actin†

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ABSTRACT: The smooth muscle tropomyosin isoforms β and γ were isolated in pure form and labeled with *N*-(1-pyrenyl)iodoacetamide (PIA) on the cysteine residues at either the N- or the C-terminal region (Cys-36 and Cys-190 of β- and γ-isoforms, respectively). The effect of caldesmon (CaD) on local conformational changes in different regions of the tropomyosin molecule was determined on the basis of changes in the excimer fluorescence (excited dimer of pyrene) formed in homodimers of tropomyosin isoforms. In the absence of actin, excimer fluorescence from the pyrene at Cys-190 of γ-tropomyosin homodimer decreased in a simple manner on the addition of CaD, whereas the excimer from the Cys-36 of β-tropomyosin homodimer exhibited a biphasic change, suggesting that additional weak binding sites exist near Cys-36. In the presence of actin, CaD-induced changes in the excimer fluorescence of pyrene−tropomyosin were observed only with Cys-36, and this change was associated with an inhibition of actin-activated myosin ATPase. A competition study with unlabeled tropomyosin isoforms indicated that the different excimer changes exhibited by β- and γ-tropomyosin in the presence of CaD were due to conformational changes in different regions of the tropomyosin molecule and not to differences in their affinities for CaD. Experiments with recombinant CaD mutants derived using the baculovirus expression system showed that the inhibition of tropomyosin potentiation of actomyosin ATPase by CaD requires the regions between residues 728−756 and 718−727 on the CaD molecule, although the latter region was sufficient for direct interaction with tropomyosin.

Smooth muscle tropomyosin plays a major role in the regulation of actin-activated ATP hydrolysis by phosphorylated myosin (Chacko et al., 1977; Sobieszek & Small, 1977; Chacko & Eisenberg, 1990). Chicken gizzard tropomyosin has two major isoforms, β and γ, and exists predominantly as a $\beta\gamma$-heterodimer *in vivo* (Sanders et al., 1986). These isoforms have the same polypeptide chain length (284 residues), and both polypeptides have a single cysteine residue, at position 36 on β and at position 190 on γ (Sanders & Smillie, 1985). Despite these differences in the amino acid sequence of smooth muscle tropomyosin isoforms, there is very little difference in their ability to bind to F-actin (Sanders & Smillie, 1985). While there is a high degree of homology, some sequence differences exist between smooth and skeletal muscle tropomyosins, particularly in the C-terminus. These differences may account for the stronger head-to-tail polymerization and the weaker binding of smooth muscle tropomyosin to skeletal muscle troponin and the chymotryptic T1 fragment of troponin T (Sanders & Smillie, 1984).

It has been reported that skeletal muscle tropomyosin specifically labeled at cysteine groups with *N*-(1-pyrenyl)-iodoacetamide (PIA)[1] exhibits excimer fluorescence (excited dimer) due to intramolecular interactions between two pyrene groups attached to cysteine residues on adjacent polypeptide chains (Ishii & Lehrer, 1990). Excimer fluorescence is sensitive to local conformational changes, and thus serves as an excellent tool to monitor such changes in tropomyosin by protein−protein interactions (Ishii & Lehrer, 1990, & 1993). Although native smooth muscle tropomyosin labeled with pyrene does not exhibit excimer fluorescence because it forms a heterodimer (Lehrer et al., 1984), it reveals excimer fluorescence when labeled under denaturing conditions or after separation into individual isoforms (Burtnick et al., 1988).

Tropomyosin amplifies the inhibition of actin-activated myosin ATPase by caldesmon (CaD), a component of the thin-filament of smooth muscle (Sobue et al., 1985; Marston et al., 1986; Dabrowska et al., 1985; Horiuchi et al., 1986; Lehman et al., 1989). This inhibition is associated with conformational changes in both CaD and tropomyosin (Horiuchi & Chacko, 1988). Sequences in the C-terminal region of the CaD molecule are responsible for the inhibition of actomyosin ATPase and the ability of CaD to bind to actin, calmodulin, and tropomyosin (Fujii et al., 1987). A 38-kDa CaD chymotryptic fragment from the C-terminus retains the same ability as the intact molecule to modulate actomyosin ATPase (Szpacenko & Dabrowska, 1986; Horiuchi & Chacko, 1989; Velaz et al., 1990). The region of the CaD molecule responsible for actin binding and the inhibition of actomyosin ATPase has been localized to 99 amino acids (residues 658−756) at the C-terminus, on the basis of studies using CaD fragments expressed in a bacterial system and those obtained by proteolysis (Hayashi et al., 1991; Wang et al., 1991; Redwood & Marston, 1993; Bartegi et al., 1990).

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[1] Abbreviations: PIA, *N*-(1-pyrenyl)iodoacetamide; PIA-β- or -γ-tropomyosin, PIA-labeled β- or γ-isoform of smooth muscle tropomyosin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CaD, caldesmon.

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Experiments using pyrene-labeled CaD and tropomyosin mutants and fragments suggested that the CaD binding site on tropomyosin is located in residues 142−227 (Watson et al., 1990a). On the basis of sequence homology of CaD with the tropomyosin binding site on troponin T, Bryan et al. (1989) proposed the amino acid residues 508−565 on CaD as the possible tropomyosin binding site. However, there is no direct evidence for an interaction between the tropomyosin residues 142−227 and the CaD residues 508−565.

In this study, we isolated two isoforms of smooth muscle tropomyosin and fluorescently labeled them with pyrene at each cysteine residue. This enabled us to monitor local conformational changes near the N-terminus (around Cys-36 of the β-isoform) or the C-terminus (around Cys-190 of the γ-isoform) in the tropomyosin molecule when it interacted with CaD. Furthermore, we used recombinant mutant CaD constructs to delineate the region of CaD responsible for the conformational changes in tropomyosin, which are associated with the inhibition of actin-activated myosin ATPase.

MATERIALS AND METHODS

CaD was prepared from chicken gizzard smooth muscle (Horiuchi & Chacko, 1989). Recombinant full-length CaD and mutant CaDs (CaD_{1-717} and CaD_{1-727}) were constructed by a PCR cloning strategy using chicken gizzard *h*-caldesmon cDNA as template (a gift from Dr. Joseph Bryan). The same forward primer, 5′-GTCAGGATCCCGGATACATCTCC-3′, was used for both CaD_{1-717} and CaD_{1-727}. The reverse primers for CaD_{1-717} and CaD_{1-727} were 5′-GTCAGGATC-CCTAGCCGGATACATCTCC-3′ and 5′-GTCAGGATC-CCTAAACTGACTGCTTCTC-3′, respectively. The recombinant CaD inserts were introduced into the Pvl941 baculovirus vector, these mutant CaDs were expressed in Sf9 cells, and the proteins were purified as described (Wang et al., 1994). Actin was prepared from acetone powder of rabbit skeletal muscle (Pardee & Spudich, 1982). Smooth muscle myosin was prepared from chicken gizzard and was fully phosphorylated using endogenous kinase, followed by chromatography on a Sepharose CL-4B column (Chacko, 1981). Smooth muscle tropomyosin was prepared from alcohol−ether powder of chicken gizzard without heat treatment (Eisenberg & Kielley, 1974). Two isoforms of tropomyosin were separated by chromatofocusing on a Mono P column (Pharmacia-LKB) in the presence of 9 M urea, according to described methods (Sanders et al., 1986). Each isoform was fluorescently labeled with PIA at the cysteine residue according to Ishii and Lehrer (1990). The labeling ratio of pyrene to tropomyosin was determined using $\epsilon_{344} = 2.2 \times 10^4$ (Ishii & Lehrer, 1990) and was found to be 1.7−1.9.

Fluorescence was measured using a photon counting system (M series, Photon Technology International Inc.). Protein concentrations were determined by either the method of Lowry et al. (1951) or by absorbance using the following extinction coefficients: actin, $E^{1\%}_{290} = 6.3$; tropomyosin, $E^{1\%}_{277} = 1.9$.

Binding of CaD to actin was determined by cosedimentation assay. Briefly, the protein mixture was sedimented using an airfuge (Beckman), and pellets were subjected to SDS−PAGE (Laemmli, 1970). Binding levels were estimated by scanning densitometry (Ultroscan XL, LKB) of gels after Coomassie Blue staining (Horiuchi & Chacko, 1995).

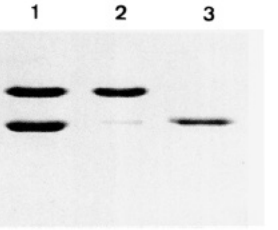

FIGURE 1: SDS−PAGE pattern of smooth muscle tropomyosin isoforms. Lane 1, purified chicken gizzard native tropomyosin; lane 2, isolated γ-isoform of tropomyosin; lane 3, isolated β-isoform of tropomyosin.

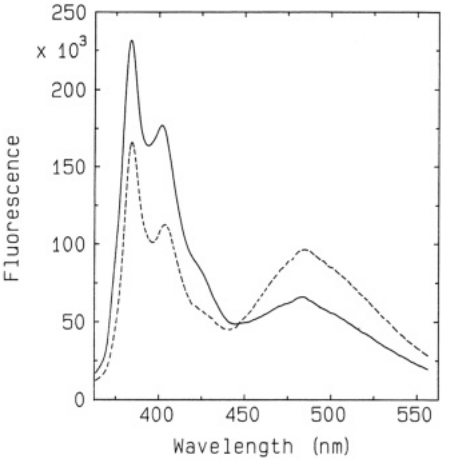

FIGURE 2: Fluorescence emission spectra of PIA-labeled β (- - -) and γ (—) tropomyosin. PIA-labeled tropomyosin (0.15 μM) is in 10 mM MOPS (pH 7.0) and 2 mM DTT at 25 °C. The excitation wavelength was 340 nm. Excimer peaks were observed in both tropomyosin isoforms at 485 nm.

Actin-activated ATPase activity of smooth muscle myosin was measured as described (Horiuchi et al., 1991) under conditions similar to those of the fluorescence measurements.

The affinity of caldesmon binding, which is associated with a conformational change in tropomyosin, was estimated from changes in excimer fluorescence using the nonlinear least-squares program as reported by Watson et al. (1990b). Estimation using this procedure is based on the assumption that the relationship between fluorescence change and binding is linear.

RESULTS

The individual β- and γ-isoforms from chicken gizzard tropomyosin were separated by chromatofocusing and resolved electrophoretically (Figure 1). Figure 2 shows the fluorescence spectra of PIA attached to the cysteine residues of purified tropomyosin isoforms (Cys-36 for β- and Cys-190 for γ-isoforms). Excimer fluorescence was observed in both labeled tropomyosin isoforms due to the formation of homodimers as reported (Burtnick et al., 1988).

To analyze CaD-induced conformational changes in the tropomyosin isoforms, either $\beta\beta$- or $\gamma\gamma$-homodimers labeled with PIA at the cysteine residues were mixed with increasing concentrations of CaD while keeping the tropomyosin concentration constant, and excimer fluorescence from these isoforms was measured. Since the excimer fluorescence is stable and more sensitive to local conformational change than the monomer fluorescence (Ishii & Lehrer, 1990), the change in the excimer fluorescence was monitored. Excimer fluorescence from PIA-γ-tropomyosin decreased upon ad-

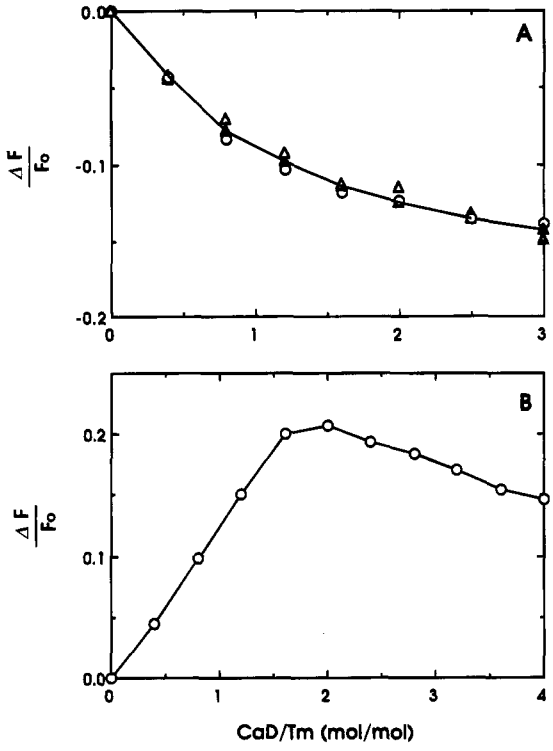

FIGURE 3: (A) Changes in excimer fluorescence from PIA-γ-tropomyosin upon addition of CaD and competition of tropomyosin isoforms. CaD was titrated as indicated to 0.25 μM PIA-γ-tropomyosin in the absence (○) or presence of 0.25 μM of nonlabeled β-tropomyosin (▲) or γ-tropomyosin (△). The abscissa indicates the CaD to total tropomyosin molar ratio. (B) Changes in excimer fluorescence from PIA-β-tropomyosin (0.25 μM) upon addition of CaD. Conditions in A and B were 10 mM NaCl, 10 mM MOPS (pH 7.0), and 2 mM DTT at 25 °C, and the excitation and the emission wavelengths were 340 and 485 nm, respectively.

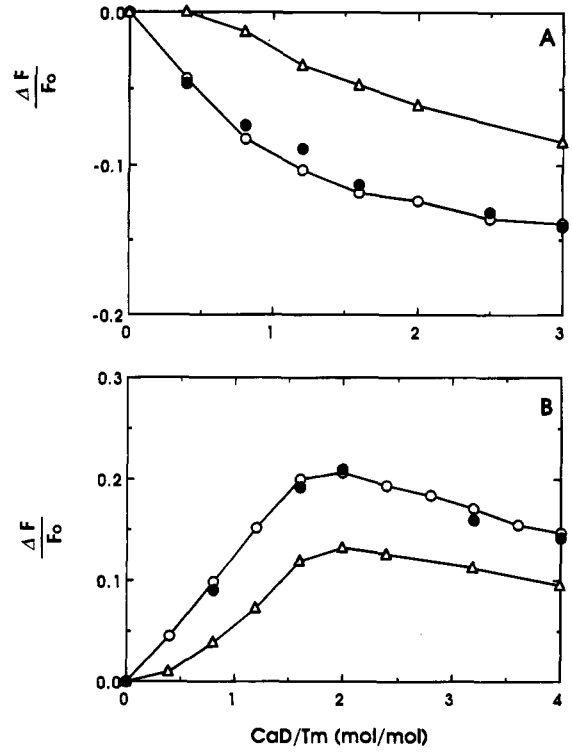

FIGURE 4: Changes in excimer fluorescence from (A) PIA-γ- and (B) β-tropomyosin on the addition of CaD mutants. Conditions were the same as in Figure 3. Native CaD (○; data taken from Figure 3A,B), mutant CaD_{1-727} (●), and mutant CaD_{1-717} (△).

dition of caldesmon in a simple manner, and this decrease started to level off at a CaD to tropomyosin molar ratio of 2:1 (Figure 3A). By contrast, the change in the excimer fluorescence from PIA-β-tropomyosin increased upon addition of CaD until a CaD:tropomyosin molar ratio of about 2:1 was reached. Further addition of CaD caused a decrease in the excimer fluorescence (Figure 3B). This biphasic change in the fluorescence of β-tropomyosin induced by CaD suggests the existence of a second weak binding site for CaD, which is saturated at a high molar ratio of CaD to tropomyosin. In agreement with previous reports (Horiuchi & Chacko, 1988; Watson et al., 1990a), no change in the excimer fluorescence was observed for either isoform at ionic strength above 50 mM.

To determine whether the difference between PIA-$\beta\beta$- and -$\gamma\gamma$-homodimers in excimer fluorescence upon interaction with CaD was due to a difference in the affinity of these isoforms for CaD, a constant amount of PIA-γ-tropomyosin was mixed with an equal amount of either β- or γ-unlabeled tropomyosin to compete for binding to CaD and the changes in excimer fluorescence upon addition of CaD were measured. The change in excimer fluorescence was similar in both cases and compatible with the changes observed for labeled tropomyosin alone, when adjusted for the molar ratio of total tropomyosin (labeled and unlabeled) to CaD (Figure 3A). The binding constant estimated using a nonlinear least-squares program for caldesmon, in the absence of unlabeled tropomyosin, was 3.95×10^6 M^{-1}; in the presence of unlabeled β- or γ-tropomyosin, the binding constant was 3.89 $\times 10^6$ or 3.81×10^6 M^{-1}, respectively. Thus the difference in the fluorescence changes from the two isoforms is not due to a difference in their binding affinity for CaD but instead to conformational changes in different regions of the tropomyosin molecule induced by the interaction with caldesmon.

To further understand the interaction between CaD and tropomyosin, CaD mutants lacking either 29 (CaD_{1-727}) or 39 (CaD_{1-717}) amino acids from the C-terminus were utilized. Interaction of the CaD_{1-727} mutant or native CaD with PIA-γ-tropomyosin caused a similar change in excimer fluorescence (Figure 4A), indicating that the 29 amino acids at the C-terminus of CaD are not involved in inducing a conformational change in tropomyosin. However, the extent of change in excimer fluorescence upon interaction with the CaD_{1-717} mutant, which lacks an additional 10 amino acids, was less than that obtained for native CaD or CaD_{1-727} (Figure 4A). The apparent binding constants estimated for CaD_{1-727} and CaD_{1-717} were 3.75×10^6 and 0.36×10^6 M^{-1}, respectively. The low level of change in excimer fluorescence of CaD_{1-717} was not entirely due to a difference in the affinity of this mutant for tropomyosin, since further addition of the mutant did not result in excimer fluorescence changes comparable to those obtained with CaD or CaD_{1-727} (data not shown). Interaction between CaD_{1-717} and PIA-β-tropomyosin also showed a low level of change in excimer fluorescence (Figure 4B).

The effect of actin on the CaD-induced changes in excimer fluorescence was determined using PIA-β- or -γ-tropomyosin bound to actin prior to the addition of CaD. Binding to actin caused a 10% decrease in excimer fluorescence of PIA-γ-tropomyosin, and addition of CaD induced no further change in the fluorescence (data not shown). In contrast, excimer fluorescence from PIA-β-tropomyosin was decreased by

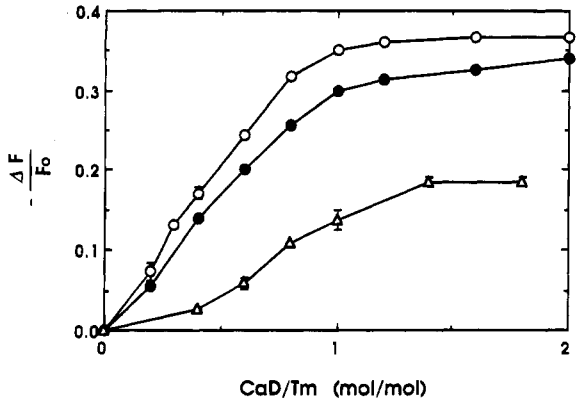

FIGURE 5: Decreases in excimer fluorescence from PIA-β-tropomyosin in the presence of actin. The ordinate indicates relative decrease in excimer fluorescence. Conditions were 30 mM NaCl, 3 mM $MgCl_2$, 10 mM MOPS (pH 7.0), and 2 mM DTT at 25 °C. Native CaD (○), mutant CaD_{1-727} (●), or mutant CaD_{1-717} (△) was added as indicated to 0.25 μM PIA-β-tropomyosin in the presence of 2.5 μM actin.

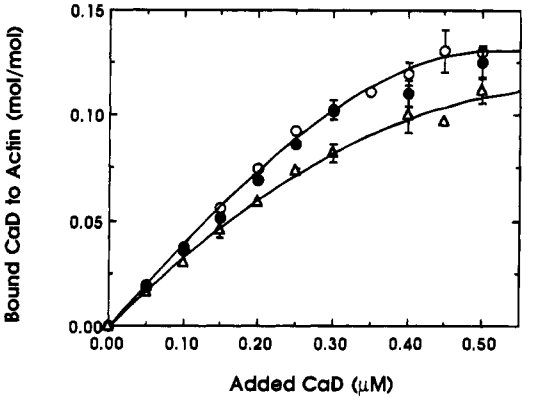

FIGURE 6: Binding of caldesmon mutants to actin in the presence of bound β-tropomyosin. Conditions and protein concentrations were the same as in Figure 5. Each assay mixture was centrifuged in an airfuge, and supernatant and pellet were subjected to SDS–PAGE separately. Bound fractions to actin were estimated by scanning densitometry of gels. Native CaD (○), mutant CaD_{1-727} (●), and mutant CaD_{1-717} (△).

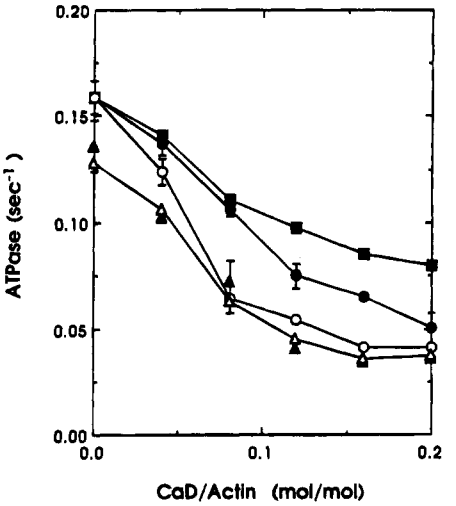

FIGURE 7: Effect of CaD on the potentiation of actin-activated myosin ATPase by tropomyosin isoforms. Conditions were the same as in Figure 5 except for the presence of 2 mM MgATP which was mixed with [γ-^{32}P]ATP. Fully phosphorylated smooth muscle myosin was added to 1–20 μM actin in the presence of 6.7 μM native tropomyosin ($\beta\gamma$) (○), β-tropomyosin (▲), or γ-tropomyosin (△), and CaD was added as indicated. Native tropomyosin ($\beta\gamma$) was used for the inhibition by mutant CaD_{1-727} (●) and mutant CaD_{1-717} (■).

20%–30% upon binding to actin, and CaD induced a further decrease in the fluorescence, until a CaD to tropomyosin molar ratio of 1:1 was reached (Figure 5). In competition experiments, such as those shown in Figure 3A using either unlabeled β- or γ-tropomyosin but performed in the presence of actin, the affinity of CaD for β- or γ-tropomyosin was similar even in the presence of actin (data not shown). When mutant CaD_{1-727} was added to PIA-β-tropomyosin bound on actin filament, excimer fluorescence also decreased, although the degree of change was slightly lower than that caused by native CaD (Figure 5). The apparent binding constants for native CaD and CaD_{1-727} were, respectively, 8.45×10^6 and 7.74×10^6 M^{-1}, values about two times higher than those in the absence of actin. Addition of mutant CaD_{1-717} induced a change in excimer fluorescence that reached only one-half the level induced by native CaD, and the apparent binding constant was 1.1×10^6 M^{-1} (estimated from Figure 5).

Since direct interaction between native CaD and tropomyosin in the absence of actin was negligible at an ionic strength close to 50 mM (conditions as in Figure 5), we examined the possibility that a difference in the affinity of mutant and native CaD for actin accounts for the observed change in excimer fluorescence in the presence of actin. Thus, the binding of the CaD mutants to actin-β-tropomyosin was determined under conditions similar to those in Figure 5 using cosedimentation techniques. Binding of native CaD to actin was the same in the presence of β-, γ-, or native tropomyosin (data not shown). As shown in Figure 6, mutant CaD_{1-727} bound to actin–tropomyosin to the same extent as did the native CaD with binding constants of 2.83×10^6 and 2.97×10^6 M^{-1} for mutant and native CaD, respectively. Mutant CaD_{1-717} gave a binding constant of 1.33×10^6 M^{-1}, which is slightly lower than those for native CaD and CaD_{1-727}. In neither case did the binding level off at a 1:1 molar ratio of CaD to tropomyosin, indicating that the excimer change did not correlate directly with CaD binding to actin.

The effects of tropomyosin isoforms on the actin-activated ATPase of smooth muscle myosin and on CaD inhibition were studied to identify any functional differences between the isoforms. The ATPase assay was performed by mixing actin with either native ($\beta\gamma$-heterodimer), β-, or γ-homodimers of tropomyosin. The potentiation of actin-activated myosin ATPase by β- or γ-tropomyosin was slightly lower than that by native tropomyosin (Figure 7), possibly due to the reduced ability of homodimers to form an end-to-end polymerization as compared with the native heterodimer (Graceffa, 1992). However, the level of CaD-induced inhibition of the potentiation of actin-activated myosin ATPase activity by β- or γ-homodimer tropomyosin was similar to that by native tropomyosin (Figure 7). These data argue against any functional difference between tropomyosin isoforms. Similar results were obtained using labeled tropomyosin isoforms (data not shown). Analyses to compare the effects of CaD and the CaD mutants on inhibition of tropomyosin-potentiated actin-activated myosin ATPase activity revealed 50% inhibition with native ($\beta\gamma$-heterodimer), β-homodimer, or γ-homodimer at a CaD to

actin molar ratio of 1:14 and about 70% inhibition when the molar ratio of CaD to actin was increased to 1:5. By contrast, inhibition by both CaD mutants was only half of that observed for native CaD at a 1:14 molar ratio of CaD to actin, which was increased to 90% when the molar ratio was increased to 1:5 with CaD_{1-727}, but the level of inhibition caused by CaD_{1-717} remained around 60% of that caused by native CaD. These results indicate that the level of conformational changes in tropomyosin induced by the native and mutant CaDs (see Figure 5) is associated with the inhibition of actin-activated ATPase activity.

DISCUSSION

The finding that both pyrene-labeled β- and γ-homodimers of tropomyosin exhibit excimer fluorescence agrees with a previous report (Burtnick et al., 1988). When adjusted for the concentration of PIA-labeled tropomyosin in the mixture, there was no difference in the CaD-induced decrease in the excimer fluorescence of PIA-tropomyosin and the apparent binding constant in the competition study with unlabeled tropomyosin isoforms (Figure 3A). These data show that there is no difference between two tropomyosin isoforms in the affinity for CaD. This finding indicates that the difference in fluorescence change from the two PIA-labeled tropomyosin isoforms caused by the addition of CaD reflects conformational changes in either the N-terminal (around Cys-36) or C-terminal region (around Cys-190) of tropomyosin. The estimated apparent binding constant for caldesmon of around 4×10^6 M^{-1} agreed with our previous data from experiments done in the presence of 10 mM NaCl (Horiuchi & Chacko, 1988). Watson et al. (1990b) obtained a value of around $(5-7) \times 10^6$ M^{-1}, a value slightly higher than that from our study. This difference may be due to the difference in the fluorescence probe utilized and the separation of fluorescence signal emitted from the cysteine at two different positions in the present study. The biphasic nature of the fluorescence change in the N-terminal region observed upon interaction with CaD indicates the existence of a second CaD binding site. This finding is in agreement with the report by Watson et al. (1990a) that a weak binding site for caldesmon is present in the N-terminal half of the tropomyosin molecule.

While both N-terminal and C-terminal regions of tropomyosin change their conformations upon addition of CaD in the absence of actin (Figure 3A,B), only the N-terminal region (around Cys-36) shows a change in its conformation in the presence of actin (Figure 5). One possible explanation is that the regions on tropomyosin which interact with CaD in the absence and the presence of actin are different. It is also possible that caldesmon interacts with the same region of tropomyosin both in the presence and the absence of actin but that this tropomyosin region becomes very rigid when it binds to actin. Consequently, unlike the Cys-190 region, the Cys-36 region of tropomyosin may have some flexibility in the presence of actin to allow a change in its conformation upon interaction with CaD. An association of the conformational change in this region of tropomyosin with the inhibition of actin-activated ATPase activity of myosin suggests that this conformational change is compatible with the inhibition of ATPase activity.

At a 1:1 molar ratio of PIA-tropomyosin to CaD, the change in excimer fluorescence leveled off in the presence of actin (Figure 5). Since no excimer change was observed in the absence of actin under this condition (close to 50 mM ionic strength), the increase in the affinity of tropomyosin for CaD is likely due to actin. The possibility that the conformational change in tropomyosin occurred through actin cannot be ruled out. In skeletal muscle, it has been reported that the cooperative interactions between adjacent troponin–tropomyosin complexes may be transmitted through the actin filament (Butters et al., 1993). In the present study, the conformational change in tropomyosin leveled off at a 1:1 molar ratio of CaD to tropomyosin (Figure 5), while the binding of CaD to actin-tropomyosin did not (Figure 6). A comparison of the apparent binding constants for tropomyosin (8.45×10^6 M^{-1} from Figure 5) and actin-tropomyosin (2.97×10^6 M^{-1} from Figure 6) suggests that these bindings are independent, if the conformational change in tropomyosin by the addition of caldesmon directly correlates with the interaction between caldesmon and tropomyosin on the actin filament. As reported by Smith et al. (1987), the apparent binding constant of caldesmon for actin is an average of the high (4×10^7 M^{-1}) and low (5×10^5 M^{-1}) affinities. However, this high-affinity binding is too high to consider that this binding caused the conformational change in the tropomyosin molecule. Therefore, it is more likely that CaD interacts directly with tropomyosin on the actin filament, although it is difficult to distinguish the conformational change in tropomyosin induced by the direct interaction with CaD from an actin-mediated change. Interestingly, the apparent binding affinity of CaD_{1-717} for tropomyosin estimated from fluorescence changes is 7-fold weaker than that of the native molecule, whereas the affinity of CaD_{1-717} for actin is weakened only 2-fold, resulting in a similar affinity for both tropomyosin and actin (1.1×10^6 M^{-1} from Figure 5 and 1.33×10^6 M^{-1} from Figure 6, respectively). It is possible that the deletion of amino acid sequence 718–727 resulted in a caldesmon mutant devoid of specific binding site for tropomyosin.

In the absence of actin, the conformational change in tropomyosin induced by mutant CaD_{1-717} was only one-half of that shown by CaD_{1-727} and native CaD. Further deletion of 90 residues from the C-terminus of CaD_{1-717} does not significantly change the interaction between CaD and tropomyosin (Z. Wang, K. Y. Horiuchi, and S. Chacko, unpublished observation); thus, we conclude that the major tropomyosin binding site on CaD is located in residues 717–727. Redwood and Marston (1993) reported that a bacterially expressed CaD fragment from the C-terminal region containing 99 amino acids, including the residues 718–756, did not interact with tropomyosin. This apparent discrepancy may be caused by the inability of the bacterially expressed fragment to associate properly with the tropomyosin. It is also possible that a difference between the bacterially expressed fragment and the bacculovirus-expressed protein, which was subjected to post-translational modification in the Sf9 insect cells, in the folding could account for this discrepancy. The possibility that the diminished ability of our CaD mutant to induce conformational change is due to a modified folding of CaD_{1-717}, caused by the deletion of amino acid residues 718–727, is not ruled out. In this case, the residues that compose the site for tropomyosin binding in CaD is still intact, but they are unable to function properly because of modified folding. Residues 508–565 have been proposed as a possible tropomyosin-binding region, on the

basis of their sequence homology with the tropomyosin-binding region of troponin T (Bryan et al., 1989). However, our present data argue against the presence of a major tropomyosin binding site in residues 508−565. In this regard, it is interesting that residues 89−147 of troponin T, which have homology with CaD, react weakly with smooth muscle tropomyosin as compared with skeletal muscle tropomyosin (Sanders & Smiley, 1984). Thus the residues 508−565 of CaD may bind weakly to smooth muscle tropomyosin, possibly as a second weak binding site for tropomyosin.

CaD_{1-727} caused conformational change in tropomyosin both in the presence and the absence of actin, although it was slightly less in the presence of actin compared to that induced by native CaD. On the other hand, CaD_{1-717} caused only one-half of the conformational change exhibited by native CaD, either in the presence or the absence of actin. The partial conformational changes in tropomyosin bound on actin caused by both CaD mutants were associated with poor inhibitions of actin-activated ATPase activity of myosin (Figures 5 and 7). This result indicates that CaD_{1-727} contains a direct tropomyosin-binding region but lacks some region responsible for the full conformational change in tropomyosin that is bound to actin and the inhibition of ATPase activity.

In this study, we focused on the CaD−tropomyosin interaction in the presence and absence of actin. Although we identified a direct interaction between residues 718−727 of CaD and the Cys-190 region of tropomyosin, it is still unclear whether this is the only region that interacts with CaD in the presence of actin. Further analyses using cross-linking and/or point mutation studies are necessary to elucidate the mechanism for the tropomyosin-mediated enhancement of the inhibition of actin-activated ATPase activity by CaD.

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