Modulation of Actin Conformation and Inhibition of Actin Filament Velocity by Calponin†

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ABSTRACT: Calponin, an actin/calmodulin-binding protein present in smooth muscle thin filaments, modulates the actin−myosin interaction and actomyosin ATPase activity of smooth muscle myosin II. Binding of myosin heads to actin under conditions that produce weak or strong binding induces conformational changes in actin. Polarized fluorimetric measurements of rhodamine−phalloidin complex and 1,5-IAEDANS specifically linked to actin in myosin-free muscle fibers (ghost fibers) and to Cys-707 in myosin head, respectively, revealed conformational changes, as determined from the changes in orientation and mobility of fluorescent probes, upon addition of calponin to ghost fibers. The effect of calponin on conformational changes produced upon binding of phosphorylated or dephosphorylated heavy meromyosin (HMM) was also determined. Subfragment-1 preparations modified with NEM (NEM-S1) or pPDM (pPDM-S1) were used as models of strong and weak binding, respectively. Calponin changed both the orientation of fluorophores on the actin and the flexibility of the actin filaments, as determined from the angle between an actin filament and the fiber axis. Changes in the flexibility of actin filaments and the orientation of fluorophores produced by phosphorylated smooth muscle HMM were similar to those seen with NEM-S1, which formed a strong-binding association with actin and caused the transition of actin monomers to the "on" state; calponin markedly inhibited this effect. In contrast, pPDM-S1 and dephosphorylated HMM induced weak binding and the transition of actin monomers to the "off" state, and these effects were enhanced by calponin. Furthermore, calponin decreased the velocity of actin filament movement over skeletal muscle myosin Oγ phosphorylated smooth muscle myosin heads in an *in vitro* motility assay. These results suggest that calponin induces modulation of smooth muscle contraction by inhibiting the force-producing (strong-binding) state of cross-bridges and involves changes in actin conformation.

A major regulatory mechanism of actomyosin ATPase and contraction in smooth muscle is mediated by thick filaments via the Ca^{2+}-calmodulin-dependent phosphorylation of myosin regulatory light chains (LC_{20})[1] [Gorecka et al.,1976; Sobieszek & Small, 1977; Chacko et al., 1977; Dillon et al., 1981; Butler & Siegman, 1982; Barany et al., 1979; for a review, see Kamm and Stull (1985)]. Myosin light chain phosphorylation accelerates actin-activated myosin ATPase activity and the velocity of force generation by enhancing both the release of P_i from the AM-ADP-P_i complex and the cross-bridge cycling in smooth muscle cells (Sellers, 1985; Itoh et al., 1989). It has been proposed that two populations of myosin are present during the development and maintenance of force in smooth muscle: myosin heads with phosphorylated LC_{20} and high rates of ATP hydrolysis and cross-bridge cycling, and myosin heads with dephosphorylated LC_{20}, characterized by low rates of ATP hydrolysis and cross-bridge cycling (Butler & Sigman, 1982). The cross-bridges formed by the phosphorylated myosin molecules are in a force-producing state and form strong binding with actin, whereas deposphorylated myosin molecules form a non-force-producing, weak-binding state with actin. The change in the relative ratio of the two populations is considered to be an essential event in this regulation [Dillon et al., 1981; for review, see Kamm and Stull (1985), Hai and Murphy (1988), Walsh (1991), and Trybus (1991)]. However, there is some evidence that regulation of smooth muscle contraction does not depend entirely on phosphorylation of LC_{20} (Gerthoffer, 1987; Hai & Murphy, 1988; Siegman et al., 1989; Washabau et al., 1994). Thus, it has been suggested that other regulatory mechanisms such as the thin filament-associated mechanism act in concert with the LC_{20} phosphorylation system in smooth muscle contraction [for review, see Marston (1989), Chalovich (1992), and Chacko and Longhurst (1994)].

Calponin, a component of the thin filament in the smooth muscle cell (Takeuchi et al., 1991; Walsh et al., 1993), has been proposed to play a regulatory role in smooth muscle contraction. This protein binds with actin, tropomyosin, calmodulin, and myosin (Takahashi et al., 1986; Vancom-

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[1] Abbreviations: HMM, heavy meromyosin; S1, subfragment-1; LC_{20}, 20 000-dalton light chain of smooth muscle myosin; NEM, *N*-ethylmaleimide; pPDM, *N,N′-p*-phenylenedimaleimide; 1,5-IAEDANS, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid; DTT, dithiothreitol; AM, actomyosin.

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pernolle et al., 1990; Szymanski & Tao, 1993); inhibits the actin-activated Mg-ATPase activity (the cross-bridge cycling rate) of skeletal muscle myosin (Makuch et al., 1991), phosphorylated smooth muscle myosin (Winder & Walsh, 1990; Abe et al., 1990; Marston, 1991), and heavy meromyosin (HMM) (Horiuchi & Chacko, 1991); and decreases the actin filament movement over immobilized myosin in an *in vitro* motility assay (Shirinsky et al., 1992; Haeberle, 1994). Furthermore, calponin has an inhibitory effect on contraction of permeabilized smooth muscle strips from rabbit mesenteric arteries (Itoh et al., 1994), and it appears to decrease the rigidity of skinned muscle fibers during both rigor and relaxed states (Heizmann et al., 1994). Calponin-mediated inhibition of the cross-bridge cycling rate results from its interaction with actin, and is due to a reduction in the V_{max} for actin-activated Mg-ATP hydrolysis rather than an effect on the affinity of actin for myosin (Nishida et al., 1990; Horiuchi & Chacko, 1991; Miki et al., 1992). However, the molecular mechanism(s) for the regulation (or modulation) of the cross-bridge cycling rate by calponin in smooth muscle cells is not well understood.

Polarized fluorimetry has been demonstrated to be a highly sensitive method for monitoring conformational changes that occur in actin (Borovikov et al., 1974; 1991; Yanagida & Oosawa, 1978), myosin (Aronson & Morales, 1969; Borejdo & Putnam, 1977; Borovikov & Levitsky, 1989; Burghard & Ajtai, 1992; Andreev et al., 1995), and tropomyosin (Szczesna et al., 1989; Borovikov et al., 1993) in the course of muscle contraction. In this study, we used polarized fluorimetry of ghost fibers from skeletal muscle, free of myosin and other regulatory proteins but containing F-actin filaments organized as in the intact muscle cells, to determine the effects of calponin binding to actin in the thin filament on the conformational state of actin and myosin during their interaction. In addition, the effects of calponin binding to F-actin on the *in vitro* motility of actin filaments over skeletal and smooth muscle myosins were determined. We demonstrate that the binding of calponin to actin filaments changes the orientation of fluorophores on the actin and the flexibility of the actin filaments, as determined from the angle between an actin filament and the fiber axis. Changes in the flexibility of the actin filaments and the orientation of fluorophores produced by phosphorylated smooth muscle HMM are similar to those seen with NEM-S1, which forms strong binding with actin (Nagashima & Asakura, 1982) and causes the transition of actin monomers to the "on" state; calponin markedly inhibits this effect. In contrast, pPDM-S1 and dephosphorylated HMM induce the formation of weak binding (Chalovich et al., 1983) and the transition of actin monomers to the "off" state, and these effects are enhanced by calponin. Furthermore, calponin decreases the velocity of actin filament movement over skeletal muscle myosin Oγ phosphorylated smooth muscle myosin heads in the *in vitro* motility assay.

MATERIALS AND METHODS

Protein Preparation. Smooth muscle myosin was prepared from chicken gizzards and was thiophosphorylated using endogenous kinase followed by gel filtration on a Sepharose CL-4B agarose column (Chacko, 1981). Dephosphorylated myosin was purified as above after incubating the myosin at 25 °C for 5 min in phosphorylation buffer (60 mM KCl, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$, 15 mM imidazole-HCl, pH 7.1, and 2 mM DTT) in the absence of ATP. HMM was purified according to Kaminski and Chacko (1984). The level of phosphorylation of column-purified myosin and HMM were determined by urea gel electrophoresis (Perrie & Perry, 1970), and all of the preparations of myosin and HMM used for this study were found to be 95−97% phosphorylated. The residual phosphorylation in preparations of dephosphorylated myosin was 0−0.02 mol P_i per mg of LC. Tropomyosin and calponin were prepared from chicken gizzard smooth muscle (Chacko, 1981; Horiuchi & Chacko, 1991). Actin was purified from acetone powder of rabbit skeletal muscle by the methods of Pardee and Spudich (1982). Tropomyosin was premixed with actin at a molar ratio of 1:4 and dialyzed overnight. Rabbit skeletal muscle subfragment-1 (S1) was prepared according to Margossian and Lowey (1982) and chemically modified to produce analogues of the strong and weak actin-binding conformations. S1 that binds strongly to actin was made by labeling with *N*-ethylmaleimide (NEM), which induces loss of ATPase activity but leaves actin-binding ability intact (Reisler, 1982; Nagashima & Asakura, 1982). S1 that binds weakly to actin was prepared by labeling with *N*,*N*′-*p*-phenylenedimaleimide (pPDM) following the method of Chalovich et al. (1983). Subfragment-1 was also labeled with 1,5-IAEDANS for 24 h at 0 °C to obtain a reagent to protein molar ratio of 1:1 (Borejdo & Putnam, 1977). Calmodulin was purified from bovine brain powder (Sigma) as described (Dedman & Kaetzel, 1983). Protein concentration was determined by the method of Lowry et al. (1951) or by absorbance using the following extinction coefficients: $E_{290} = 0.63$ (mg/mL)$^{-1}$ cm^{-1} for actin and $E_{277} = 0.19$ (mg/mL)$^{-1}$ cm^{-1} for tropomyosin.

Preparation of Ghost Fibers. Ghost fibers were prepared from single glycerinated fibers of rabbit skeletal muscle by extraction of myosin and regulatory proteins as described (Borovikov & Gusev, 1983). Modification of F-actin in ghost fibers with fluorescent dye was carried out during incubation of the fibers in a standard solution containing 20 mM KCl, 1 mM $MgCl_2$, 6.7 mM phosphate buffer, pH 7.0, and 40 μM rhodamine−phalloidin. Unbound dye was removed by washing the fibers in standard solution.

Binding of Calponin, Tropomyosin, HMM, and S1 to Ghost Fibers. Proteins were incorporated by immersion of ghost fibers into buffer containing 20 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl buffer, pH 6.8 and 1−2.5 mg/mL of the respective proteins (Nowak et al., 1989). To eliminate possible interaction of calponin with HMM, calponin was incorporated into the fibers before HMM. Incorporation of each protein was confirmed by SDS−PAGE (Laemmli, 1970). The molar ratios of calponin, tropomyosin, NEM-S1, pPDM-S1, and HMM bound to actin in ghost fibers were approximately 1:2.5 (±0.5), 1:6.5 (±0.5), 1:5 (±2), 1:5 (±2), and 1:10 (±2), respectively, as determined by densitometric scanning of the gels (UltroScan XL, Pharmacia LKB).

In Vitro Motility Assay. The *in vitro* motility assay was performed according to Warshaw et al. (1990) using a computer-assisted video imaging system for tracking filaments (Vermont Tech). Myosin was used in monomeric form (0.3 M KCl) at 250 μg/mL. The assay was performed in 25 mM KCl, 1 mM EGTA, 4 mM $MgCl_2$, 1 mM ATP, 25 mM imidazole (pH 7.4), 5 mM DTT, and 0.5% methyl cellulose to prevent Brownian motion. An oxygen scavenger

system (0.1 mg of glucose oxidase/mL, 0.018 mg of catalase/mL, and 2.3 mg of glucose/mL) was employed to reduce fluorescence photobleaching (Kishino & Yanagida, 1988). Before the assay, actin or actin−tropomyosin filaments (40 μg of actin/mL) were fluorescently labeled overnight with an equal molar ratio of tetramethylrhodamine isothiocyanate−phalloidin (TRITC−phalloidin: Sigma Chemical Co., St. Louis, MO) following the method of Kron and Spudich (1986). Calponin was added to actin filaments to obtain a molar ratio of 0.4 followed by overnight labeling with the fluorescent compound. The stoichiometries of calponin and tropomyosin bound to actin filaments were confirmed as described (Horiuchi & Chacko, 1995).

Fluorescence Polarization Measurements. The polarized fluorescence from 1,5-IAEDANS-labeled S1 was recorded at 480−550 nm after excitation at 365 ± 5 nm, and the fluorescence from rhodamine−phalloidin-labeled F-actin was recorded at 500−600 nm after excitation at 489 ± 5 nm. The intensities of the four components of polarized fluorescence were measured in parallel ($_{\parallel}I_{\parallel}$, $_{\parallel}I_{\perp}$) and in perpendicular ($_{\perp}I_{\perp}$, $_{\perp}I_{\parallel}$) orientation of the fiber axis to the polarization plane of the exciting light. From these four components, we calculated the degree of fluorescence polarization ($P_{\parallel}$ and $P_{\perp}$), the angles of the absorption (Φ_A), and emission (Φ_E) dipoles of the fluorophore relative to the long axis of F-actin and the average angle between the F-actin axis and the fiber long axis ($\Theta_{1/2}$). The theoretical basis for these calculations has been described (Yanagida & Oosawa, 1978; Wilson & Mendelson, 1983; Kakol et al., 1987; Andreev et al., 1995). Changes in the polarization parameters (Φ_A, Φ_E, and $\Theta_{1/2}$) are interpreted in terms of structural alteration of proteins in the region of fluorophore localization. The type of Φ_A changes was similar to the corresponding changes of Φ_E in all experiments; therefore, the values of Φ_A are not presented.

The significance of the differences observed was determined by Student's *t*-test.

RESULTS

Conformational Changes of Rhodamine−Phalloidin-Labeled F-Actin in Ghost Muscle Fibers

Ghost fibers were characterized by high levels of polarized fluorescence anisotropy of rhodamine−phalloidin complex bound to F-actin. The degree of fluorescence polarization was a high positive value ($P_{\parallel}$) when a fiber was oriented parallel to the polarization plane of the exciting light ($P_{\parallel} = 0.512 \mp 0.03$, $n = 60$), and a high negative value ($P_{\perp}$) when a fiber was oriented perpendicularly to this plane ($P_{\perp} = -0.422 \mp 0.02$, $n = 60$). These findings are in agreement with previous reports indicating that the rhodamine−phalloidin molecules are fixed on actin filaments in an ordered helical array and that the rigidity of the rhodamine−phalloidin-bound actin filaments is high (Kakol et al., 1987; Galazkiewicz et al., 1987).

The angles Φ_E and $\Theta_{1/2}$ in actin fibers ($n = 23$) were 39.0 ± 0.1° and 13.4 ± 0.2°, respectively. Incorporation of calponin into actin filaments of the ghost fiber (calponin:actin = 1:2.5) resulted in increases of Φ_E and $\Theta_{1/2}$ values to 40.7 ± 0.1° and 14.5 ± 0.2°, respectively (Figure 1A,B). Maximum changes in those polarization parameters were observed when the calponin-to-actin molar ratio was 1:7. Further increases in the calponin concentration up to a molar ratio of 1:1 did not cause additional changes in the polarization parameters (data not shown).

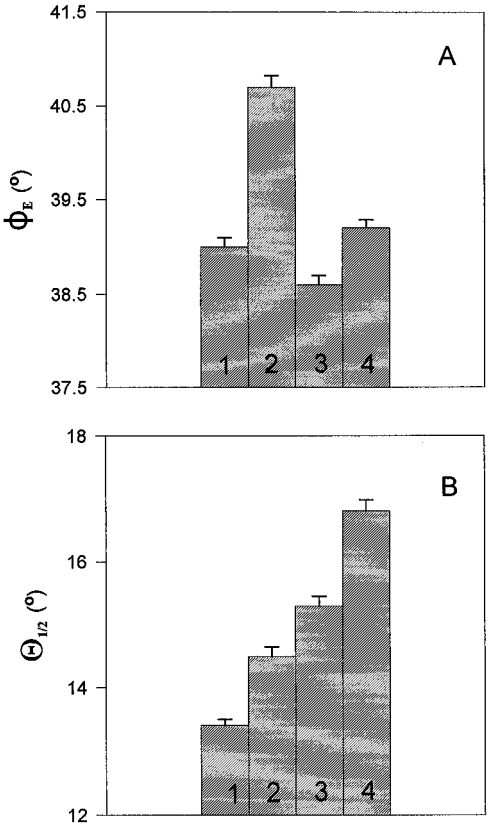

FIGURE 1: Effect of calponin on the conformational state of rhodamine−phalloidin-labeled F-actin in the presence or absence of tropomyosin in ghost fibers. Angle of the emission dipole of the dye relative to the F-actin axis (Φ_E) and the average angle between the F-actin axis and the fiber long axis ($\Theta_{1/2}$) were calculated as described in Materials and Methods. The preparation of the fibers, their composition, and the conditions of the experiments are given in Materials and Methods. Data represent means of at least 15 ghost fibers for each experimental condition. Bars 1−4 are F-actin, F-actin and calponin, F-actin and tropomyosin, and F-actin and tropomyosin and calponin, respectively. Error bars indicate ±SEM. There is a significant difference between F-actin (lane 1) and F-actin plus tropomyosin (lane 3) in the values of Φ_E and ($\Theta_{1/2}$), and these values are altered significantly by calponin (P is <0.05).

In the presence of tropomyosin, the flexibility of the thin filament increased, as indicated by a $\Theta_{1/2}$ value of 15.3 ± 0.2° compared with 13.4 ± 0.2° in the absence of tropomyosin (Figure 1B), but the value of Φ_E decreased from 39.0 ± 0.1° to 38.6 ± 0.1° in the presence of tropomyosin (Figure 1A). Calponin increased the flexibility of actin filaments even in the presence of tropomyosin, as indicated by an increase in $\Theta_{1/2}$ to 16.8 ± 0.2°. This was accompanied by an increase in Φ_E value both in the presence or the absence of tropomyosin (Figure 1A), indicating a low-level inhibition of the calponin-induced effect in the presence of tropomyosin.

In other experiments, actin filaments in ghost fibers were labeled with either NEM-S1 or pPDM-S1, respectively, to produce the strong or weak binding of myosin heads to actin. These fiber preparations were used to determine the effect of calponin on the strong binding (force-producing) and weak binding (non-force-producing) between myosin and actin. In the presence of NEM-S1 (NEM-S1:actin = 1:5), the Φ_E angle decreased by 1.5° and the value for $\Theta_{1/2}$ angle

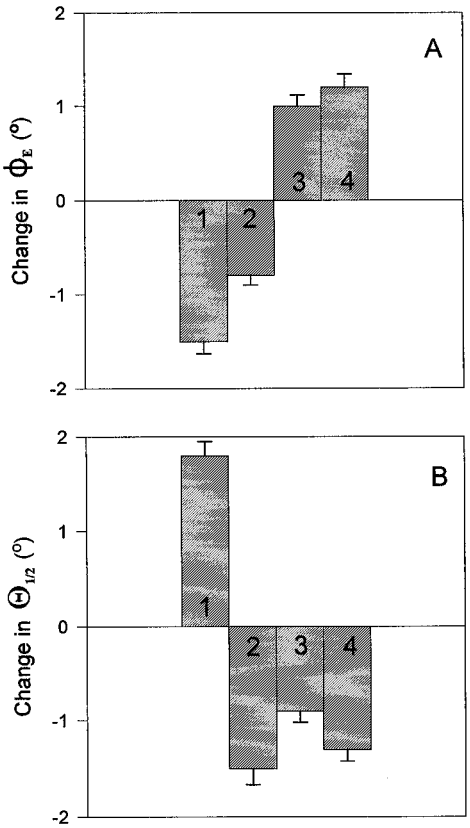

FIGURE 2: Effect of calponin on NEM-S1- or pPDM-S1-induced structural rearrangement of rhodamine−phalloidin-labeled F-actin in ghost fibers. Changes in the values of Φ_E (A) and $\Theta_{1/2}$ (B) were calculated as the difference between the respective values determined for F-actin before and after the incorporation of NEM-S1 or pPDM-S1 to ghost fibers with or without bound calponin. Data represent means of at least 25 ghost fibers for each experimental condition. Conditions are given in Materials and Methods. Bars 1−4 are NEM-labeled myosin subfragment-1, NEM-labeled myosin subfragment-1 in the presence of calponin, pPDM-labeled myosin subfragment-1, and pPDM-labeled myosin subfragment-1 in the presence of calponin, respectively. Error bars indicate ±SEM. The molar ratios of NEM-S1, pPDM-S1, and calponin to actin were in the range of 1:5 (±2), 1:5 (±2), and 1:2.5 (±0.5), respectively. Alterations in the values of Φ_E and $\Theta_{1/2}$ in all cases, except the increase in the value of Φ_E for pPDM-labeled subfragment 1 in the presence of calponin (lanes 3 and 4 in Figure 2A), are significant (P is <0.05).

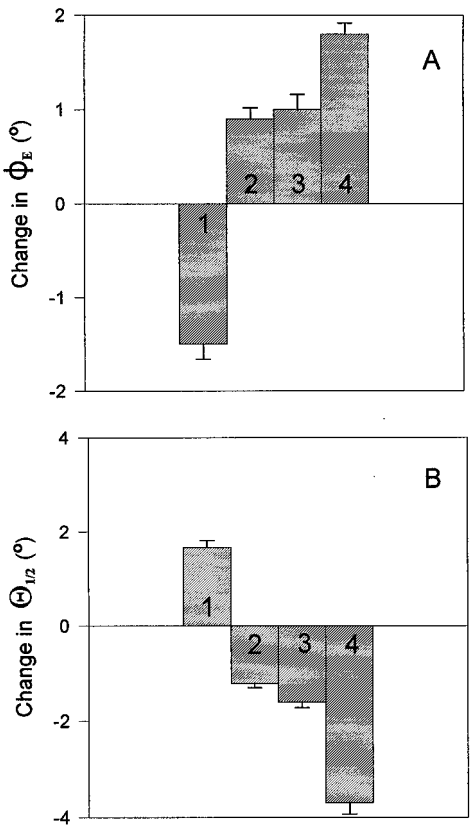

FIGURE 3: Effect of calponin on phosphorylated HMM- or dephosphorylated HMM-induced structural rearrangement of rhodamine−phalloidin-labeled F-actin in ghost fibers. Changes in Φ_E and $\Theta_{1/2}$ were calculated as the difference between the respective values determined for F-actin before and after incorporation of phosphorylated HMM (pHMM) or dephosphorylated HMM (dpHMM) in ghost fibers with or without bound calponin. Data represent means of at least 25 ghost fibers for each experimental condition. For conditions, see Materials and Methods. Bars 1−4 represent pHMM, pHMM in the presence of calponin, dpHMM, and dpHMM in the presence of calponin, respectively. Error bars indicate ±SEM. The molar ratios of pHMM, dpHMM and calponin to actin were in the range of 1:10 (±2), 1:10 (±2), and 1:2.5 (±0.5), respectively. The alterations in the values of Φ_E and ($\Theta_{1/2}$) with pHMM and dpHMM induced by calponin are significant (P is <0.05).

increased by about 1.8° (Figure 2), indicating that the labeling of thin filaments with NEM-S1 results in strong binding between actin and myosin heads and an increase in the relative number of actin monomers in the "on" state (Yanagida & Oosawa, 1978; Dobrowolski et al., 1989). In the presence of calponin, actin filaments decorated with NEM-S1 showed a 2-fold decrease in the Φ_E as compared to the corresponding value without calponin. The change in the $\Theta_{1/2}$ value showed a complete reversal of the effect of NEM-S1 on the actin filament in the presence of calponin, indicating a decrease in strong binding.

On the other hand, binding of pPDM-S1 (pPDM-S1:actin = 1:5) to actin in ghost fibers induced an increase in Φ_E by 1.0°, whereas $\Theta_{1/2}$ decreased by 0.9° (Figure 2A,B), indicating an increase in the relative number of actin monomers in the "off" state in thin filaments (Borovikov et al., 1991). Calponin slightly increased the changes in these parameters.

Since phosphorylation of the myosin light chain accompanies force development and cross-bridge cycling [Dillon et al., 1981; Butler & Siegman, 1983; for review, see Kamm and Stull (1985)], the phosphorylation of myosin light chain is likely to produce strong binding of myosin heads to actin [for review, see Trybus (1991) and Chalovich (1992)]. Phosphorylated HMM induced a decrease in Φ_E by 1.5° and an increase in $\Theta_{1/2}$ values by 1.7° (Figure 3), similar to the changes observed with NEM-S1. In the presence of calponin, Φ_E increased by 0.9° and $\Theta_{1/2}$ decreased by 1.2° (Figure 3), suggesting that calponin reverses the changes in polarization parameters characteristic of strong binding induced by phosphorylated HMM. The changes in Φ_E and $\Theta_{1/2}$ in the presence of calponin are typical of those found with pPDM-S1 (Figure 2), which produces weak binding.

In contrast, binding of dephosphorylated HMM to actin produced an increase in Φ_E of 1.0° and a decrease in $\Theta_{1/2}$ of 1.6°, similar to the values obtained with pPDM-S1 (Figure 3). In the presence of calponin, changes in Φ_E and $\Theta_{1/2}$ were 2-fold higher and lower, respectively, as compared with dephosphorylated HMM without calponin.

In another series of experiments, tropomyosin was first bound to actin filaments, and the effects on the polarized fluorescent parameters upon binding of NEM-S1, pPDM-

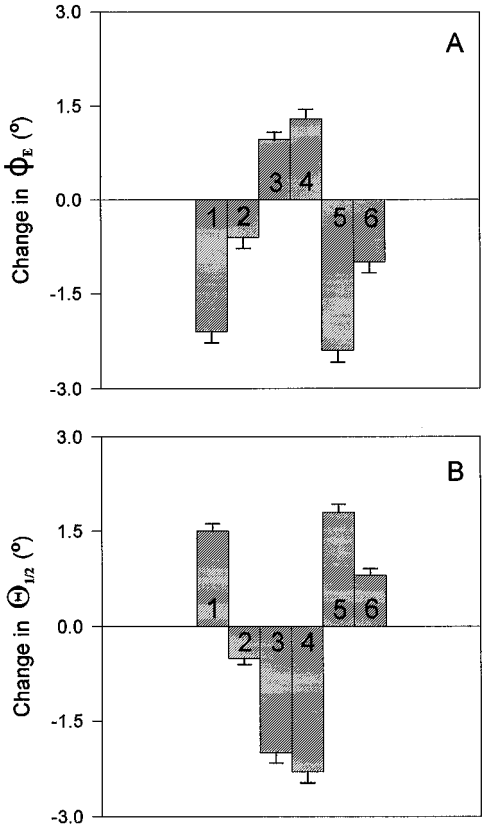

FIGURE 4: Effect of calponin on myosin head-induced structural rearrangement of fluorescein-5-maleimide-labeled F-actin in ghost fibers in the presence of tropomyosin. Changes in Φ_E and $\Theta_{1/2}$ were calculated as the difference between the respective values determined for F-actin before and after incorporation of NEM-S1, pPDM-S1, or pHMM in ghost fibers with or without bound calponin. Data represent means of at least 25 ghost fibers for each experimental condition. Bars 1−6 are NEM-S1, NEM-S1 in the presence of calponin, pPDM-S1, pPDM-S1 in the presence of calponin, pHMM, and pHMM in the presence of calponin, respectively. Error bars indicate ±SEM. The values of Φ_E and ($\Theta_{1/2}$) with NEM-S1, pPDM-S1, and pHMM were significantly altered by calponin (P is <0.05). The molar ratios of NEM-S1, pPDM-S1, pHMM, and calponin to actin were the same as in Figures 2 and 3.

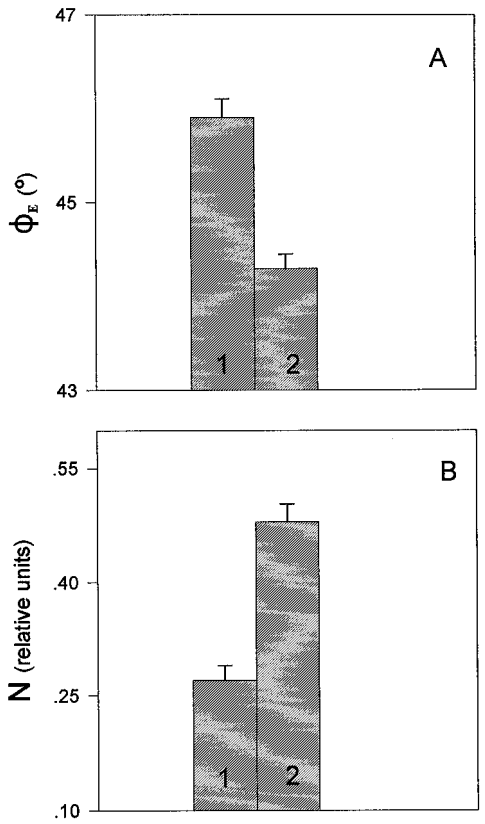

FIGURE 5: Effect of calponin on the conformational state of 1,5-IAEDANS-labeled myosin subfragment-1 in ghost fibers. Angle of the emission dipole of the dye relative to the F-actin axis (Φ_E) and amount of the randomly oriented fluorophores (N) were calculated as described in Materials and Methods. Data represent means of at least 25 ghost fibers for each experimental condition. Bars 1 and 2 represent F-actin labeled with 1,5-IAEDANS−labeled S1 in the absence or in presence of calponin, respectively. Error bars indicate ±SEM. The alterations in the values of Φ_E and ($\Theta_{1/2}$) induced by calponin are significant (P is <0.05). Molar ratios of 1,5-IAEDANS−S1 and calponin to actin were in the range of 1:5 (±2) and 1:2.5 (±0.5), respectively.

S1, or phosphorylated HMM to actin in the presence or absence of calponin were tested. In the presence of tropomyosin, calponin had only a minimal effect on reversing the strong binding by NEM-S1, since the change in Φ_E induced by calponin in the presence of tropomyosin was less than that in its absence (compare Figure 2A with 4A), whereas it modulated pPDM-S1-induced effects regardless of tropomyosin. When calponin and tropomyosin were present, Φ_E decreased (1.0°) with phosphorylated HMM, whereas in the absence of tropomyosin, an increase (0.8°) was observed (compare Figure 3A with 4A). Thus, calponin in the presence of tropomyosin does not induce the same pronounced inhibition of strong binding between myosin heads and actin as it does in the absence of tropomyosin.

Effect of Calponin on the Conformational State of the Actomyosin Complex

The conformational state of myosin in the actomyosin complex has been studied previously using polarized fluorescence of 1,5-IAEDANS which specifically binds to the reactive thiol group (Cys-707) in the heavy chain of myosin subfragment-1 (Borejdo & Putnam, 1977; Nowak et al., 1989). The angles of absorption (Φ_A) and emission (Φ_E) dipoles of the fluorophore relative to the muscle fiber axis and the number of randomly oriented fluorophores (N) can be used to test the type of binding between the myosin head and actin and the rigidity of actomyosin cross-bridges. High Φ_E and low N values have been taken as evidence for the high rigidity of actomyosin cross-bridges, resulting from the formation of the strong binding (rigor state) between actin and S1 (Nowak et al., 1989). In contrast, formation of the weak binding of myosin head to actin and the corresponding high flexibility of actomyosin cross-bridges result in decreased Φ_E angles and increased N values (Borovikov et al., 1991). The 1,5-IAEDANS−S1 has been shown to form strong binding with actin (rigor state) in the absence of ATP (Borejdo & Putnam, 1977; Nowak et al., 1989).

The fluorescence polarization of 1,5-IAEDANS−S1-labeled ghost fibers is characterized by pronounced anisotropy, since $P_{||}$ and $P_{\perp}$ are different ($P_{||} = 0.411 \pm 0.003$ and $P_{\perp} = 0.132 \pm 0.002$, $n = 25$). The values of Φ_E and N in ghost fibers labeled with 1,5-IAEDANS−S1 (molar ratio 1:5) were 45.9 ± 0.2° and 0.27 ± 0.02, respectively (Figure 5A,B). In the presence of calponin, the value of Φ_E decreased to 44.3 ± 0.2° and the value of N increased to 0.479 ± 0.024 (about 2-fold) as shown in Figure 5A,B. This

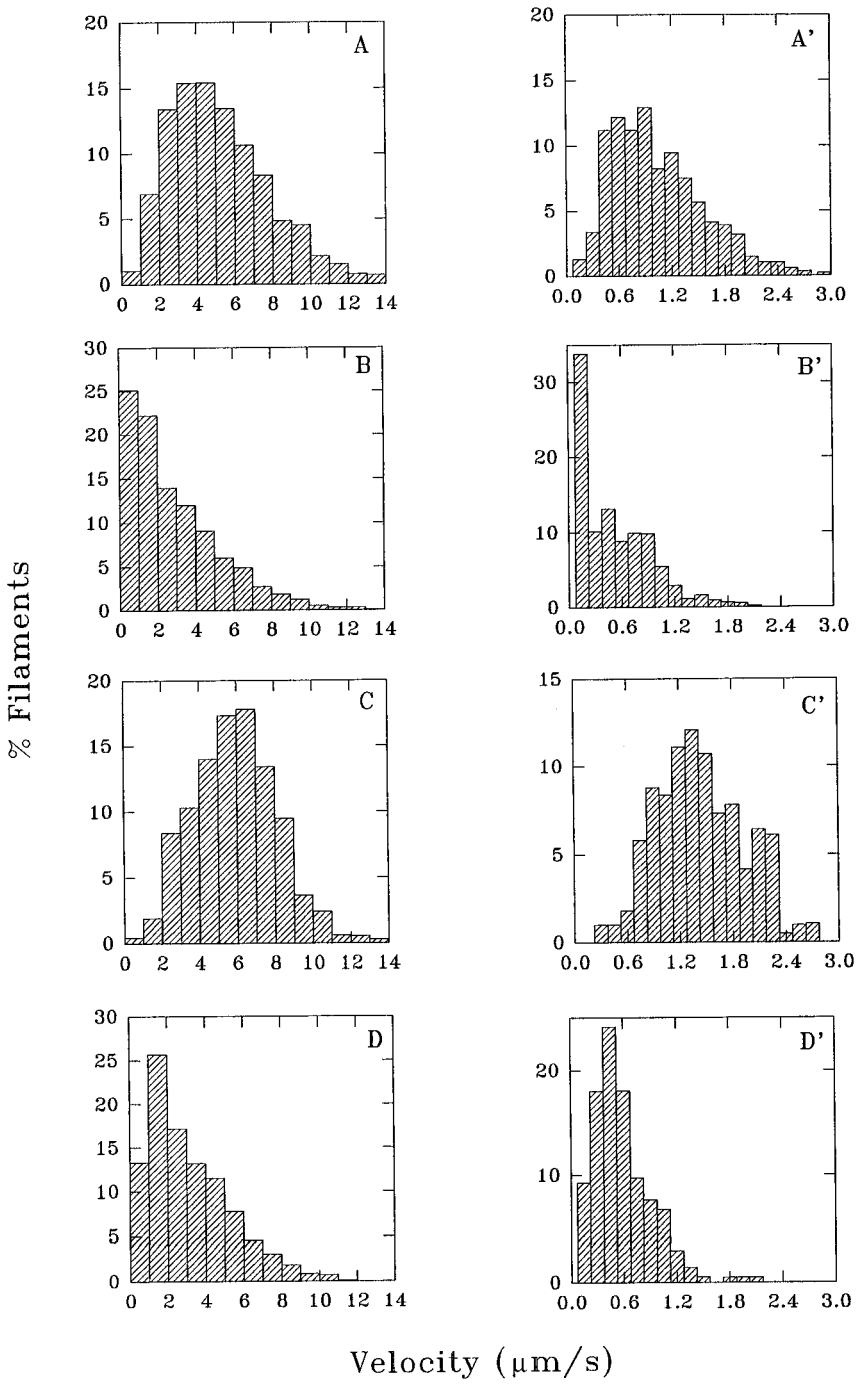

FIGURE 6: Distribution of velocities of actin filaments sliding over skeletal muscle myosins (left panel) and smooth muscle myosin (right panel). Histograms of the velocity of the distribution of actin filaments sliding in the absence or in presence of tropomyosin and/or calponin are shown. Data represent means of at least 2000 actin filaments for each experimental condition with standard deviation and standard error less than ±1.7 and ±0.05 μm/s, respectively (left panels), and means of at least 1800 actin filaments with standard deviation and error less than ±0.5 and ±0.01 μm/s, respectively (right panels). Conditions were 25 mM KCl, 1 mM EGTA, 4 mM $MgCl_2$, 1 mM ATP, 25 mM imidazole (pH 7.4), 5 mM DTT, 0.1 mg of glucose oxidase/mL, 0.018 mg of catalase/mL, 2.3 mg of glucose/mL, and 0.5% methyl cellulose (30 °C). The molar ratios of tropomyosin and calponin bound to actin were in the range of 1:6.5 (±0.5) and 1:2.5 (±0.2), respectively. Panels A and A', F-actin; panels B and B', F-actin plus calponin; panels C and C', F-actin plus tropomyosin; and panels D and D', F-actin plus calponin and tropomyosin.

indicates that calponin switches the binding of myosin heads to actin from strong to weak binding.

Effect of Calponin on the Velocity of Actin Filaments in the in Vitro Motility Assay

Figure 6A and A' show the distribution of the velocities of actin filaments in the *in vitro* motility assay using skeletal muscle myosin and phosphorylated smooth muscle myosin, respectively. The average moving velocity was 4.79 ± 1.73 μm/s (n = 2117) with skeletal muscle myosin and 0.99 ± 0.57 μm/s (n = 2050) with smooth muscle myosin, in agreement with published data (Umemoto & Sellers, 1990; Warshaw et al., 1990; Haeberle, 1994). The effect of calponin on the velocity of actin filaments at a 1:2.5 molar ratio of calponin to actin is depicted in Figure 6B and B'. In the presence of calponin, the average moving velocities decreased to 2.38 ± 0.67 μm/s (n = 2907) for skeletal muscle myosin and 0.45 ± 0.32 μm/s (n = 2071). Hence, calponin

inhibits the *in vitro* motility of actin filaments with both skeletal muscle myosin and phosphorylated smooth muscle myosin.

Tropomyosin increased the velocity of actin filaments in the absence of calponin (Figure 6C and C') in both skeletal and smooth muscle myosin. The average filament moving velocity increased from 4.79 ± 1.73 μm/s (n = 2117) to 5.40 ± 1.85 μm/s (n = 2457) for skeletal myosin and from 0.99 ± 0.57 μm/s (n = 2050) to 1.55 ± 0.59 μm/s (n = 2673) for smooth muscle myosin. Tropomyosin slightly weakened the effect of calponin (compare Figure 6B and D with B' and D').

DISCUSSION

Calponin, a thin filament-associated protein, is thought to modulate the regulation of smooth muscle contraction via phosphorylation−dephosphorylation, since it inhibits the actin-activated ATPase activity of isolated myosin (Nishida et al., 1990; Winder & Walsh, 1990; Abe et al., 1990; Horiuchi & Chacko, 1991; Makuch et al., 1991) and decreases the velocity of the actin filament sliding over myosin heads (Shirinsky et al., 1992; Haeberle, 1994). Thus, it becomes important to determine whether it exerts conformational changes in the actin filament and causes the "on/off" transition of actin monomers that is associated with weak- and strong-binding of myosin heads to actin.

A rise in Φ_E and $\Theta_{1/2}$ indicates a calponin-induced conformational change in actin monomers, resulting from the alterations in fluorophore orientation and from an increase in actin filament flexibility; these conformational changes occur at sites within and among actin monomers (Yanagida & Oosawa, 1978; Prochniewicz-Nakayama et al., 1983; Galazkiewicz et al., 1987). The binding of smooth muscle tropomyosin to actin also changes the polarized fluorescence parameters; an increase in thin filament flexibility ($\Theta_{1/2}$ increased by 14.2%) and a decrease in the value of Φ_E (by 1.0%) were observed, rather than the marked increase seen with calponin (Figure 1).

Tropomyosin does not alter the type of polarization parameters observed with calponin, indicating that the structural state of actin in the presence of calponin is not altered. In the presence of tropomyosin, calponin increased the flexibility of actin filaments ($\Theta_{1/2}$ increased by 9.8%) but not the Φ_E value (Figure 1). Apparently, conformational changes in actin caused by calponin are similar in the presence or absence of tropomyosin. A difference in the conformational changes in actin filaments observed upon addition of calponin and tropomyosin may reflect a difference in the binding sites of these proteins on the actin filaments, although these binding sites partially overlap (Kabsch et al., 1990; Mezgueldi et al., 1992).

Comparative analysis of Φ_E and $\Theta_{1/2}$ changes (Figures 2 and 3) indicated that both NEM-S1 and phosphorylated HMM induce a decrease in the Φ_E angle (by 4.0% and 4.2%, respectively) and an increase in $\Theta_{1/2}$ (by 13% and 12.4%, respectively) upon binding to actin in ghost fibers. Thus, the labeling of actin filaments with phosphorylated HMM or NEM-S1 results in a strong-binding interaction between actin and myosin heads. Furthermore, the relative number of actin monomers in the "on" state in the fiber is increased with both NEM-S1 and phosphorylated HMM. Calponin inhibited the strong binding between myosin heads and actin, whereas tropomyosin did not (Figure 2). Tropomyosin does hasten the conformational changes in actin monomers induced by the strong binding between actin and myosin (Dobrowolski et al., 1988). These conformational changes are accompanied by a pronounced increase in the relative amount of actin monomers in the "on" state, a conformational state that is thought to underlie the potentiation of actin-activated ATPase of myosin in the presence of tropomyosin (Eaton et al., 1975; Chacko et al., 1977; Small & Sobieszek, 1977; Chacko, 1981; Chacko & Eisenberg, 1990).

In the experiments with NEM-S1, the changes in Φ_E in the presence of calponin were about one-half the magnitude of those found in its absence. The value of $\Theta_{1/2}$ was decreased (by 11.0%), in contrast to the increase seen when calponin was absent (Figure 2). The calponin-induced inhibition of the polarization parameter changes typical of strong binding suggests a decreased ability of some NEM-modified myosin heads to form a strong-binding complex with actin, and thus the weakened ability of these preparations to raise the relative amount of actin monomers in thin fibers to the "on" state. Thus, calponin appears to transform the binding of myosin heads to actin from a strong-binding state to a state compatible with weak binding.

As a model for the weak-binding state, we used the F-actin complex with pPDM-S1. A report by Levitsky et al. (1992) shows that pPDM cross-linked to the SH1 and SH2 groups (Cys-707 and Cys-697, respectively) of S1 heavy chain, as well as the presence of ATP or ADP-P_i at the active site of S1, may cause structural changes at the region that is responsible for strong binding to actin, thus allowing only weak binding between myosin heads and actin. The structural changes in the strong-binding site induced by pPDM are local and most probably concern solely this site, whereas those induced by nucleotides result in conformational changes of the whole S1 molecule (Levitsky et al., 1992). Although pPDM-S1 itself may not be a good structural analogue of the S1-ATP or S1-ADP-P_i complexes (Levitsky et al., 1992), pPDM-S1 binds weakly to F-actin (Chalovich et al., 1983), and the orientation of pPDM-modified S1 relative to actin filament is the same as that of native S1 in the presence of ATP (Flicker et al., 1991). Therefore, we utilized pPDM-S1 as a model for weak binding between myosin heads and actin. To be consistent in using modified S1s for both weak and strong binding, the NEM-S1 was used, instead of S1, for strong-binding conditions.

In the experiments with pPDM-S1 and dephosphorylated HMM, the Φ_E values increased by 2.6% and 3.0%, respectively, and the value of $\Theta_{1/2}$ decreased (by 6.8% and 12.0%, respectively) (Figures 2 and 3), in contrast to the changes observed with NEM-S1. These results indicate that pPDM-S1 and dephosphorylated HMM induce an increase in the relative amount of actin monomers in the "off" state in thin filaments. It is possible that only weak binding of myosin heads with actin occurs in this case. Dephosphorylation of the myosin light chain may affect the conformation of the hinge region of myosin, favoring weak binding between myosin heads and actin monomers, whereas phosphorylation of the regulatory light chains significantly changes the type of actin−myosin interaction from weak to strong binding.

Calponin appears to exert a marked inhibitory effect on strong myosin binding to actin but does not affect the weak binding between these proteins, since the changes in Φ_E and $\Theta_{1/2}$ obtained with pPDM-S1 in the presence or absence of

calponin (Figure 3) were typical of the weak-binding complex of myosin heads and actin.

When calponin was bound to actin in ghost fibers decorated with S1 modified with 1,5-IAEDANS, the value of Φ_E decreased (by 3.5%) and the value of *N* markedly increased (by 177%) (Figure 5). Similar types of changes in Φ_E and *N* were observed previously with relaxed skeletal muscle fibers, in which myosin heads form weak-binding complexes with actin (Borejdo & Putnam, 1977; Borovikov et al., 1991). Thus, the conformational changes in actin induced by calponin (Figures 2 and 3) might be transmitted to S1, causing conformational changes in its heavy chain and, in turn, changing the flexibility of the actin−myosin bonds. Apparently, in the presence of calponin, only weak binding (or a binding compatible with weak binding) between myosin heads and actin takes place.

Calponin decreased the velocity of thin filament sliding over myosin (Figure 6), possibly by modifying the kinetics of actin-activated ATP hydrolysis by myosin. Since the presence of calponin alters the actin−myosin interaction, some cross-bridges that form act as an internal load, slowing down the fast-cycling phosphorylated cross-bridges (Washaw et al., 1990). Because weak binding switches actin monomers to the "off" state and these cross-bridges cannot produce force, the velocity of the thin filament decreases. Thus, the calponin-induced decrease in filament velocity may rest in formation of weak-binding complexes between myosin heads and actin. Upon binding to actin filaments that have formed cross-bridges with dephosphorylated myosin heads, calponin caused a remarkable increase in the relative amount of actin monomers in the "off" state (Figure 4). Since switching the actin monomers to the "off " state is followed by a marked increase in the rigidity of thin filaments (as shown by a decrease in $\Theta_{1/2}$), it is likely that the cross-bridges leaning against a rigid thin filament will develop more strength than those braced against a flexible one. An increase in isometric force (strength) in the presence of calponin has been reported (Haeberle, 1994).

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