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Calponin Binds to the 20-Kilodalton Regulatory Light Chain of Myosin[†]

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ABSTRACT: Calponin (CaP) is a 34 kDa smooth muscle-specific protein that has been implicated in regulation of smooth muscle contractility. Two CaP binding sites on smooth muscle myosin rod have been recently described [Szymanski and Tao (1997) *J.Biol.Chem. 272*, 11142−11146]. We used a combination of cosedimentation, overlay, and fluorescence assays to determine the interaction between CaP and both subfragment 1 of myosin and isolated 20 kDa regulatory light chain of myosin (RLC). Subfragment 1, which was generated by cleavage of myosin with *Staphylococcus aureus* protease (myosin $S1_{SA}$) inhibits cosedimentation of CaP with myosin filaments. Fluorescence assay showed that CaP labeled with fluorescent label (DAN−CaP) interacts with myosin $S1_{SA}$ in solution via a single class of binding sites. The binding constant (k_{aff}) of this interaction at 50 mM NaCl is $(2.1 \pm 0.2) \times 10^6\ M^{-1}$ ($n = 3$). The interaction between DAN−CaP and myosin $S1_{SA}$ depends on ionic strength, and the EC_{50} of inhibition of this interaction occurs at about 130 mM NaCl. In contrast, the subfragment 1 that was generated by papain digestion (myosin $S1_{PA}$), which cleaves RLC 4 kDa away from the NH_2-terminal end of the molecule, does not interact with DAN−CaP. Overlay and fluorescent assay in solution showed that CaP binds to isolated RLC, suggesting that the interaction between CaP and subfragment 1 of myosin is due to a direct binding of CaP to RLC. CaP binding to myosin $S1_{SA}$ is stronger than to subfragment 2 in physiological salt concentrations. CaP binding to myosin head strengthened upon phosphorylation of RLC by Ca^{2+}/calmodulin-dependent myosin light chain kinase. We suggest that CaP binds to subfragment 1 of myosin, exclusively via the NH_2-terminal end of RLC, and this interaction could play a role in regulation of the actin−myosin interaction in smooth muscle contractility.

Calponin (CaP) is a 34 kDa smooth muscle-specific protein that may be involved in regulation of smooth muscle contractility. Recently Malmqvist et al. (*1*) have shown that smooth muscle cells with mutated 20 kDa regulatory light chain of myosin (RLC), which cannot be phosphorylated, shorten and develop isometric force. Addition of CaP to the cells with mutated RLC produced complete relaxation of these cells. These authors suggested that CaP suppresses slowly cycling unphosphorylated myosin cross-bridges leading to the relaxed state of the muscle. The inhibitory action of CaP on smooth muscle contraction is also supported by the observations that CaP pretreatment in permeabilized ferret aortic smooth muscle cells blocked contraction due to phenylephrine or protein kinase C (PKCϵ) activation that caused smooth muscle contraction in the absence of any change in RLC phosphorylation (*2*).

In contrast to mutated RLC, CaP does not appear to affect force generation in cells with irrreversibly phosphorylated RLC (*1*). Moreover, in permeabilized smooth muscle fibers, which are precontracted with phosphorylation of myosin by ATPγS, CaP causes a decrease in shortening velocity but not in the generation of isometric force (*3*, *4*). It was suggested that in the presence of RLC phosphorylation, CaP causes force maintenance at a disproportionately reduced actomyosin ATPase activity—a phenomenon that is characteristic for latch contraction. These conclusions are supported by studies of Haeberle (*5*), who has shown that in an in vitro motility assay, with thiophosphorylated myosin, CaP decreases filament velocity and increases force exerted on stationary actin filaments. It was suggested that CaP may act to inhibit the rate of dissociation of higher affinity actomyosin complexes (*5*). Thus, it appears that whether CaP causes smooth muscle relaxation or force maintenance depends on the state of RLC phosphorylation.

CaP has been shown to bind to actin and inhibit the actin-activated Mg^{2+}ATPase activity of myosin (*6*−*11*), probably by causing conformational change in the actin molecule (*12*). This view is supported by the observation that CaP suppresses V_{max} rather than decreases actin's affinity to myosin heads (*5*, *7*, *13*). It has also been shown that binding of one CaP molecule to every three actin monomers causes maximal (80%) inhibition of the actin-activated myosin subfragment 1 Mg^{2+}-ATPase, without interfering with binding of subfragment 1 to F-actin (*14*). Moreover, the binding of CaP to actin and its inhibitory effect on Mg^{2+}-ATPase are not enhanced by tropomyosin (*13*), and this mechanism of CaP inhibition of Mg^{2+}-ATPase of actomyosin is different from that of troponin−tropomyosin and caldesmon. All these observations raise the possibility that CaP may interact with actin as well as myosin. Our recent studies showed the

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interaction between CaP and smooth muscle myosin (*15*) and indicated that CaP binding to subfragment 2 of myosin and light meromyosin (LMM) may be involved in myosin filament assembly (*16*). CaP has also been reported to stimulate, albeit slightly, myosin Mg^{2+}-ATPase activity in the absence of actin (*17*), suggesting that CaP may cause disinhibition of RLC, due to interaction with myosin head. However, in our previous study, we did not find that CaP interacted with subfragment 1 that was generated by cleavage of myosin with papain (*16*), an enzyme that produces partial degradation of the NH_2-terminal end of RLC (*18*−*22*).

The purpose of the present study was to examine a possible interaction between CaP and the myosin head and the isolated RLC. We isolated myosin head using *Staphylococcus aureus* protease. This protease produces subfragment 1 of myosin (myosin $S1_{SA}$) that retains undegradaded RLC component (*20, 23*). Using a combination of cosedimentation, fluorescence, and overlay assays, we found that chicken gizzard CaP binds to myosin $S1_{SA}$. Our data show that CaP binding to myosin head occurs via interaction with RLC, and most likely the NH_2-terminal end of RLC is involved in this process. The CaP binding to myosin head is preferential over its binding to subfragment 2, and the binding of CaP to phosphorylated myosin head is preferential over the unphosphorylated subfragment 1. We suggest that CaP binding to myosin head via RLC may play a role in regulation of the actin−myosin interaction in smooth muscle contractility.

MATERIALS AND METHODS

Materials

Reagents. All commonly used reagents were from Sigma. Materials for gel electrophoresis were from Bio-Rad. Chicken gizzard myosin light chain kinase (MLCK) was a generous gift from Dr. Primal de Lanerolle (Department of Physiology, University of Illinois Medical School, Chicago, IL).

Proteins. CaP (*24*) and smooth muscle myosin (*25*) were isolated from chicken gizzard. Fragmentation of myosin into subfragment 1 by digestion with *S. aureus* protease (myosin $S1_{SA}$) (*20*) and papain (myosin $S1_{PA}$) (*23*) were carried out according to well-established procedures. Subfragment 2 of myosin was generated from heavy meromyosin (HMM) by cleavage with α-chymotrypsin (*26*). Full-length RLC (RLC) and RLC lacking 4 kDa fragment at the NH_2-terminal end (RLC_{PA}) were isolated from native chicken gizzard myosin (*27*) or papain-generated subfragment 1 (*23, 27*). Labeling of CaP with *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine (DAN−CaP) (Aldrich, Milwaukee, WI) was carried out as described previously (*16*). Protein concentration was determined either by the BCA method (Pierce, Rockford, IL), with bovine serum albumin (BSA) as a standard, or spectrophotometrically with *A*(1%, 1 cm) values of 11.3 at 277 nm for CaP (*10*), 4.5 for myosin (*28*), 7.7 and 7.0 for subfragment 1 and subfragment 2 of myosin, respectively (*14*), and 1.5 for RLC (*29*), the last three at 280 nm.

Methods

Preparation of Subfragment 1 of Myosin with Irreversibly Phosphorylated 20-kDa Regulatory Light Chain ($S1_{SA-P_i}$). Myosin was irreversibly phosphorylated to about 1.9 mol of P_i/mol of myosin by incubating 2−4 mg of myosin/mL for 30 min at 25 °C in a “phosphorylation solution” consisting of 1 mM adenosine 5′-*O*-3-thiotriphosphate (ATPγS), 30 mM tris(hydroxymethyl)aminomethane, pH 7.5, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM NaN_3, 0.5 mM dithiothreitol (DTT), 20−40 μg/mL myosin light chain kinase (MLCK), 5 μg/mL calmodulin, and 100 μM $CaCl_2$, as previously described (*30*). Reaction was stoped by addition of ethylene glycol bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) to a final concentration of 5 mM. Then the reaction mixture was extensively dialyzed against 0.6 M KCl, 30 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, and 2 mM EGTA. Subfragment 1 with thiophosphorylated RLC was prepared by digestion of previously thiophosphorylated myosin with *S. aureus* protease (*20*). The level of RLC phosphorylation was controlled as follows. Phosphorylation of myosin was performed under identical conditions, with the exception that our initiating solution contained 1 mM ATP with radioactive tracer [γ-^{32}P]ATP to yield a specific activity of 1500−2000 counts min^{-1} (pmol of ATP)$^{-1}$. After each 5 min of incubation time, 80 μL of reaction mixture was removed. Incorporation of ^{32}P into RLC of myosin was measured by filter paper (Whatman 3 MM) methodology (*31*). Radioactivity of the samples were measured with an automatic liquid scintillation counter, Taurus model (ICN Biomedicals Inc.).

Competition Assay. Smooth muscle myosin and CaP at final concentrations of 4.5 μM each were incubated together for 20 min at 4 °C in 3 mL of 50 mM NaCl, 10 mM Hepes, pH 7.5, 0.5 mM DTT, then the increasing concentrations of myosin $S1_{SA}$ (0−6 μM) were added. After 20 min of incubation, samples were centrifuged at 130,000 x g for 20 min at 4 °C in Sorvall RC-80 ultracentrifuge, using rotor TFT 80.4. Aliquots of the incubation mixtures prior to centrifugation, supernatant and solubilized pellets were all subjected to a 6−20% polyacrylamide gradient SDS−PAGE (*32*). The amounts of material in the samples were quantified by gel densitometry using the program Image from National Institute of Health, Research Series Branch as described (*15*). Standard curve for CaP was constructed to establish the linear concentration range.

Overlay Assay. Myosin (10 μg/lane) was separated on a 6−20% polyacrylamide continuous gradient SDS−PAGE and then electophoretically transferred onto Immobilon-P transfer membranes (Millipore Corp., Bedford, MA). Membranes were blocked with 50 mM NaCl and 25 mM Tris−glycine, pH 7.2 (TBS buffer) supplemented with 0.5% poly(oxyethylene) sorbitan monolaurate (Tween-20) and 5% BSA for 1 h at room temperature and then extensively washed (3 times, 15 min each) in the same TBS−Tween buffer without BSA. Then, membranes were incubated in 20 mL of TBS−Tween buffer supplemented with CaP (1.9 μg/mL) for 1 h at room temperature. After extensive washes, membranes were incubated in TBS−Tween buffer containing mouse monoclonal anti-CaP antibody (Sigma, St. Louis, MO) at a dilution of 1:2000 for 1 h at room temperature. After removal of the latter solution, membranes were extensively washed in TBS−Tween buffer and then incubated in TBS−Tween buffer supplemented with peroxidase-conjugated AffinitiPure goat anti-mouse antibody (Immuno Research Lab, Inc., West Grove, PA), at a dilution of 1:50 000 for 1 h at room temperature. Following extensive washes in TBS-Tween buffer, substrates were visualized by enhanced chemiluminescence Western blotting detection methodology (WB-ECL), according to the protocol of the manufacturer (Amersham Corp., Arlington Heights, IL), via exposure of the membranes to autoradiographic film (Kodak X-OMAT, XAR-5).

Emission Spectra. Fluorescence emission spectra of DAN−CaP alone (0.5 μM) and DAN−CaP (0.5 μM) plus myosin subfragment 1 (0.5 μM) or isolated RLC (0.5 μM) were obtained with the Spex 1681 0.22 spectrometer (Industries, Inc., Edison, NY), with an excitation wavelength of 377 nm and emission wavelength from 400 to 650 nm. All spectra were recorded at room temperature in solutions containing 50 mM NaCl, 10 mM Hepes, pH 7.5, 0.1 mM DTT, and 2 mM NaN_3.

Binding Assay in Solution. The binding constants (k_{aff}) of the interactions between CaP and subfragment 1 of myosin and between CaP and isolated RLC were determined as follows. DAN−CaP (0.5 μM) was incubated with increasing concentrations of subfragments-1 (0−3.6 μM) or isolated RLC (0−2.5 μM) in 50 mM NaCl, 10 mM Hepes, pH 7.5, 0.1 mM DTT, and 2 mM NaN_3 for 20 min at room temperature, and then fluorescence intensity measurements were recorded, with wavelengths of 377 and 490 nm for excitation and emission, respectively. The strength of the interactions between CaP and myosin $S1_{SA}$ and between CaP and subfragment 2 of myosin was determined as follows. DAN−CaP (0.5 μM) was incubated with myosin $S1_{SA}$ or myosin subfragment 2 (each at 0.5 μM) in 50−400 mM NaCl, 10 mM Hepes, pH 7.5, 0.1 mM DTT, and 2 mM NaN_3 for 20 min at room temperature, and then fluorescence intensity measurements was recorded.

Statistical Analysis. Student's *t*-test was used for statistical analysis, and a confidence level of $P < 0.05$ was chosen as an indication of a statistically significant difference.

RESULTS

Myosin $S1_{SA}$ Inhibits Binding of CaP to Filamentous Myosin. To determine whether CaP interacts with subfragment 1 of myosin, we examined the ability of subfragment 1, generated by cleavage of myosin with *S. aureus* protease (myosin $S1_{SA}$), to extract CaP from preformed complexes between CaP and unphosphorylated smooth muscle myosin filaments. Increasing concentrations of myosin $S1_{SA}$ (0−6 μM) were added to preformed complexes between CaP and smooth muscle myosin filaments (each protein at 4.5 μM). These were followed by a high-speed centrifugation. The amount of CaP that sedimented together with the myosin filaments was determined by densitometry following SDS−PAGE. We found that the amount of CaP that sedimented together with myosin filaments in the absence of myosin $S1_{SA}$ was 75.4% ± 7.1% ($n = 3$) and this value decreased to 13.6% ± 2.0% ($n = 3$) in the presence of 2.5 μM myosin $S1_{SA}$ (Figure 1). Higher concentrations of myosin $S1_{SA}$ did not further decrease CaP sedimentation together with the myosin filaments. This observation suggests that myosin $S1_{SA}$ interacts with CaP and thus removes CaP from preformed complexes between CaP and filaments of unphosphorylated myosin.

Calponin Binding to Myosin $S1_{SA}$. To determine the interaction between CaP and myosin head, the fluorescence emission spectrum of DAN−CaP was studied at 50 mM NaCl before and after addition of subfragment 1 generated by cleavage of myosin with *S. aureus* protease (myosin $S1_{SA}$). The fluorescence emission spectrum of DAN−CaP alone (0.5 μM) showed a broad peak with a maximum fluorescence at 490 nm (Figure 2, dotted line). Addition of myosin $S1_{SA}$ (0.5 μM) caused a 20% ± 2.0 ($n = 3$) increase in fluorescence intensity, without any shift in fluorescence maximum (Figure 2, solid line). The increase in fluorescence intensity of DAN−CaP upon addition of myosin $S1_{SA}$ indicates that CaP interacts indeed with myosin $S1_{SA}$. This observation supports our findings that myosin $S1_{SA}$ removes CaP from preformed complexes between CaP and myosin filaments due to a direct interaction between CaP and myosin head.

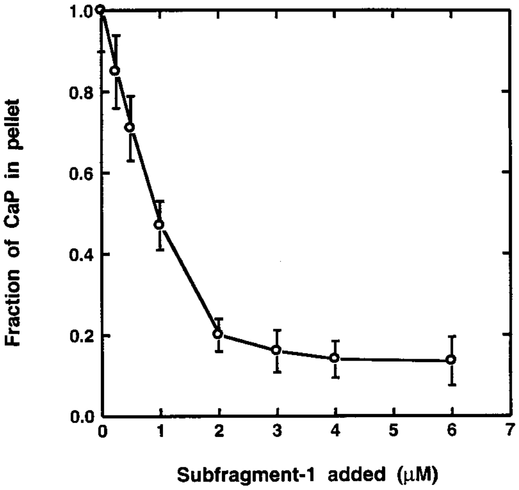

FIGURE 1: Sedimentation of CaP (4.5 μM) in the presence of fixed concentrations of myosin filaments (4.5 μM) and the increasing concentrations of subfragment 1 (0−6 μM), which was generated by cleavage of myosin with *S. aureus* protease (myosin $S1_{SA}$). The ordinate is the amount of CaP in the pellet divided by the total amount.

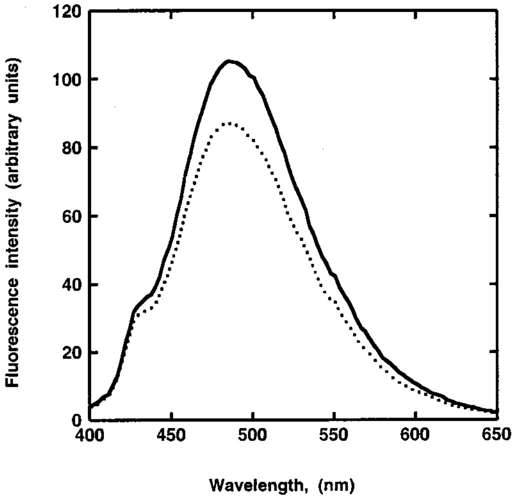

FIGURE 2: Fluorescence emission spectrum of DAN−CaP in the (—) presence and (···) absence of subfragment 1, generated by cleavage of myosin with *S. aureus* protease (myosin $S1_{SA}$). Spectra were recorded in 50 mM NaCl, 10 mM Hepes, pH 7.5 and 0.5 mM DTT, at room temperature. The concentration of each protein was 0.5 μM.

The strength of the CaP−myosin $S1_{SA}$ interaction was examined in the presence of increasing NaCl concentrations from 50 to 400 mM NaCl and compared to the interaction between CaP and subfragment 2 of myosin. Buffer solution

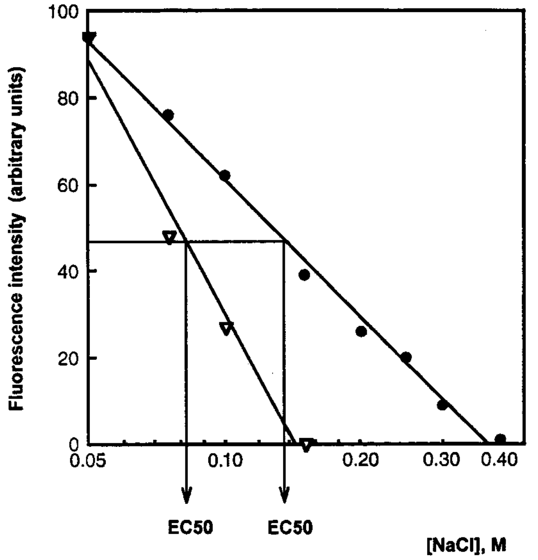

FIGURE 3: Salt-dependent interaction between (●) DAN−CaP and subfragment 1 generated by cleavage of myosin with *S. aureus* protease (myosin $S1_{SA}$) and (▽) DAN−CaP and subfragment 2 of myosin. Each value represents the average of three separate experiments. The line represents the best fit obtained by linear regression. The concentration of each protein was 0.5 μM.

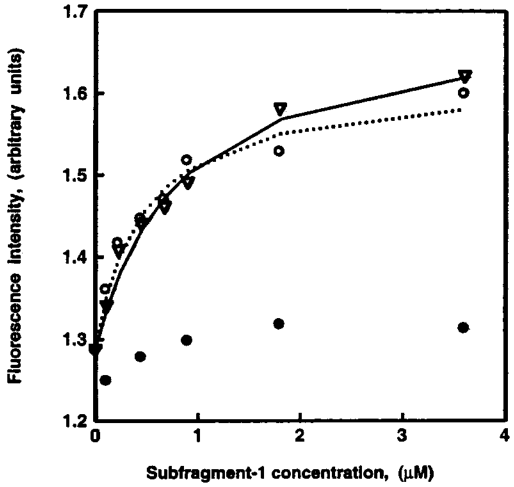

FIGURE 4: Fluorometric titration of DAN−CaP with thiophosphorylated (▽) and unphosphorylated (○) subfragment 1, both generated by cleavage of myosin with *S. aureus* protease (myosin $S1_{SA-Pi}$ and myosin $S1_{SA}$, respectively), and subfragment 1, generated by papain digestion (myosin $S1_{PA}$, ●). Increasing concentrations (0−3.6 μM) of myosin S1 were added to DAN−CaP (0.5 μM). Each point represents the average of three independent experiments. The line represents the best fit obtained by nonlinear regression (*33*).

of the 50 mM NaCl concentration was chosen as the lowest salt concentration, since CaP has a tenedency to aggregate below this ionic strength. As shown in Figure 3, the increasing NaCl concentrations weaken the interactions between DAN−CaP (0.5 μM) and subfragment 2 of myosin (0.5 μM) and between DAN−CaP (0.5 μM) and myosin $S1_{SA}$ (0.5 μM). The interaction between CaP and subfragment 2 of myosin disappears nearly completely at about 150 mM NaCl, whereas the binding of CaP to myosin $S1_{SA}$ is virtually abolished at about 400 mM NaCl. When data from both experiments were fitted by a linear regression program, the EC_{50} of inhibition of the interaction between CaP and subfragment 2 of myosin was found to occur at 84.4 ± 9.3 mM NaCl ($n = 3$), and for CaP and myosin $S1_{SA}$ at 134.3 ± 9.5 mM NaCl ($n = 3$).

To determine a binding constant (k_{aff}) of the CaP−myosin $S1_{SA}$ interaction, increasing concentrations of myosin $S1_{SA}$ (0−3.6 μM) were added to solutions contained fixed concentrations of DAN−CAP (0.5 μM), and fluorescence intensities of DAN−CaP were measured. Addition of the increasing concentrations of myosin $S1_{SA}$ to a solution containing constant concentrations of DAN−CaP produced a concentration-dependent and saturable increase in the label's fluorescence intensity (Figure 4, ○). Fits of the data by nonlinear regression analysis (*33*) yielded an apparent binding constant (k_{aff}) of $(2.1 \pm 0.2) \times 10^6\ M^{-1}$ ($n = 3$). Using the same experimental protocol, we found that addition of increasing concentrations (0−3.6 μM) of subfragment 1 of myosin, generated by cleavage with *S. aureus* protease, that contained irreversibly phosphorylated RLC (myosin $S1_{SA-P_i}$) to solutions containing constant concentrations of DAN−CaP (0.5 μM) also produced concentration-dependent and saturable increases in the label's fluorescence intensity (Figure 4, ▽). Fits of the latter data by nonlinear regression analysis (*33*) yielded an apparent binding constant (k_{aff}) of this interaction to be $(1.3 \pm 0.1) \times 10^6\ M^{-1}$ ($n = 4$). This interaction is significantly ($P < 0.05$) stronger as compared to that between CaP and unphosphorylated myosin $S1_{SA}$ and suggests that phosphorylation of RLC strengthens the interaction between CaP and myosin head. It is interesting that the binding data from these two experiments could be fitted with a single binding constant, suggesting that CaP interacts with a single class of binding sites on myosin head, regardless of the phosphorylation status of the RLC component. Figure 4 (●) shows that addition of increasing concentrations of subfragment 1 generated by cleavage of myosin with papain (myosin $S1_{PA}$) does not change the fluorescence intensities of DAN−CaP. We also found that addition of myosin $S1_{PA}$ (0.5 μM) to DAN−CaP (0.5 μM) does not shift the peak of the emission spectrum of DAN−CaP from 490 nm (data not shown). These observations indicate that CaP does not interact with subfragment 1 of myosin with degraded RLC and suggest that CaP binding to subfragment 1 of myosin takes place in the presence of undegraded, full-length RLC or that CaP binds to myosin head via interaction with RLC.

Calponin Binding to Isolated 20-kDa Regulatory Light Chain of Myosin. To test the hypothesis that CaP interacts with myosin head via binding to the RLC component, RLC was separated from the 17 kDa essential light chain and heavy chains of myosin by the 6−20% polyacrylamide continuous gradient SDS−PAGE and immobilized by transblotting onto Immobilon-P membranes. Then, membranes with immobilized myosin components were incubated in solution containing CaP. CaP bound to myosin components was determined by overlay assay with antibodies directed against CaP and WB-ECL. We found that the anti-CaP antibody colocalizes with myosin heavy chains and RLC but not with the 17 kDa essential light chain (Figure 5). This observation indicates that CaP binds to myosin heavy chains, and isolated RLC, but not to the 17 kDa essential light chain. The capability of CaP to bind to myosin heavy chains is consistent with our previous report that CaP binds to myosin rod, and specifically to subfragment 2 of myosin and light meromyosin (LMM) (*16*). However, these results demonstrate CaP binding to RLC for the first time.

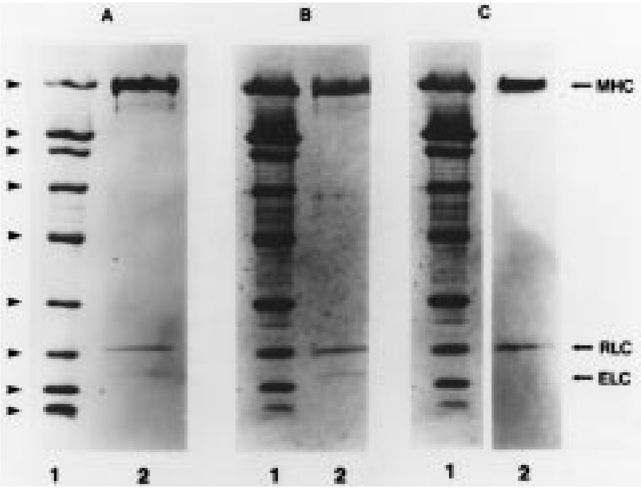

FIGURE 5: Identification of CaP binding components on smooth muscle myosin using an overlay assay. (panel A, lane 2) Coomassie blue-stained smooth muscle myosin (10 μg/lane) separated on the 6−20% polyacrylamide continuous gradient SDS−PAGE, and (panel B, lane 2); Coomassie blue-stained smooth muscle myosin (10 μg/lane) separated on the 6−20% SDS−PAGE and transblotted into Immobilon-P membranes. (panel C, lane 2) WB-ECL film image of immunoblot of myosin, which was incubated in 20 mL of solution containing CaP (1.9 μg/mL), prior staining with antibody against CaP (for details see Materials and Methods). Lane 1 in each panel shows molecular masses of protein standards: 200, 116.3, 97.4, 66.2, 45.0, 31.0, 21.5, 14.4, and 6.5 kDa, corresponding to myosin, β-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and aprotinin, respectively, on the Coomassie blue-stained the 6−20% SDS−PAGE (panel A), and on Immobilon-P membranes (panels B and C). MHC, RLC, and ELC indicate positions of myosin heavy chains and the 20 kDa regulatory and 17 kDa essential light chains of myosin, respectively.

Next we studied the interaction between CaP and isolated RLC in solution. This was considered to be physiologically important because binding in solution may better mimic conditions in situ, rather than binding of CaP to RLC that was immobilized on membranes after denaturation by SDS−PAGE. We compared the fluorescence emission spectra of DAN−CaP alone and DAN−CaP together with isolated full-length RLC and RLC lacking 4 kDa fragment of the NH_2-terminal end (each proteins at 0.5 μM). Addition of intact, full-length isolated RLC to solution containing DAN−CaP results in a (15.0% ± 0.7%) ($n = 3$) significant increase ($P < 0.05$) in the fluorescence intensity as compared to DAN−CaP alone, without any shift of the fluorescence maximum (Figure 6, solid and dotted lines, respectively). Interestingly, however, addition of isolated RLC isolated from papain generated subfragment 1, lacking the 4 kDa NH_2-terminal fragment, results in only a 2.0% ± 0.2% ($n = 3$) increase in the fluorescence intensity without any shift of the fluorescence maximum (Figure 6, dashed−dotted line versus dotted lines). These data together indicate that CaP interacts with isolated intact, full-length RLC in solution but not with isolated RLC, whose NH_2-terminus was partially degraded during myosin digestion with papain.

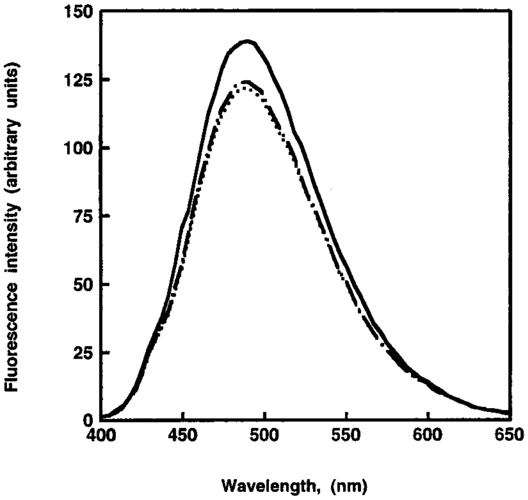

FIGURE 6: Fluorescence emission spectra of (···) DAN−CaP alone, (—) DAN−CaP in the presence of isolated full-length 20 kDa regulatory light chain of smooth muscle myosin (RLC), and (−•−) DAN−CaP together with isolated RLC lacking 4 kDa fragment of the NH_2-terminal end. Spectra were recorded in 50 mM NaCl, 10 mM Hepes, pH 7.5, and 0.5 mM DTT, at room temperature. The concentration of each protein was 0.5 μM.

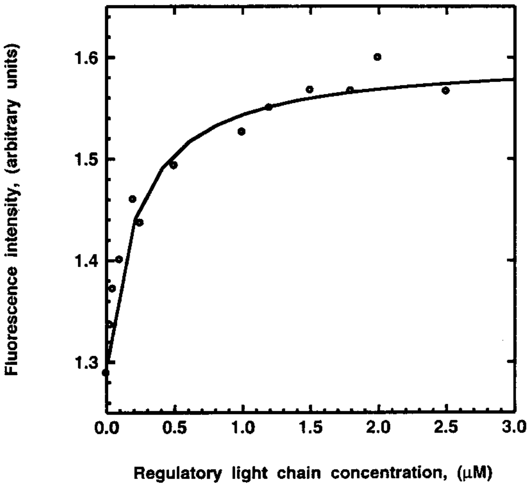

FIGURE 7: Fluorometric titration of DAN−CaP with isolated full-length 20 kDa regulatory light chain (RLC). Increasing concentrations of RLC (0−2.5 μM) were added to DAN−CaP (0.5 μM). Each data point represents the average of four independent experiments. The line represents the best fit obtained by nonlinear regression (*33*).

Next we determine the binding constant (k_{aff}) of the interaction between CaP and isolated, full-length RLC. We titrated isolated native chicken gizzard RLC (0−2.5 μM) into constant concentrations of DAN−CaP (0.5 μM) and measured fluorescence intensities. As shown in Figure 7, addition of increasing concentrations of full-length RLC to the solution containing DAN−CaP produced a concentration-dependent and saturable increase of the label's fluorescence intensity. When the experimental data were fitted by nonlinear regression analysis (*33*), we obtained a single class of binding sites with an apparent binding constant (k_{aff}) of $(4.4 \pm 0.5) \times 10^6\ \mathrm{M}^{-1}$ ($n = 4$). On the basis of the data, utilizing three methodologies, we conclude that CaP binding to myosin head exclusively occurs via CaP interaction with RLC.

DISCUSSION

These data show, for the first time, that CaP binds to intact myosin head as a result of interaction with the 20 kDa regulatory light chain of myosin and suggest that the NH_2-terminal end of RLC is involved in this interaction.

The cleavage of smooth muscle myosin with *S. aureus* protease produces subfragment 1 of myosin (myosin $S1_{SA}$)

with a full-length RLC component (*20, 23*). In contrast, digestion of myosin with papain produces subfragment 1 of myosin (myosin $S1_{PA}$), which retains the capability of myosin head to bind to actin and hydrolyze ATP but destroys its ability to be phosphorylated by Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK). This is due to the loss of the phosphorylable Ser^{19} (*34−36*) that localizes within 4 kDa fragment of the NH_2-terminal end of RLC (*18−22*). Our findings that CaP interacts both with myosin $S1_{SA}$ and isolated full-length RLC, but does not bind to myosin $S1_{PA}$ suggest that CaP binding to myosin head occurs exclusively via the RLC component and that the 4 kDa NH_2-terminal end of RLC is involved in this process.

The EC_{50} of inhibition of the interaction between CaP and myosin $S1_{SA}$ occurs at approximately 130 mM NaCl, a salt concentration which is close to the ionic strength of cellular solutions. In contrast, the EC_{50} of inhibition of the binding of CaP to subfragment 2 of myosin occurs at approximately 80 mM NaCl, suggesting that under physiological conditions myosin head is a more favorable binding site for CaP than subfragment 2. The affinity of CaP binding to unphosphorylated [$k_{aff} = (2.1 \pm 0.2) \times 10^6\ M^{-1}$, $n = 3$] and phosphorylated myosin head [$k_{aff} = (1.3 \pm 0.1) \times 10^6\ M^{-1}$, $n = 4$] is 2.2- and 3.6-fold stronger than to subfragment 2 of myosin [$k_{aff} = (4.7 \pm 0.4) \times 10^6\ M^{-1}$, $n = 3$] (*16*) ($P < 0.05$ for both). The suggestion that subfragment 1 is the most favorable binding site for CaP can also be supported by the findings that the fraction of CaP bound at high concentrations of added proteins is lowest for LMM, intermediate for myosin rod, and highest for intact myosin (*16*). Previously, we also found that CaP interacts with heavy meromyosin (HMM) in solution that was prepared by digestion with α-chymotrypsin (*16*). The overall binding constant (k_{aff}) of this interaction was $(4.0 \pm 0.3) \times 10^6\ M^{-1}$ ($n = 3$) (*16*). The affinity of CaP binding to HMM is significantly lower than its affinity to myosin $S1_{SA}$ ($P < 0.05$) but was not significantly different then its binding to subfragment 2 ($P > 0.05$). The reason for the masking of the high-affinity binding site for CaP on RLC in HMM is not clear. However, it is possible that HMM prepared by digestion with α-chymotrypsin may result from partial degradation of RLC (*23, 37, 38*). This may explain the absence of high-affinity CaP−RLC interaction in HMM.

The physiological significance of the interaction between CaP and its three myosin binding sites [subfragment 1, subfragment 2, and light meromyosin (LMM)] in regulation of contractility in smooth muscle is not fully understood. It has been suggested that CaP binding to myosin rod (subfragment 2 and LMM) is involved in thick filament assembly (*8, 16*), similar to that of caldesmon (*39*) and telokin (*40*). On the other hand, the interaction between CaP and myosin head may lead to formation of complexes between actin, subfragment 1 of myosin, and CaP. It has been shown that binding of one CaP to every three actin monomers is sufficient for inhibition of the actin-activated myosin Mg^{2+}-ATPase activity, but it does not influence the binding of subfragment 1 of myosin to F-actin (*14*). This putative complex between CaP−myosin head and F-actin could also account for inhibition of actin movement over myosin heads in the in vitro motility assay, due to increased affinity between myosin head and actin (*5*). CaP has also been found to inhibit unloaded shortening velocity but not isometric force generation in contracted, phosphorylated smooth muscle skinned fibers (*3, 4*). These data indicate that CaP influences the kinetics of the interaction between actin and myosin head. The observation that CaP causes a gradual and a dose-dependent shift in the velocity distribution toward lower velocities, rather than introducing a population of nonmoving filaments, in an on-or-none fashion suggests that the effects of CaP can be due to an introduction of an internal load during filament sliding, possibly by decreasing the detachment rates and increasing the cross-bridge time spent in the attached state (*3*).

The effect of CaP on smooth muscle contractility appears to depend on phosphorylation state of RLC. Malmqvist et al. (*1*) have shown that addition of CaP to a single smooth muscle cell containing unphosphorylatable RLC mutant inhibited isometric force generation. However, addition of CaP to identical cells supplemented with phosphorylated RLC (ATPγS was used as a substrate) does not inhibit force production. The authors (*1*) suggested that in cells reconstituted with mutated RLC, which has kinetic properties in vitro similar to those of dephosphorylated RLC (*41*), CaP mostly remains bound to actin filaments and therefore inhibits isometric force generation. In contrast, in cells supplemented with phosphorylated RLC, CaP fails to inhibit isometric force generation (*1*). Our data show that binding of CaP to subfragment 1 of myosin increases upon phosphorylation of RLC. The affinity of CaP binding to phosphorylated subfragment 1 is similar to that between CaP and F-actin ($k_{aff} = 1 \times 10^6\ M^{-1}$) (*14*). Studies of Malmqvist et al. (*1*) also show that incorporation of CaP is higher into the cells supplemented with thiophosphorylated RLC than into the cells with mutated and unphosphorylated RLC. It is possible that higher incorporation of CaP into cells with thiophosphorylated RLC and the lack of inhibitory effect of CaP on force generation is due to the CaP interaction with phosphorylated RLC as well as F-actin. Thus CaP may bind to rapidly cycling cross-bridges and act by slowing their detachment rate as suggested by Haeberle (*5*), thereby leading to a latch contraction. Further studies are needed to fully define the physiological role of the CaP−RLC interaction in smooth muscle contractility.

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