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Calcium-Induced Quenching of Intrinsic Fluorescence in Brain Myosin V Is Linked to Dissociation of Calmodulin Light Chains

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Myosin V isolated from chick brain (BM V) is a multimeric protein of about 640 kDa consisting of two intertwined heavy chains of 212 kDa and multiple light chains of 10 to 20 kDa. A distinctive feature of the heavy chain is an extended neck region with six consensus IQ sites for the binding of calmodulin (CaM) and myosin light chains. The actin-activated MgATPase has been shown to require ≥1 μM Ca^{2+} for full activity, and evidence points to a myosin-linked regulatory system where the CaM light chains participate as modulators for the Ca^{2+} signal. Still, the precise mechanism of Ca^{2+} regulation remains unknown. In the present study we have used the intrinsic tryptophan fluorescence of native BM V to monitor conformational changes of BM V induced by Ca^{2+}, and we relate these changes to CaM dissociation from the BM V molecule. The fluorescence intensity decreases ~17% upon addition of sub-micromolar concentrations of Ca^{2+} ($K_{0.5}$ = 0.038 μM). This decrease in fluorescence, which is dominated by a conformational change in the heavy chain, can be reversed by addition of 1,2-di(2-aminoethoxy)ethane-*N,N,N′,N′*-tetraacetic acid (EGTA) followed by an excess of CaM, but not by addition of EGTA alone. Gel filtration of native BM V using HPLC shows that CaM is partially dissociated from the heavy chain in EGTA and dissociates further upon addition of sub-micromolar concentrations of Ca^{2+}. These observations suggest that the affinity of CaM for at least one of the IQ sites on the BM V heavy chain decreases with Ca^{2+} and that the Ca^{2+} concentration required for this effect is lower than that needed to activate acto-BM V. Using a cosedimentation assay in the presence of actin, we also observe partial dissociation of CaM when Ca^{2+} is absent, but now the addition of Ca^{2+} has a biphasic effect: sub-micromolar Ca^{2+} concentrations lead to reassociation of CaM with the heavy chain, followed by dissociation when Ca^{2+} exceeds 5–10 μM. Thus, the binding of CaM to BM V is affected by both actin and Ca^{2+}.

Key Words: **brain myosin V; calmodulin; light chains; intrinsic fluorescence.**

The first myosins recognized as being directly regulated by Ca^{2+} were identified in scallop striated muscles (1), and regulation has since been shown to involve subunits (light chains) of ~20 kDa that share homologies with the EF-hand family of proteins [for reviews, see (2, 3)]. In the past few years, evidence has been presented to show that mechanochemical cycles in a number of nonmuscle myosins of classes I and V may be regulated in different ways by calmodulin (CaM)[3] and other light-chain components that modulate the activity of the heavy chains [for a review, see (4)]. Brain myosin V (BM V), an unconventional myosin purified from chick brain [for reviews, see (5–7)], is a multimeric protein containing both endogenous CaM (8) and other light chains (9) associated with dimerized

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[3] Abbreviations used: BBM I, brush border myosin I; BM V, chick brain myosin V; CaM, calmodulin; DTT, dithiothreitol; EGTA, 1,2-di(2-aminoethoxy)ethane-*N,N,N′,N′*-tetraacetic acid.

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myosin heavy chains. The heavy chain (212 kDa) has a tail with globular regions of unknown function and a head with binding sites for ATP and actin. Between the head and tail is an extended neck containing six IQ sites, which are consensus binding sites for CaM and other light chains. The stoichiometry of CaM (16.8 kDa), light chains (23 and 17 kDa), and heavy chain has been reported as 4.0:0.3:0.7:1.0 (10), suggesting that each molecule of BM V has 10–12 light chains, of which 8 are probably CaM. Espreafico *et al.* (11) clearly demonstrated that CaM binds to the neck domain, and no CaM interactions were detected elsewhere in the molecule.

Purified BM V requires actin and Ca^{2+} for maximum MgATPase activity (8, 10, 12). In the presence of actin, addition of micromolar Ca^{2+} causes an increase in the ATPase activity but a decrease in motility (10). Nascimento *et al.* (12) showed a very sharp Ca^{2+} concentration dependence for the actin-activated ATPase of BM V, with no effect up to 1 μM Ca^{2+} and a maximum effect at 3 μM. The mechanism of Ca^{2+} regulation is not known, but it is thought to require the binding of Ca^{2+} to CaM. The addition of micromolar Ca^{2+} to BM V in the presence of actin caused about 30% of the CaM to dissociate from the heavy chains (12). The authors postulated a suppressive effect by the neck domain akin to that seen with other Ca^{2+}-regulated myosins, where Ca^{2+} binding to light chains in the neck relieves the inhibition of ATP hydrolysis (13–16).

Each BM V heavy chain has 13 tryptophan residues (11), 8 of which are located in the head and neck. In this study, we use intrinsic fluorescence, HPLC, and cosedimentation with actin to obtain evidence for Ca^{2+}-induced changes in BM V in the absence and presence of actin. In both cases, changes occur at very low (sub-micromolar) Ca^{2+} concentrations, lower than those previously shown to be required for activation of hydrolysis (12).

EXPERIMENTAL

Reagents. ATP, buffers, electrophoresis reagents, and protease inhibitors were purchased from Sigma Chemical Co. Resins were purchased from Pharmacia. All other reagents were analytical grade, from Brazilian suppliers. All solutions were prepared with Milli-Q (Millipore Corp., Bedford, MA) deionized water.

Proteins. Brain myosin V was purified according to Cheney *et al.* (10), with modifications: 200 chick brains (~200 g wet wt) were homogenized in 100 mM Tris–HCl, pH 7.7, 10 mM EDTA, 10 mM ATP, 2 mM 2-mercaptoethanol, 300 μM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 40,000g_{max} (40 min). NaCl was added to the supernatant to a final concentration of 600 mM and the suspension was centrifuged at 40,000g_{max} (20 min). The pellet was resuspended in 25 mM Tris–HCl, pH 7.2, containing 5 mM EDTA and 5 mM EGTA and centrifuged at 40,000g_{max} (60 min). The pellet was resuspended in 25 mM Tris–HCl, pH 7.2, and centrifuged at 40,000g_{max} (20 min). Myosin in the pellet was solubilized in 25 mM Tris–HCl, pH 8.2, 600 mM NaCl, 1 mM EGTA, 10 mM ATP, 20 mM $MgCl_2$, and 2 mM DTT. After 15 min the suspension was centrifuged at 250,000g_{max} (30 min) and the supernatant was collected. The pellet was resuspended in the same buffer and the centrifugation was repeated (15 min). The two supernatants were pooled and concentrated by ultrafiltration under N_2 using an Amicon YM 300 membrane. On SDS–PAGE, no BM V was detected in the filtrate. The concentrated protein was centrifuged at 250,000g_{max} (15 min) and the supernatant was applied on a Sephacryl S 400 HR column (1.5 × 100 cm) equilibrated in the same buffer except that it contained 5 mM ATP and 10 mM $MgCl_2$. Fractions of 2 ml were collected at a flow rate of 15 ml/h. Those enriched in BM V were pooled and diluted to a final NaCl concentration of 250 mM using the same buffer but without NaCl. The pool was titrated to pH 7.4 and applied on a Q Sepharose fast-flow column (3 × 1 cm) preequilibrated in the same buffer (25 mM Tris–HCl, pH 7.4, 250 mM NaCl, 1 mM EGTA, 2 mM DTT, 5 mM ATP, 10 mM $MgCl_2$). The column was washed with 3 vol of this buffer followed by 3 vol of the same buffer in the absence of ATP. The protein was eluted using a linear gradient of NaCl (0.25–1.00 M, 10–15 vol) in the buffer without ATP. The fractions containing protein were dialyzed overnight against 2 × 400 vol 25 mM Tris–HCl, pH 7.0, 1 mM EGTA, and 1 mM DTT and stored on ice. With this procedure, as much as 4 mg of purified protein could be obtained from 200 chick brains.

Calmodulin was purified using the method of Gopalakrishna and Anderson (17). F-Actin was prepared as described by Pardee and Spudich (18); just before use, it was centrifuged and resuspended in 25 mM Tris–HCl, pH 7.0, 5 mM EGTA, and 1 mM DTT. Protein concentrations were determined by the Hartree method (19) or by absorbance at 280 nm, using extinction coefficients of 1.04 for BM V (1 mg/ml) (10) and 0.21 for CaM (20). Molar concentrations of the proteins were calculated using 640 kDa for BM V and 16.8 kDa for CaM.

Fluorescence measurements. Fluorescence emission was measured using spectrofluorometers from Hitachi (Model F-4500, Tokyo) or ISS Inc. (Model ISS K2, Champaign, IL). The samples were equilibrated at room temperature and emission spectra were recorded upon excitation at 290 nm (bandpass 1–2 nm for excitation and 1–5 nm for emission). The choice of 290 nm (rather than 295 nm) is based on a compromise between the need for sensitivity with small amounts of BM V and the need to reduce interference from Rayleigh scattering. Calcium was added to the cuvette using a micropipet and then mixed in manually by taking up and ejecting the solution several times. The integrated fluorescence intensity was calculated between 315 and 420 nm and corrected for buffer baseline. Calcium concentrations were calculated using the Schwarzenbach association constants for Ca^{2+}, H^+, and EGTA (21) and a program modified from that used by Kosk-Kosicka *et al.* (22, 23).[4] The Ca^{2+} concentration dependence was analyzed by nonlinear regression through the experimental points, using the equation $\Delta F/\Delta F_{max} = Ca^n/\{(K_{0.5})^n + Ca^n\}$, where ΔF is the change in fluorescence compared to the value in EGTA, ΔF_{max} is the maximal change in fluorescence in the presence of Ca^{2+}, $K_{0.5}$ is the Ca^{2+} concentration for the half-maximal effect, Ca is the calculated free Ca^{2+} concentration, and n is the Hill coefficient.

HPLC experiments. High-performance liquid chromatography (Shimadzu LC-10A, Kyoto) was carried out at room temperature using a TSK G3000 SW column (Toya Soda, Tokyo) at a flow rate of 1 ml/min. The column was equilibrated using 25 mM Tris–HCl, pH 7.0, 1 mM EGTA, 1 mM DTT, and 50 mM NaCl. Titration with Ca^{2+} was carried out by adding $CaCl_2$ to the buffer and the protein sample and reequilibrating the column. Protein samples injected were typically 35 μg, in 0.02–0.10 ml. Protein elution was monitored by measuring absorbance at 280 nm, and peak areas were calculated using software supplied by the manufacturer.

[4] In a previous report (12), this program (MCALC) was attributed to Fabiato (24). In fact, Fabiato's program is more versatile and also more complex.

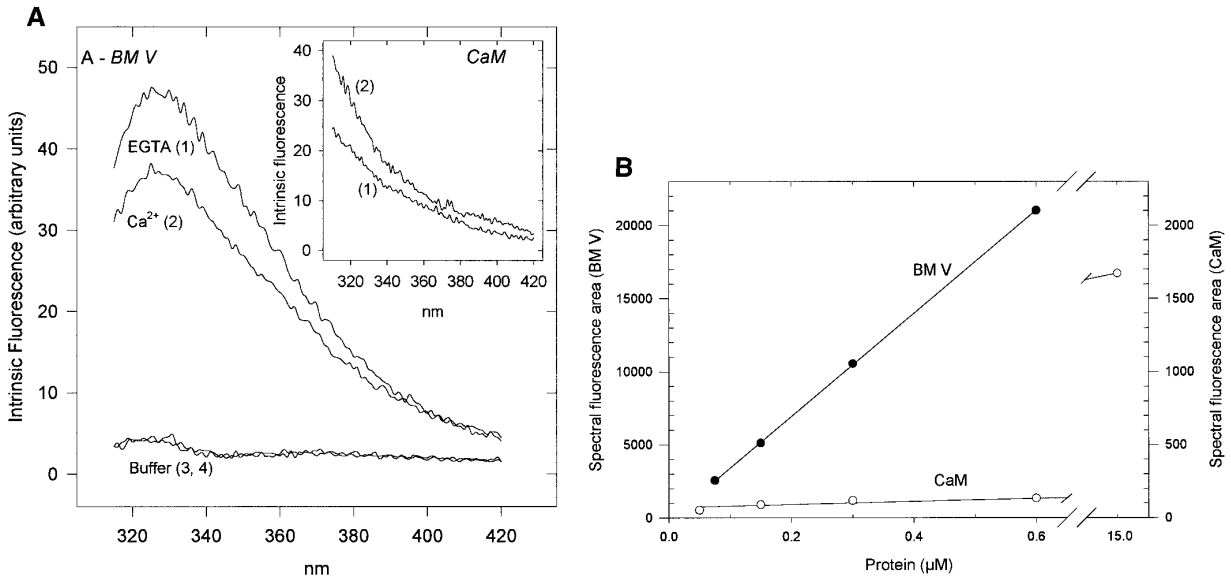

FIG. 1. Effect of Ca^{2+} on the intrinsic fluorescence spectrum of BM V. In A, native BM V (0.3 μM) was excited at 290 nm and its emission spectrum was measured in a buffer containing 25 mM Tris–HCl, pH 7.0, 1 mM DTT, 1 mM EGTA, and either 0 (curve 1) or 0.2 μM free Ca^{2+} (curve 2), at room temperature. Curve 3, buffer alone; curve 4, buffer + Ca^{2+}. Inset shows the spectra of purified CaM under the same conditions. In B, the area under the fluorescence peak (315–420 nm) is correlated with the concentration of native BM V (●, left axis) and purified CaM (○, right axis), in the presence of EGTA.

Electrophoresis. Purification of proteins and cosedimentation experiments were monitored by SDS–PAGE (25) on minigels of 12 or 17% acrylamide. Gels were stained with Coomassie blue or silver (26), the latter after fixation overnight in 5% (v/v) glutaraldehyde.

Cosedimentation assay. BM V and a 15-fold molar excess of actin were incubated for 30 min at 35°C in the presence of 25 mM Tris–HCl, pH 7.0, 5 mM K_2EGTA, 1 mM DTT, and $CaCl_2$ to obtain the indicated concentrations of free Ca^{2+}. The samples were centrifuged at 100,000*g* for 1 h. The supernatants and pellets were separated and the pellets resuspended to the same volume as the supernatant. Samples for SDS–PAGE (100 μl) were diluted in sample buffer (50 μl) containing 5 mM EDTA, 200 mM Tris–HCl, pH 6.8, 5% (v/v) 2-mercaptoethanol, 6% (w/v) SDS, and 50% (v/v) glycerol, and 10-μl aliquots were applied to the gel.

RESULTS

Intrinsic fluorescence of BM V. When excited at 290 nm in the presence of EGTA, BM V shows a characteristic tryptophan fluorescence emission spectrum, with a peak at 325–330 nm (Fig. 1A). Addition of Ca^{2+} to BM V causes a reproducible decrease in its intrinsic fluorescence, with no shift in the emission spectrum (Fig. 1A). This change is complete in the time required for mixing (10–15 s) (see Experimental). Since the CaM light chains on BM V are the probable receptors for Ca^{2+}, we tested the effect of adding Ca^{2+} to purified CaM alone, under the same conditions. The inset to Fig. 1A shows that Ca^{2+} causes an increase rather than a decrease in the intrinsic fluorescence of CaM, in accordance with previous reports (20). The integrated area of the BM V spectrum (315 to 420 nm) is directly and linearly related to the concentration of the protein (Fig. 1B). Under these conditions, the spectral emission of an equimolar concentration of CaM, which has no tryptophan, is only 1–2% of the signal obtained from BM V. Furthermore, Fig. 1B shows that the fluorescence from a 25-fold molar excess of CaM (15 μM, right axis) is less than 10% of the fluorescence from BM V (0.6 μM, left axis). Thus, for a sample of BM V excited at 290 nm, the contribution to its spectrum from endogenous CaM is negligible. Taken together, these data indicate that the quenching of BM V fluorescence by Ca^{2+} reflects primarily changes in the environment of tryptophan residues in the BM V heavy chain. A contribution from other (non-CaM) light chains cannot be ruled out, but the available data (see Introduction) indicate that they are a minor component compared to CaM, and their primary sequence is not known.

The Ca^{2+} concentration dependence of the quenching effect was determined by measuring the decrease in area of the fluorescence spectrum (315–420 nm) as the free Ca^{2+} concentration was increased in steps from 0 to 7.6 μM, using a CaEGTA buffer. Figure 2A shows that the decrease in fluorescence (~17%) is essentially complete with 1–2 μM Ca^{2+}; little further change occurs as Ca^{2+} is raised to higher values. Ca^{2+} concentrations as high as 100–500 μM were used in some experiments, with no additional effect on fluorescence (data not shown). The half-maximal effect in these experiments can be seen to occur at Ca^{2+} concentrations between 0.03 and 0.2 μM. Addition of 4 mM EGTA at the end of the Ca^{2+} titration reduced the

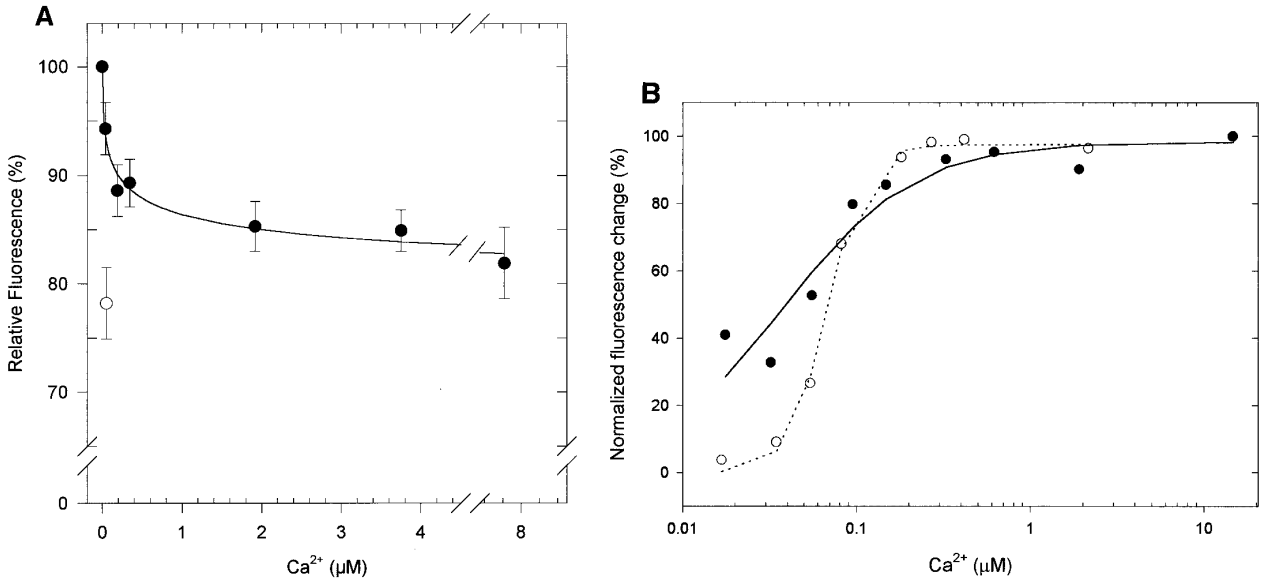

FIG. 2. Ca^{2+} concentration dependence of the intrinsic fluorescence of BM V. In A, BM V (0.3 μM) in the buffer described for Fig. 1 (1 mM EGTA) was excited at 290 nm and the area of the emission spectrum (315–420 nm) recorded as in Fig. 1. $CaCl_2$ was added to give the free Ca^{2+} concentrations shown on the abscissa and the area was recorded after each addition (●). Finally, 4 mM EGTA (○) was added in order to return the free Ca^{2+} to 0.05 μM. Controls showed that the change in pH during the experiment was ~0.1 units. The data for each preparation were normalized to the fluorescence observed before initiating the Ca^{2+} titration and are given as means ± SE of 5–11 preparations. The maximal extent of quenching varied from 10 to 34% in different preparations. In B, the averaged data from two preparations of BM V (●, fluorescence decrease) and two preparations of CaM (○, fluorescence increase) were normalized and plotted against free Ca^{2+} on a log scale. Values for $K_{0.5}$, calculated using the equation given under Experimental, were 0.031 and 0.044 μM for BM V (average, 0.038 μM) and 0.064 and 0.091 μM for CaM (average, 0.076 μM).

Ca^{2+} concentration to 0.05 μM, but did not reverse the Ca^{2+} effect on fluorescence (n = 9; open circle, Fig. 2A).

Two preparations of BM V were titrated with Ca^{2+} in small increments from 0.015 to 15 μM. In parallel experiments, titrations were also carried out on purified CaM alone, where the increase in fluorescence reflects changes in C-domain tyrosine fluorescence. The titration profile for BM V (closed circles, Fig. 2B) yields a $K_{0.5}$ equal to 0.038 μM Ca^{2+}, an index of the apparent binding constant. The Hill n values for the two preparations of BM V were 0.7 and 1.6, not unusually high.

The fluorescence quenching of BM V by Ca^{2+} is not due to aggregation of the heavy chains, since this would result in an increase in light scattering with an asymmetrical effect on the fluorescence spectrum at wavelengths to the left and right of the peak. On the contrary, the changes in fluorescence at ±5 nm on either side of the peak were symmetrical throughout the Ca^{2+} titration curve (n = 3; data not shown).

Dissociation of calmodulin. In a previous report, partial dissociation of CaM from BM V was observed in cosedimentation experiments when micromolar concentrations of Ca^{2+} were added in the presence of actin (12). Calcium addition also causes one or two CaM light chains to dissociate from the heavy chain of brush border myosin-I (BBM I) (15, 16). It is not known how Ca^{2+} affects BM V in the absence of actin. To examine the possibility that CaM dissociation could account for the decrease in fluorescence, the Ca^{2+} titration of BM V was performed in conjunction with size-exclusion HPLC. For this purpose we chose a resin that excludes proteins >300 kDa and includes proteins as small as 10 kDa, in order to separate native BM V (≥425 kDa, with or without light chains) from free CaM (16.8 kDa). When BM V is injected into the column after equilibration with EGTA, two absorption peaks can be seen at 280 nm: a large peak corresponding to BM V in the void volume, at 5 min, and a small peak at 8.4 min (Fig. 3A). Injection of purified protein identifies the second peak as CaM (dashed line, Fig. 3A), indicating that even in the absence of Ca^{2+} (1 mM EGTA), CaM is partially dissociated from the heavy chain. In different preparations, the ratio of the areas of the two peaks (CaM/BM V) varied from 0.04 to 0.16, suggesting that BM V is isolated with varying amounts of CaM accompanying it. Considerable variability in CaM content has also been reported for BBM I (15, 16).

In the presence of low concentrations of Ca^{2+}, the BM V peak decreases and the peak corresponding to endogenous CaM increases. Figure 3B shows these changes for Ca^{2+} concentrations below 0.1 μM. Part of the elution profile from another preparation showing the increase in CaM on an expanded scale is seen at the

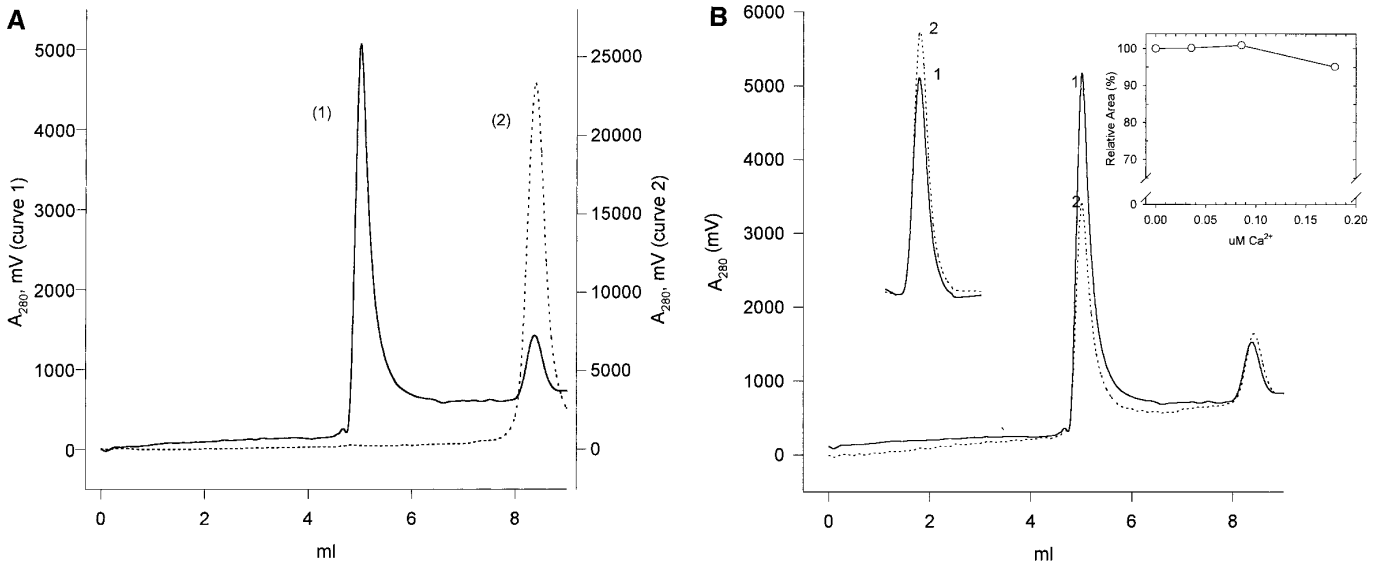

FIG. 3. Size-exclusion HPLC of native BM V. In A (curve 1), BM V (0.78 nmol) was dialyzed at 4°C overnight against the buffer described for Fig. 1 (1 mM EGTA) and then injected into a TSK 3000 column after equilibration at room temperature in the same buffer. The elution profile at 1 ml/min was monitored by absorbance at 280 nm. Curve 2 shows the profile of purified CaM (2 nmol) treated in the same way for comparison. (B) The center graph shows the elution of native BM V without (curve 1) and with 0.053 μM free Ca^{2+} (curve 2). In the presence of Ca^{2+}, the area of the BM V peak decreased by 22%, and the CaM peak increased by 14%. Inset on the left shows the CaM peaks from another preparation (EGTA and 0.036 μM free Ca^{2+}) on an expanded scale. The increase in area was 18%. Inset on the right shows the control, where identical concentrations of purified CaM were injected in the presence of the concentrations of free Ca^{2+} shown on the abscissa.

left in Fig. 3B. These are changes that would be expected if Ca^{2+} causes an increase in the amount of free CaM—i.e., a transfer of mass from the heavy-chain peak to the CaM peak. To rule out a change in the molar absorption coefficient, which could also affect the CaM peak, purified CaM was injected into the column after equilibration with different concentrations of Ca^{2+}. Figure 3B (inset) shows that the area of the peak does not change as Ca^{2+} is increased from 0 to 0.085 μM and decreases slightly (5%) at higher Ca^{2+} concentrations (0.18 μM). Therefore, we conclude that the increase in area of the CaM peak shown in the main panel of Fig. 3B is due to an increase in the amount of free CaM.

Changes in the BM V peak are more complex, since heavy chains both without and with associated CaM migrate in this position. However, it is clear that the decrease in total area of the BM V peak in Fig. 3B is much larger (9- and 13-fold in the two examples) than the increase in area of the corresponding CaM peak. This suggests that in addition to the loss of mass as CaM dissociates, the BM V heavy chain undergoes a change in conformation that alters its spectroscopic properties.

If indeed Ca^{2+} induces the release of CaM subunits from BM V, the failure to reverse the decrease in fluorescence by EGTA in the experiments of Fig. 2A may be due to the concentration of free CaM being too low after its release from BM V to promote reassociation when Ca^{2+} is removed. To test this idea we added an excess of exogenous CaM (14 μM) to BM V after Ca^{2+} titration and the addition of EGTA, under the same conditions as in Fig. 2A. In five of seven preparations, the Ca^{2+} effect on the fluorescence of BM V was reversed, leading to a fluorescence intensity even greater than the original value in EGTA before the Ca^{2+} titration. After subtraction of the fluorescence due to the added CaM, the final values ranged from 1.1 to 2.1 times the original reading for BM V (1.5 ± 0.2; $n = 5$). The variability in this experiment as well as the observation that with added CaM the fluorescence intensity can exceed the original BM V value may mean that BM V as originally isolated is partially depleted of CaM. Repletion of lost light chains has been proposed to account for the increase in the Ca^{2+}-activated enzymatic activity (20 to 56%) that occurs when CaM is added to acto-BM V *in vitro* (10, 12).

Dissociation of calmodulin in the presence of actin. In a previous report using cosedimentation of BM V with actin, partial dissociation of CaM (1–1.3 mol/mol heavy chain) was observed at Ca^{2+} concentrations above 5 μM (12). Here we have presented evidence that sub-micromolar concentrations of Ca^{2+} ($K_{0.5} \sim 0.04$ μM) induce dissociation of CaM from BM V, in experiments performed in the absence of actin. This raises the question of whether actin has an effect on the affinity of CaM for BM V. Thus, a

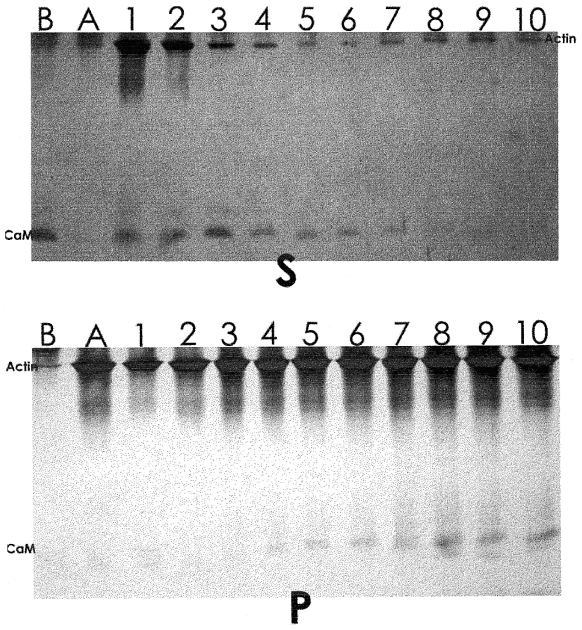

FIG. 4. Cosedimentation assay for Ca^{2+}-induced changes in binding of calmodulin to BM V in the presence of actin. BM V (1.5 μM) and actin (25 μM) were incubated for 30 min in the presence of 25 mM Tris–HCl, pH 7.0, 5 mM K_2EGTA, 1 mM DTT, and $CaCl_2$ to obtain the free Ca^{2+} concentrations of (in nM) 0, 25, 55, 95, 150, 220, 330, 1985, 4430, and 9860, indicated above each lane as 1–10, respectively. After centrifugation at 100,000*g* for 1 h, the supernatants (top) and pellets (bottom) were separated and the pellets resuspended to the same volume as the supernatants. Aliquots of 100 μl were mixed with 50 μl SDS–PAGE sample buffer, and 10-μl samples were separated by SDS–PAGE (17%). After electrophoresis the gels were silver stained. BM V alone (lane B) and actin alone (lane A) were run as controls, in the absence of Ca^{2+}.

cosedimentation assay with actin was performed under rigor conditions, using Ca^{2+} concentrations ranging from 0.025 to 9.9 μM (Fig. 4). The heavy chain of BM V (not shown in the figure) cosediments with actin in the pellet at all Ca^{2+} concentrations tested. In the presence of EGTA, a significant fraction of the endogenous CaM is dissociated from BM V and thus appears in the supernatant, in accordance with the data in Fig. 3 for BM V alone. However, in contrast to the observations with isolated BM V, increasing the Ca^{2+} concentration causes CaM to disappear from the supernatant and appear in the pellet, indicating that CaM has reassociated with the heavy chain. At Ca^{2+} concentrations between 0.33 and 9.9 μM, essentially all of the CaM is associated with the heavy chain and cosediments with actin (Fig. 4). At higher Ca^{2+} concentrations (>10 μM in this preparation and as low as 7 μM in others, not shown), CaM once again begins to appear in the supernatant, as reported previously (12). Thus, the effect of Ca^{2+} on the binding of CaM to BM V is more complex than originally perceived, and it is altered by the binding of actin.

DISCUSSION

The aim of this study was to determine how Ca^{2+} affects the association between CaM and BM V, an association that has previously been studied only in the presence of actin (12). We show that sub-micromolar concentrations of Ca^{2+} quench the intrinsic fluorescence of native BM V, which has the spectral characteristics of Trp, and that this quenching is correlated with the dissociation of CaM from the BM V heavy chain. The data suggest that significant changes in conformation of the native molecule accompany the release of CaM. Furthermore, the binding of F-actin to BM V, as occurs in the cosedimentation assay, alters the affinity between BM V and CaM. This coupling may reflect properties of the structural and functional cross-talk between the CaM-binding neck domain and the actin-binding head domain that are related to the Ca^{2+} dependence of the actin-activated ATPase activity of BM V (12).

Ca^{2+}-induced conformational changes. In heavy meromyosin from vertebrate skeletal muscle, Ca^{2+} causes a 6% decrease in tryptophan fluorescence (27), as well as a small blue shift (28). Since neither of these effects occurred with the isolated heavy chains (29) and the quenching was accentuated with the isolated light chains, the authors concluded that the fluorescence changes in heavy meromyosin were caused by conformational changes in the light chains only.

The situation is different for BM V. Calmodulin, the principal light-chain component, lacks tryptophan, and we have shown that CaM fluorescence does not contribute significantly to the effects observed here. Based on the deduced amino acid sequence, the first and fifth IQ motifs on each BM V heavy chain include a tryptophan (11). Thus, release of CaM from these IQ sites could potentially expose these Trp residues to the aqueous medium, causing a decrease in fluorescence. However, the fact that there is no shift in the spectral center of mass when Ca^{2+} is added (Fig. 1A) leads us to believe that exposure of buried Trp residues to the medium is not a primary cause of the quenching. We speculate that the changes in fluorescence may be due to alterations in heavy-chain conformation that affect these and other Trp residues. This interpretation is reinforced by the HPLC data of Fig. 3, which shows that Ca^{2+} induces a decrease in A_{280} for the peak that contains the BM V heavy chain. In this regard, it is of interest that dissociation of light chains from molluscan myosins leads to a partial collapse in the neck region, so that the distance from the tail to the far end of the head becomes shorter (30, 31). A collapse in the neck region when CaM dissociates from BM V might

account for the difficulty encountered in reversing the dissociation (fluorescence decrease) induced by Ca^{2+} (Fig. 2A).

By analogy with other myosins, the IQ sites in the neck region of BM V are likely to be part of a long α helix that is stabilized by the binding of CaM and the other light chains (2, 32). It can be estimated from structural data that the last IQ site at the C-terminal end of the neck region lies at a distance of ~25 nm from the actin-binding region of the head domain, nearly twice as far as in skeletal myosin II (10). A substantial conformational change in the neck region with subsequent repercussions on the actin-binding region would be consistent with a mechanism in which Ca^{2+} binding to one or more light chains somehow regulates the activity at the rather distant catalytic site. Since calpain cleavage of BM V at the head–neck junction mimics the effect of Ca^{2+} in turning on the acto-BM V ATPase (12), continuity of the heavy chain in this region appears to be essential for regulation. Precise localization of the structural changes that lead to the observed decrease in fluorescence will require further experiments, such as those that have been done with proteolytic fragments of molluscan myosin (33).

Ca^{2+} concentration dependence and effect of actin. The Ca^{2+} concentrations that cause CaM to dissociate from isolated BM V (0.03–0.05 μM, Fig. 3B) are more than an order of magnitude lower than those required for actin-activated ATPase activity (1–3 μM, Ref. 12). In addition, Ca^{2+} titration of the intrinsic fluorescence (Fig. 2B) does not show the high Hill *n* value associated with Ca^{2+} activation of acto-BM V ATPase activity (12). Thus, the effects of Ca^{2+} on the intrinsic fluorescence and on the actin-activated ATPase activity are not directly correlated. Our experiments were necessarily performed under rigor conditions (absence of MgATP), in order to avoid dynamic changes associated with ATP hydrolysis and the mechanochemical cycle. However, it appears that the association of BM V with actin does alter its binding affinity for CaM and the effect of Ca^{2+} under rigor conditions.

In the F-actin cosedimentation assay, binding of CaM to BM V is promoted by Ca^{2+} concentrations that cause dissociation of CaM from BM V alone, whereas higher concentrations of Ca^{2+} ($\geq$5–10 μM) cause CaM to dissociate in the cosedimentation assay and have no further effect on BM V alone. This shift in the presence of F-actin to a biphasic effect of Ca^{2+} implies that binding with actin profoundly affects the affinity of one or more IQ sites for CaM in its Ca^{2+}-bound form. Possibly the binding of Ca^{2+} at sub-micromolar concentrations (in the range of those in a resting cell) ensures that a critical IQ site(s) is unoccupied by CaM. In the presence of actin, CaM binding to the empty site may be a precondition for activation, providing BM V with a "hair-trigger" response so that only a small increment in Ca^{2+} is needed to activate it sharply.

Other myosins that have been shown to be regulated directly by Ca^{2+} include scallop myosin II and the vertebrate BBM I [for a review, see (34)]. In the molluscan myosin, Ca^{2+} binding to the light chains of the neck domain (32) leads to activation of the mechanochemical activity. Calcium induces conformational changes (32, 35) and in heavy meromyosin either no change (36) or a very small (<5%) increase (33) in Trp fluorescence. However, there is no evidence that either of the scallop myosin light chains dissociates on binding Ca^{2+}; on the contrary, dissociation requires EDTA (37). In BBM I, micromolar Ca^{2+} induces dissociation of CaM light chains, and this event was originally perceived as being responsible for inhibition of actin activation and motility (15, 38). However, Ca^{2+} also inhibits mechanochemical activity when excess CaM is present in the medium (16), so it is still somewhat uncertain whether CaM dissociation is essential for regulation. In the case of acto-BM V, micromolar Ca^{2+} inhibits motility but not actin activation (10, 12), and CaM dissociation induced by 5–10 μM Ca^{2+} is not correlated with changes in activity. The binding of CaM induced by submicromolar Ca^{2+} concentrations (Fig. 4) has no counterpart in BBM I. Thus, BM V differs from other Ca^{2+}-regulated myosins in several important respects, and in none of these cases is it yet entirely clear how the light chains contribute to regulation.

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REFERENCES

1. Kendrick-Jones, J., Lehman, W., and Szent-Györgyi, A. G. (1970) *J. Mol. Biol.* **54,** 313–326.
2. Trybus, K. M. (1994) *J. Musc. Res. Cell Motil.* **15,** 587–594.
3. Szent-Györgyi, A. G. (1996) *Biophys. Chem.* **59,** 357–363.
4. Sellers, J. R., and Goodson, H. V. (1995) *Protein Profile* **2,** 1323–1423.
5. Titus, M. A. (1993) *Cell* **75,** 9–11.
6. Mooseker, M. S., and Cheney, R. E. (1995) *Annu. Rev. Cell Dev. Biol.* **4,** 27–35.
7. Larson, R. E. (1996) *Braz. J. Med. Biol. Res.* **29,** 309–318.

8. Espindola, F. S., Espreafico, E. M., Coelho, M. V., Martins, A. R., Costa, F. R. C., Mooseker, M. S., and Larson, R. E. (1992) *J. Cell Biol.* **118,** 359–368.
9. Espindola, F. S., Cheney, R. E., King, S. M., Sutter, D. M., and Mooseker, M. S. (1996) *Mol. Biol. Cell.* **7,** 372a.
10. Cheney, R. E., O'Shea, M. K., Heuser, J. E., Coelho, M. V., Wolenski, J. S., Espreafico, E. M., Forscher, P., Larson, R. E., and Mooseker, M. S. (1993) *Cell* **75,** 13–23.
11. Espreafico, E. M., Cheney, R. E., Matteoli, M., Nascimento, A. A. C., De Camilli, P. V., Larson, R. E., and Mooseker, M. S. (1992) *J. Cell Biol.* **119,** 1541–1557.
12. Nascimento, A. A. C., Cheney, R. E., Tauhata, S. B. F., Larson, R. E., and Mooseker, M. S. (1996) *J. Biol. Chem.* **271,** 17561–17569.
13. Wells, C., and Bagshaw, C. R. (1985) *Nature* **313,** 696–697.
14. Szent-Györgyi, A. G., and Chantler, P. D. (1994) *in* Myology (Engel, A., and Franzini-Armstrong, C., Eds.), 2nd ed., pp. 506–528, McGraw-Hill, New York.
15. Swanljung-Collins, H., and Collins, J. H. (1991) *J. Biol. Chem.* **266,** 1312–1319.
16. Wolenski, J. S., Hayden, S. M., Forscher, P., and Mooseker, M. S. (1993) *J. Cell Biol.* **122,** 613–621.
17. Gopalakrishna, R., and Anderson, W. (1985) *Biochem. Biophys. Res. Commun.* **104,** 830–836.
18. Pardee, J. D., and Spudich, J. A. (1982) *Methods Enzymol.* **85,** 164–181.
19. Hartree, E. F. (1972) *Anal. Biochem.* **48,** 422–427.
20. Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., and Means, A. R. (1977) *J. Biol. Chem.* **252,** 8415–8422.
21. Schwarzenbach, G., Senn, H., and Anderegg, G. (1957) *Helv. Chim. Acta* **40,** 1886–1890.
22. Kosk-Kosicka, D., Kurzmack, M., and Inesi, G. (1983) *Biochemistry* **22,** 2559–2567.
23. Sorenson, M. M., Coelho, H. S. L., and Reuben, J. P. (1986) *J. Membr. Biol.* **90,** 219–230.
24. Fabiato, A. (1988) *Methods Enzymol.* **157,** 378–417.
25. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
26. Ansorge, W. (1985) *J. Biochem. Biophys. Methods* **11,** 13–20.
27. Werber, M. M. (1978) *Experientia* **34/5,** 575–576.
28. Werber, M. M., Szent-Györgyi, A. G., and Fasman, G. D. (1973) *J. Mechanochem. Cell Motil.* **2,** 35–43.
29. Werber, M. M., and Oplatka, A. (1974) *Biochem. Biophys. Res. Commun.* **57,** 823–830.
30. Flicker, P. F., Wallimann, T., and Vibert, P. (1983) *J. Mol. Biol.* **169,** 723–741.
31. Walker, M., and Trinick, J. (1989) *J. Mol. Biol.* **208,** 469–475.
32. Xie, X., Harrison, D. H., Schlichting, I., Sweet, R. M., Kalabokis, V. N., Szent-Györgyi, A. G., and Cohen, C. (1994) *Nature* **368,** 306–312.
33. Wells, C., Warriner, K. E., and Bagshaw, C. R. (1985) *Biochem. J.* **231,** 31–38.
34. Wolenski, J. S. (1995) *Trends Cell Biol.* **5,** 310–316.
35. Hardwicke, P. M. D., Walliman, T., and Szent-Györgyi, A. G. (1983) *Nature* **301,** 478–482.
36. Chantler, P. D., and Szent-Györgyi, A. G. (1978) *Biochemistry* **17,** 5440–5448.
37. Bennett, A. J., and Bagshaw, C. R. (1986) *Biochem. J.* **233,** 179–186.
38. Collins, K., Sellers, J. R., and Matsudaira, P. T. (1990) *J. Cell Biol.* **110,** 1137–1147.