High Affinity Ca\textsuperscript{2+} Binding Sites of Calmodulin Are Critical for the Regulation of Myosin I\textbeta Motor Function*

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We coexpressed myosin I\textbeta heavy chain with three different calmodulin mutants in which the two Ca\textsuperscript{2+}-binding sites of the two N-terminal domain (E12Q), C-terminal domain (E34Q), or all four sites (E1234Q) are mutated in order to define the importance of these Ca\textsuperscript{2+} binding sites to the regulation of myosin I\textbeta. The calmodulin mutated at the two Ca\textsuperscript{2+} binding sites in N-terminal domain and C-terminal domain lost its lower affinity Ca\textsuperscript{2+} binding site and higher affinity Ca\textsuperscript{2+} binding site, respectively. We found that, based upon the change in the actin-activated ATPase activities and actin translocating activities, myosin I\textbeta with E12Q calmodulin has the regulatory characteristics similar to myosin I\textbeta containing wild-type calmodulin, while myosin I\textbeta with E34Q or E1234Q calmodulin lose all Ca\textsuperscript{2+} regulation. While the increase in myosin I\textbeta ATPase activity paralleled the dissociation of 1 mol of calmodulin from myosin I\textbeta heavy chain for both wild type (above pCa 5) and E12Q calmodulin (above pCa 6), the Ca\textsuperscript{2+} level required for the inhibition of actin-translocating activity of myosin I\textbeta was lower than that required for dissociation of calmodulin, suggesting that the conformational change induced by the binding of Ca\textsuperscript{2+} at the high affinity site but not the dissociation of calmodulin is critical for the inhibition of the motor activity. Our results suggest that the regulation of unconventional myosins by Ca\textsuperscript{2+} is directly mediated by the Ca\textsuperscript{2+} binding to calmodulin, and that the C-terminal pair of Ca\textsuperscript{2+}-binding sites are critical for this regulation.

Myosins are a large family of molecular motors responsible for diverse forms of cell contractility and motility, such as muscle contraction, chemotaxis, and phagocytosis (1). Myosin I, one of the 14 classes of this myosin superfamily, is single-headed and unable to form filaments (1–4). Based on the similarity of their primary structures in the head domain, myosin Is are further classified into four subclasses. Myosin I\textbeta is one of these subclasses (1, 4).

Myosin I\textbeta was first purified from bovine adrenal gland and brain (5). Subsequently, cDNAs encoding myosin I\textbeta were isolated from bovine adrenal gland (6), bovine brain (7), and neonatal rat brain (8). Myosin I\textbeta was found in a variety of tissues with the highest expression levels in heart, lung, adrenal gland, esophagus, and stomach (6, 7, 9, 10). Myosin I\textbeta localizes to actin-rich peripheral structures, such as filopodia and lamellipodia of culture cells (9), and it is thought to play a role in cytoskeleton rearrangement. Interestingly, myosin I\textbeta is also found in hair bundles purified from the bullfrog sacculus, suggesting that myosin I\textbeta may function as an adaptation motor which regulates the tip link-associated cation selective channels (11, 12).

Studies from both naturally isolated and recombinant myosins I\textbeta have shown that calmodulin is associated with myosin I\textbeta heavy chain (5, 13). In contrast to most calmodulin-dependent enzymes, the association of calmodulin with myosin I does not require Ca\textsuperscript{2+} binding to calmodulin. Thus, calmodulin functions as a light chain subunit. Myosin I\textbeta, like all the other vertebrate unconventional myosins, has several repeats of a 24–30-amino acid sequence called the IQ motif at the neck region between the myosin head motor domain and the tail domain. This motif has been suggested to provide the binding site for EF-hand family proteins such as calmodulin (1, 14). All vertebrate unconventional myosins that have been characterized so far contain calmodulin as light chains. For some unconventional myosins, other small proteins besides calmodulin have also been found to function as light chains (15). A common property of calmodulin targets that contain IQ motif, such as the unconventional myosins and neuro-musculin, is that they have a higher affinity for the Ca\textsuperscript{2+}-free form of calmodulin (16).

Calmodulin is one of the major intracellular Ca\textsuperscript{2+}-sensor proteins, containing four EF-hand type Ca\textsuperscript{2+}-binding loops. The N-terminal pair are linked to the C-terminal pair by a central flexible linker. Among the four Ca\textsuperscript{2+}-binding sites, the C-terminal pair of Ca\textsuperscript{2+}-binding sites have a higher Ca\textsuperscript{2+}-binding affinity than those of the N terminus (17, 18). Ca\textsuperscript{2+} binding induces structural changes in calmodulin, and it is believed that these Ca\textsuperscript{2+}-induced conformational changes allow calmodulin to activate target enzymes when the cytosolic Ca\textsuperscript{2+} concentration is elevated (reviewed in Ref. 19). Mutagenesis studies have shown that a conserved glutamic acid residue at the 12th position of each Ca\textsuperscript{2+}-binding loop is critical for Ca\textsuperscript{2+} binding, and substitution of this conserved glutamic acid with glutamine in each Ca\textsuperscript{2+}-binding site abolishes its Ca\textsuperscript{2+} binding ability (17, 20).

Both the enzymatic and mechanical activities of vertebrate myosin I have been shown to be regulated by Ca\textsuperscript{2+} (5, 13, 21–23), and this is also true for other unconventional myosins, such as myosin V (15). A member of another myosin I subclass, intestinal brush-border myosin I (BBMI), has been extensively characterized in terms of Ca\textsuperscript{2+} effects. It was found that BBMI

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§ The abbreviations used are: BBMI, brush-border myosin I; MOPS, 4-morpholinepropanesulfonic acid; 9-AC, 9-anthroylcholine.
moved actin filaments although the velocity was quite low (−0.05 μm/s), and the activity was abolished at Ca2+ concentrations above 5 μM. On the other hand, actin-activated Mg2+-ATPase activity of BBM1 increased with increasing Ca2+ concentrations (24). Interestingly, partial dissociation of calmodulin from BBM1 was observed at a Ca2+ concentration of 10 μM. Similarly, Zhu et al. (13) have shown that one of the three calmodulin molecules bound to recombinant myosin Iβ dissociated from the heavy chain at a Ca2+ concentration of 10 μM. While Mg2+-ATPase activity increased above pCa 6, actin sliding velocity of myosin Iβ was abolished at pCa 6 (13). These results suggest that Ca2+ binding to the calmodulin light chains is critical for the regulation of vertebrate myosin I motor function. However, it is unclear whether the dissociation of calmodulin is necessary to stop myosin I motor activity since the Ca2+ concentration required for the dissociation of one calmodulin from myosin Iβ heavy chain seems to be higher than the Ca2+ concentration for inhibition of the actin-translocating activity (13).

In the present study, we have examined the mechanism by which Ca2+ and calmodulin regulate myosin Iβ motor function by coexpressing various calmodulin mutants defective in Ca2+ binding with myosin Iβ heavy chain and analyzing the actin-activated ATPase activity and motor function of the expressed myosin Iβ.

MATERIALS AND METHODS

Expression of Vinculin Myosin Iβ Together with Mutant Calmodulin in Sf9 Cells—Expression of bovine myosin Iβ cDNA with calmodulin was performed as described previously (13). cDNA for wild-type calmodulin and calmodulin with the two N-terminal, two C-terminal, or all four Ca2+-binding sites mutated, termed E12Q, E34Q, and E1234Q, respectively (25), were subcloned into pBlueBacM baculovirus transfer vector at the EcoRI site in the polylinker region. Orientation and accuracy of the subcloning were examined by DNA sequencing (Sequence 2.0, U. S. Biochemical Corp.). Recombinant baculoviruses containing these mutant calmodulins cDNAs were obtained by blue plaque selection and subsequent steps of purification and amplification as described in the manual from Invitrogen, MaxBac Baculovirus Expression System. Sf9 cells were coinfected with the recombinant viruses of myosin Iβ and calmodulin mutant.

Purification of Recombinant Myosin Iβ with Mutant Calmodulin—The purification of recombinant myosin Iβ was performed as previously with slight modifications (13). Briefly, cells were harvested after 3 days of culture at 28 °C and lysed in the presence of ATP, Trition X-100, Nonident P-40, and various protease inhibitors. The supernatant (150,000 × g for 30 min) of lysed cells was incubated with 10 μM glucose and 20 units/ml hexokinase at 0 °C for 30 min in completely deoxyribosyl terminal ATP. F-actin (1 mg/ml) was added to coprecipitate the expressed myosin Iβ. The pellet was resuspended with buffer containing 5 mM MgCl2, 100 mM KCl, 25 mM Tris-HCl, pH 7.5, and 1 mM ATP was added to release myosin Iβ from the myosin I-actin complex. The sample was ultracentrifuged at 150,000 × g for 30 min to remove F-actin, and the supernatant containing the expressed myosin Iβ heavy chain and calmodulin was subjected to a DE52 column (1 × 10 cm). The protein was eluted with a linear gradient (12 ml-12 ml) of 50–250 mM KCl. Approximately 100 μg of myosin Iβ can be obtained from 800 ml of culture. Expression and Purification of Calmodulin Mutants—Wild-type calmodulin and mutant proteins were expressed by infecting Sf9 cells with recombinant virus. Cells were homogenized with 5 volumes of buffer A (30 mM Tris-HCl, pH 7.5, 8 mM urea, 5 mM dithiothreitol, 10 μg/ml leupeptin) for 5 min. After centrifugation at 35,000 × g for 15 min, 5% trichloroacetic acid was added to the supernatant. The pellets were collected and suspended with 8 mM urea, and the pH was adjusted to be neutral. The suspension was dialyzed against buffer B (30 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol) for 2 h. After insoluble material was removed by centrifugation, the sample was loaded to a DE52 anion exchange column (24 × 3 cm) equilibrated with buffer B. Calmodulin was eluted with a 0–0.4 M NaCl linear gradient. The fractions containing calmodulin were combined, concentrated, and loaded onto a Sephacryl S200 gel filtration column (100 × 3 cm). The fractions containing calmodulin were identified by SDS-polyacrylamide gel electrophoresis analysis. They were combined and concentrated. The concentrated calmodulin was dialyzed against buffer C (30 mM Tris-HCl, pH 7.5, 30 mM NaCl, 1 mM dithiothreitol) and stored in −80 °C. Bacterially expressed wild type and mutant calmodulins were prepared as described previously (25).

ATPase Assays and Other Biochemical Procedures—The effect of Ca2+ on actin-activated ATPase activity was assayed in 30 mM KCl, 2 mM MgCl2, 20 mM imidazole-HCl, pH 7.0, with or without 50 μM actin, in the presence of 1 mM EGTA or various concentrations of Ca2+ (26). All assays were initiated by adding 100 μM [γ-32P]ATP (Amersham Corp.) to the reaction mix. The liberated 32P was measured as described previously (27) to determine ATPase activity.

The effect of Ca2+ on the binding of calmodulin to myosin Iβ heavy chain was determined as follows: myosin Iβ was dialyzed against different Ca2+ buffers in the absence of ATP, and the sample was ultracentrifuged at 150,000 × g for 30 min in the presence of 1 mg/ml F-actin. Then the pellet was analyzed by SDS-polyacrylamide gel electrophoresis. The amount of the cosedimented myosin Iβ and calmodulin was determined by densitometry, using a Macintosh computer equipped with a frame grabber (LG-3, Scion Corp., Walkersville, MD) connected to a video camera (Pulnix, TM-745, Motion Analysis, Inc., Eugene, OR), and the software used was NIH Image 1.56b18 (Bethesda, MD).

All experiments were carried out at 25 °C, and all the results are represented by mean ± S.E.

In Vitro Motility Assay—In vitro motility assays were performed as described previously (28). A larger nitrocellulose-coated coverslip (24 × 30 mm) was covered by smaller coverslip (18 × 18 mm), each edge of which was applied with 0.1 g of silicon grease (Dow Corning, MI), to create a fluid-filled flow cell. After 60 μl of myosin I-containing solution was added to the flow cell, the myosin I was washed away with 150 μl of buffer A (400 mM KCl, 25 mM HEPES, pH 7.5, 4 mM MgCl2, and 10 mM dithiothreitol). Then the unoccupied nitrocellulose surface was coated with 0.5 mg/ml bovine serum albumin in buffer B (30 mM KCl, 20 mM HEPES, 1 mM MgCl2, and 1 mM EGTA, pH 7.5). The flow cell was washed with buffer B, then rhodamine-phalloidin (Molecular Probes Inc., Eugene, OR) labeled actin filaments in the motility buffer (50 mM KCl, 25 mM MgCl2, 25 mM imidazole, 1 mM EDTA, 1% 2-mercaptoethanol, 0.5% methylcellulose, 4.5 mg/ml glucose, 216 μg/ml glucose oxidase, 36 μg/ml catalase) with various concentrations of Ca2+ were introduced onto the myosin-coated coverslip. After 120 μl of motility buffer were perfused to wash out unbound actin filaments, motility buffer containing Mg2+-ATP was applied to initiate the reaction. Movements of fluorescent actin filaments were observed using an inverted fluorescence microscope (Diaphot, Nikon) with a SIT camera (VE 1000 SIT, DAGE MTI) and a video cassette recorder. The actin sliding velocity was determined as described previously (13).

RESULTS

Three different calmodulin Ca2+ binding site mutants, termed E12Q, E34Q, and E1234Q (25), were used in this study. In each protein, particular Ca2+–binding sites were inactivated by mutation of a conserved glutamate residue (at position 12 of the Ca2+–binding loops) to glutamine. In E12Q, the two N-terminal sites were mutated, in E34Q, the two C-terminal sites were mutated and in E1234Q, all four sites carried the mutation.

Wild-type calmodulin and each of the calmodulin mutants was coexpressed with myosin Iβ heavy chain in insect Sf9 cells, and the expressed myosin Iβ was isolated. All of the calmodulin mutants copurified with myosin Iβ heavy chain, suggesting that the mutations do not affect the binding of calmodulin to
myosin Iβ heavy chain. The ability of Ca\(^2+\) to increase electrophoretic mobility, a characteristic of wild-type calmodulin, was examined for each of these three mutants (Fig. 1). Wild type calmodulin migrated with an apparent molecular mass of 16 kDa in 5 mM Ca\(^{2+}\) but with an apparent molecular mass of 21 kDa in the presence of 1 mM EGTA. E12Q calmodulin mutant migrated at 22 kDa in EGTA but at 19 kDa in the presence Ca\(^{2+}\). On the other hand, E34Q migrated at 21 and 18 kDa in the absence and presence of Ca\(^{2+}\), respectively. The mobility shift by Ca\(^{2+}\) was abolished with E1234Q calmodulin mutant, which migrated at 21 kDa under both conditions. These results confirm that the calmodulin light chain associated with the myosin Iβ heavy chain in each preparation is indeed the expressed recombinant calmodulin mutant, and not endogenous calmodulin. They also suggest that the effect of mutating the two N-terminal Ca\(^{2+}\) binding sites on the conformational change of calmodulin is different from that of mutating the two C-terminal sites.

Ca\(^{2+}\) -induced conformational changes in the calmodulin mutants were further monitored as a function of Ca\(^{2+}\) concentration by use of the reporter molecule 9-AC bromide. The Ca\(^{2+}\) -induced appearance of hydrophobic sites on calmodulin is revealed by the enhanced fluorescence of 9-AC upon binding to these sites, and this technique has been used previously to examine the Ca\(^{2+}\) binding and conformational properties of Ca\(^{2+}\) binding site mutants of calmodulin (20). The 9-AC fluorescence enhancement for wild type calmodulin as a function of Ca\(^{2+}\) concentration is shown in Fig. 2. A single transition is detected, with midpoint at a Ca\(^{2+}\) concentration (10 \(^{-7}\)) that is lower than the dissociation constant for the high affinity sites on calmodulin. This finding probably reflects increased overall affinity for Ca\(^{2+}\) and increased cooperativity of Ca\(^{2+}\) binding induced by 9-AC. The hydrophobic reporter 1-anilino-8-naphthalene sulfonate has been shown previously to increase the affinity of calmodulin for Ca\(^{2+}\) (29). The curve for the E12Q (see Fig. 2) is very similar to the wild type curve at low Ca\(^{2+}\) concentrations but shows no increase in fluorescence enhancement at Ca\(^{2+}\) concentrations above pCa 6. This is consistent with induction of a conformational change as a result of Ca\(^{2+}\) binding to the two intact C-terminal sites present on this protein followed by absence of Ca\(^{2+}\) binding and the associated conformational change in the N-terminal domain. In contrast, the E34Q mutant shows no fluorescence enhancement at low Ca\(^{2+}\) and relatively minor enhancement of fluorescence with a midpoint at about pCa 6 as Ca\(^{2+}\) levels are increased. Thus a major conformational change normally associated with C-terminal high affinity sites is lost in the E34Q mutant leaving a smaller conformational change associated Ca\(^{2+}\) binding in the intact N-terminal domain. The sum of the fluorescence changes for E12Q and E34Q equals the changes for the wild type calmodulin (Fig. 2). The E1234Q mutant showed no change in 9-AC fluorescence throughout the entire pCa range tested (data not shown).

The actin-activated Mg\(^{2+}\)-ATPase activity of myosin is coupled to actomyosin cross-bridge turnover. In order to examine the effect of Ca\(^{2+}\) binding at the N- and C-terminal sites of calmodulin on myosin Iβ mechanoenzymatic function, actin-activated Mg\(^{2+}\)-ATPase activity of myosin Iβ containing the mutant calmodulins was measured as a function of Ca\(^{2+}\) (Fig. 3). For all assays, the timecourse of P\(_i\) liberation was determined and the activity was estimated from the slope of the P\(_i\) assay system (Table I). Wild-type, E12Q, E34Q, and E1234Q myosin Iβ were all able to translocate actin filament at a rate about 0.3 \(\mu\)m/s in the presence of EGTA. The value agrees well
with the one reported in the previous study (13). While switching motility buffer from 1 mM EGTA to 1 or 10 μM Ca²⁺ abolished the actin filament movement for wild-type and E12Q myosin Iβ, it had little effect on the motility activity of E34Q and E1234Q myosin Iβ. These results suggest that the Ca²⁺ regulation of motor activity of myosin Iβ is also mediated through the binding of Ca²⁺ to calmodulin. They further demonstrate that the two C-terminal Ca²⁺-binding sites but not the two N-terminal sites are critical for this regulation.

The effects of Ca²⁺ on the binding of the various calmodulins to myosin Iβ heavy chain were also examined (Fig. 4). Purified myosin 1β containing each calmodulin mutant was coprecipitated with F-actin at various Ca²⁺ concentrations, and the precipitated myosin Iβ and calmodulin were subjected to SDS-polyacrylamide gel electrophoresis followed by densitometry analysis to quantify the stoichiometry of the bound calmodulin. It is known that 3 mol of calmodulin bind to 1 mol of myosin Iβ heavy chain (13). For wild-type calmodulin, one of the three molecules of bound calmodulin was dissociated from the heavy chain above pCa 5 (Fig. 4A). On the other hand, for E12Q myosin Iβ, the dissociation of calmodulin was observed at lower Ca²⁺ (i.e. pCa 6) (Fig. 4B). In contrast, Ca²⁺ had no effect on the binding of E34Q calmodulin, i.e. all three calmodulin molecules were associated with the heavy chain even at pCa 4 (Fig. 4C). As expected, the binding of E1234Q mutant calmodulin to the heavy chain showed no Ca²⁺ sensitivity (Fig. 4D).

**DISCUSSION**

In this study, we have coexpressed myosin Iβ heavy chain with three different calmodulin mutants, in which the conserved critical glutamic acid residue at the 12th position of the two N-terminal, two C-terminal, or all four of the Ca²⁺ binding loops were substituted by glutamine. The importance of the carbonyl side chain of this glutamic acid to the Ca²⁺ coordination system was revealed by crystallographic studies of calmodulin (30). As expected, the Ca²⁺-binding abilities of the mutated sites are completely abolished, based upon the Ca²⁺ binding induced conformational changes probed by the fluorescent hydrophobic reporter molecule, 9-AC. The results are consistent with the earlier study in which the conserved glutamic acid residue in each individual Ca²⁺-binding site was mutated (20).

The fluorescence titration results show that the two C-terminal Ca²⁺-binding sites have a higher affinity for Ca²⁺ than the two N-terminal sites. The different electrophoretic mobility of calmodulin in the presence of EGTA as compared with Ca²⁺ also reflects conformational changes upon Ca²⁺ occupation. The obtained results suggest that the conformational change caused by Ca²⁺ binding to the two N-terminal sites (E34Q) is different from that caused by Ca²⁺ binding to the two C-terminal sites (E12Q); occupation of the two C-terminal Ca²⁺-binding sites has a greater impact on the overall conformational change. This is consistent with the previous findings that the Ca²⁺ binding at the C-terminal sites of calmodulin induces a larger conformational change (31). Recent structural studies suggest that the C-terminal domain of calmodulin exists in a semi-open conformation in contrast to the close conformation of the N-terminal domain in the absence of Ca²⁺, and it changes to an open conformation upon Ca²⁺ binding (32).
This may explain the difference of the mobility shift between E12Q and E34Q.

The present results clearly indicate that the effects of Ca\(^{2+}\) on the properties of myosin I\(\beta\) molecule, i.e. the change in actin-activated ATPase activity, actin sliding motor activity, and the dissociation of calmodulin light chains at high Ca\(^{2+}\)
motility was not observed with myosin I
creases basal myosin ATPase activity (33). It should be noted
result suggests that there is a cross-talk between the N-termi-
quired for calmodulin dissociation to lower concentration. This
This is consistent with the earlier finding that conforma-
binding at the N-terminal domain of calmodulin is significantly influenced by a change in the Ca²⁺
Clearly, the binding of Ca²⁺ to calmodulin at the C-terminal
sites is critical for the inhibition of actin translocating activity
myosin Iβ by Ca²⁺, since this Ca²⁺-induced inhibition of the
motility was not observed with myosin Iβ containing E344Q or
E1234Q calmodulin (Table I). However, it is more complicated
to determine whether or not the dissociation of the calmodulin
molecule from myosin Iβ heavy chain is critical for the inhibi-
tion of the motor activity. Thus, although the wild-type myosin
Iβ still binds all three calmodulin light chains at pCa 6, its
motor activity is completely inhibited at this Ca²⁺ concen-
tration. One possible explanation is that although the C-terminal
domain of the one calmodulin molecule is dissociated from
myosin Iβ heavy chain at pCa 6, the N-terminal domain is still
associated with the heavy chain at this Ca²⁺ concentration,
and further conformational change induced by Ca²⁺ binding at
the N-terminal low affinity sites of this molecule is necessary for
the complete dissociation (Fig. 5). Presumably, this incom-
plete association of calmodulin with myosin Iβ is no longer able
to support motor activity. For the E12Q mutant, the conforma-
tional change induced by the binding of Ca²⁺ to the higher
affinity sites may be sufficient to dissociate calmodulin from
myosin Iβ heavy chain. It should be noted, however, Ca²⁺
"binding to calmodulin dissociates only one of the three bound
calmodulin from myosin Iβ heavy chain. According to the
amino acid sequence, myosin Iβ has three IQ motifs, one of
which is not a completely matched IQ motif, IQXXRGXXR
(“one-letter amino acid code; X is any amino acid residue”) (6). It is
plausible that the calmodulin bound to the incomplete IQ
motif is dissociated from myosin Iβ when Ca²⁺ binds to the
C-terminal domain. Alternatively, the conformational change
in all three calmodulin upon Ca²⁺ binding to the C-terminal
domain results in the inhibition of motility and the additional
conformational change upon the Ca²⁺ binding at the N-termi-
low affinity sites destabilizes the association of one of the
bound calmodulin to the heavy chain presumably due to steric
hindrance. Further studies are needed to clarify the reason
why only one molecule of calmodulin is dissociated from myosin
Iβ.

Studies reported here with mutant calmodulin show that
motor function of myosin Iβ is regulated by Ca²⁺ binding to the
high affinity sites of calmodulin light chains. This regulatory
mechanism may also apply to those of other unconventional
myosins which contain calmodulin as their light chains.

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