THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 44, Issue of November 3, pp. 34766–34771, 2000
© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Ca^{2+}-dependent Regulation of the Motor Activity of Myosin V*

Received for publication, April 12, 2000, and in revised form, July 18, 2000
Published, JBC Papers in Press, August 16, 2000, DOI 10.1074/jbc.M003132200

Kazuaki Homma, Junya Saito, Reiko Ikebe, and Mitsuo Ikebe‡

From the Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655-0127

Mouse myosin V constructs were produced that consisted of the myosin motor domain plus either one IQ motif (M5IQ1), two IQ motifs (M5IQ2), a complete set of six IQ motifs (SHM5), or the complete IQ motifs plus the coiled-coil domain (thus permitting formation of a double-headed structure, DHM5) and expressed in Sf9 cells. The actin-activated ATPase activity of all constructs except M5IQ1 was inhibited above *p*Ca 5, but this inhibition was completely reversed by addition of exogenous calmodulin. At the same Ca^{2+} concentration, 2 mol of calmodulin from SHM5 and DHM5 or 1 mol of calmodulin from M5IQ2 were dissociated, suggesting that the inhibition of the ATPase activity is due to dissociation of calmodulin from the heavy chain. However, the motility activity of DHM5 and M5IQ2 was completely inhibited at *p*Ca 6, where no dissociation of calmodulin was detected. Inhibition of the motility activity was not reversed by the addition of exogenous calmodulin. These results indicate that inhibition of the motility is due to conformational changes of calmodulin upon the Ca^{2+} binding to the high affinity site but is not due to dissociation of calmodulin from the heavy chain.

Myosins are motor proteins that translocate actin filaments upon hydrolysis of ATP, and thus they play a critical role in diverse forms of cell contractility and motility. During the last decade a number of myosin-like proteins have been found, and the myosins are currently organized into 15 classes based upon phylogenetic sequence comparisons of the motor domains (1–5). Class V myosin was originally identified in brain as a calmodulin-binding protein that had actin-dependent ATPase activity (6). Myosin V is a member of the myosin superfamily that is expressed in variety of cell types and is involved in a variety of membrane trafficking and organelle transport functions (1–5). Myosin V has two heads that are connected with a long coiled-coil domain; however, in contrast to conventional myosin, it contains a globular C-terminal domain and does not form thick filaments (7). The head domain is composed of a globular motor domain and an elongated neck domain that is associated with a number of light chains. The sequence at the neck region contains six IQ motifs that have been implicated as calmodulin or myosin light chain binding consensus motifs as found in a variety of calmodulin-binding proteins and myosins (7). Since light chains play a critical role in the regulation of various conventional myosins, it has been proposed that the IQ domain serves as a regulatory component of myosin V. The role of the IQ motif and bound calmodulin serving as a regulatory component of unconventional myosins is best studied for mammalian myosin Is. For both brush border myosin I (8–10) and myosin Iβ (11–13), high Ca^{2+} inhibits motor activity due to Ca^{2+} binding to the calmodulin light chain. Since 1 mol of bound calmodulin dissociates from myosin I at high Ca^{2+}, it was originally thought that this dissociation of calmodulin was responsible for the inhibition of myosin I motor activity. However, since virtually no calmodulin dissociation is observed at *p*Ca 6 where the motility activity is completely abolished, this view has been questioned (13). For naturally isolated myosin V, motility activity is inhibited at high Ca^{2+}, whereas actin-activated ATPase activity markedly increases in the presence of Ca^{2+} (7). Recently it was shown (14) that a truncated recombinant monomeric myosin V with two IQ motifs had motility activity that was inhibited at high Ca^{2+}, but only in the absence of exogenous calmodulin, suggesting that the inhibition is via the physical dissociation of calmodulin. Interestingly, the truncated myosin V showed inhibition of actin-activated ATPase activity by Ca^{2+} rather than activation, as is found for naturally isolated myosin V (14). This apparent discrepancy is not understood, but there are several possible explanations. Since the two-headed structure is critical for the regulation of both conventional smooth muscle and non-muscle myosin motor function, it is plausible that the two-headed structure may play some role in the regulatory mechanisms of myosin V. Alternatively, a complete neck domain may be required for the proper regulation of myosin V.

The aim of the present study is to clarify the regulation of myosin V motor function by Ca^{2+}. To address these questions, we have produced a two-headed myosin V construct, a single-headed construct having the entire neck domain (six IQ motifs), and a truncated single-headed construct containing only two IQ motifs. These myosin V constructs were expressed, purified, and examined for motor function. It was found that although calmodulin dissociation is responsible for inhibition of actin-activated ATPase activity at high Ca^{2+}, inhibition of motility occurs at lower Ca^{2+} where calmodulin is not dissociated from myosin V heavy chain.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (15). Recombinant calmodulin from *Xenopus* oocyte (16) was expressed in *Escherichia coli* as described (17).

Generation of the Expression Vectors for Myosin V Mutants—A baculovirus transfer vector for mouse myosin V variants in pBluebac4 (Invitrogen, CA) was produced as follows. Mouse myosin V cDNA clones containing −35–1549 and 1549–3928 in pBluescript were kindly supplied by Dr. N. Jenkins (NCI, National Institutes of Health). A cDNA fragment-(1549–3928) flanked with *Eco*RI sites was ligated to the vector containing a cDNA fragment-(−35–1549) at the unique *Eco*RI site at nucleotide 1549. A unique *Nhe*I site was created at the 5′ side of the initiation codon; a unique *Kpn*I site at nucleotide 2125 was deleted

* This work was supported by National Institutes of Health Grants AR 41653, HL 60381, and GM 55834. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Physiology, University of Massachusetts Medical School, 55 Lake Ave. North, Worcester, MA 01655-0127. Tel.: 508-856-1954; Fax: 508-856-4600. E-mail: mitsuo.ikebe@umassmed.edu.

This paper is available on line at http://www.jbc.org

without changing the resulting amino acid residue, and a new *Kpn*I site was created at nucleotide 3578. A cDNA fragment was excised with *Nhe*I/*Kpn*I digestion and in-frame ligated to a PBluebac4His baculovirus transfer vector containing a hexahistidine tag sequence with a stop codon at the 3′ side of the *Kpn*I site. This construct (DHM5), containing the entire coiled-coil domain, was used to express double-headed myosin V. To obtain single-headed myosin V with complete IQ motifs, a *Kpn*I site was created at nucleotide 2744 of DHM5. The vector was digested with *Kpn*I and the nucleotide fragment-(2744–3578) was removed. The vector was self-ligated and used as a construct expressing a single-headed myosin V with complete IQ motifs (SHM5). A *Kpn*I site was also created at nucleotide 2468 or 2393 of DHM5. Vectors containing nucleotides 1–2468 and 1–2393 of myosin V were used to express single-headed myosin V that contained two IQ motifs (M5IQ2) and one IQ motif (M5IQ1), respectively. For M5IQ2, nucleotides encoding a Myc tag sequence (EQKLISEEDL) were inserted at 5′ side of the hexahistidine tag.

Preparation of Recombinant Myosin V—To express recombinant myosin V, 200 ml of Sf9 cells (about 1×10^9) were coinfected with two separate viruses expressing the myosin V heavy chain and calmodulin, respectively. The cells were cultured at 28 °C in 175-cm^2 flasks and harvested after 3 days. Cells were lysed with sonication in 30 ml of Lysis Buffer (0.15 M NaCl, 30 mM KP_i, pH 8.0, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 2 mM phenylmethanesulfonyl fluoride, 0.2 mM *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone, 0.2 mM *N*-*p*-tosyl-L-lysine chloromethyl ketone, 0.01 mg/ml leupeptin, 0.1 mg/ml trypsin inhibitor, 10% glycerol, and 2 mM ATP). After centrifugation at 140,000 × *g* for 15 min, the supernatant was incubated with 50 mM glucose and 20 units/ml hexokinase at 4 °C for 30 min to hydrolyze completely residual ATP. F-actin (0.2 mg/ml) was added to the sample and centrifuged (140,000 × *g* for 20 min) to coprecipitate the expressed myosin V. The pellets were washed once with buffer A (0.3 M NaCl, 30 mM KP_i, pH 8.0, 0.1 mM EGTA, 10 mM β-mercaptoethanol) and then resuspended with buffer A with 5 mM $MgCl_2$ and 5 mM ATP to release myosin V from F-actin. The supernatant was mixed with 0.2 ml of nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany) in a 50-ml conical tube on a rotating wheel for 30 min at 4 °C. The resin suspension was then loaded on a column (1 × 10 cm) and was washed with 10-fold volume of buffer B (0.3 M KCl, 20 mM imidazole, pH 7.5, 0.1 mM EGTA, and 10 mM β-mercaptoethanol). Myosin V was eluted with buffer C (0.1 M KCl, 0.1 mM EGTA, 10 mM β-mercaptoethanol, and 0.2 M imidazole, pH 7.5).

After SDS-polyacrylamide gel electrophoresis analysis, fractions containing myosin V were pooled and dialyzed against 150 mM KCl, 20 mM MOPS,[1] pH 7.0, and 1 mM dithiothreitol. The purified myosin V was stored on ice and used within 2 days. Typically, 1 mg of isolated myosin V was obtained.

Gel Electrophoresis and ATPase Assay—SDS-polyacrylamide gel electrophoresis was carried out on a 7.5–20% polyacrylamide gradient slab gel using the discontinuous buffer system of Laemmli (18). Molecular mass markers used were smooth muscle myosin heavy chain (204 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), myosin regulatory light chain (20 kDa), and α-lactalbumin (14.2 kDa). The amount of the myosin V heavy chain and calmodulin was determined by densitometry as described previously (13). The steady-state ATPase activity was determined by measuring liberated P_i at 25 °C as described previously (19).

In Vitro Motility Assay—The *in vitro* motility assay was performed as described previously (20). Myosin V was attached to the coverslip. For M5IQ2, a coverslip was first coated with anti-Myc antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and then the surface of the coverslip was blocked with bovine serum albumin. Myosin V was then applied to the coverslip. Actin filament velocity was calculated from the movement distance and the elapsed time in successive snapshots. Student's *t* test was used for statistical comparison of mean values. A value of $p < 0.01$ was considered to be significant.

RESULTS

Expression of Myosin V Constructs—In order to study the regulatory mechanism of myosin V motor function, various myosin V constructs were produced and expressed in Sf9 insect cells. The DHM5 construct contains the entire coiled-coil domain in addition to the complete head domain, so it is anticipated that it has a double-headed structure (Fig. 1). SHM5 has

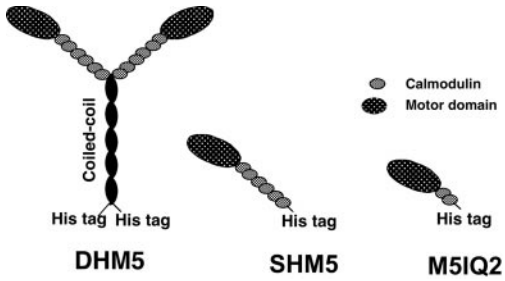

FIG. 1. **Schematic drawing of myosin V constructs.** The motor domain of myosin V is indicated by the *filled black ovals.* Calmodulin light chains are indicated by *dotted shapes.* The coiled-coil domain is indicated by a *chain figure.*

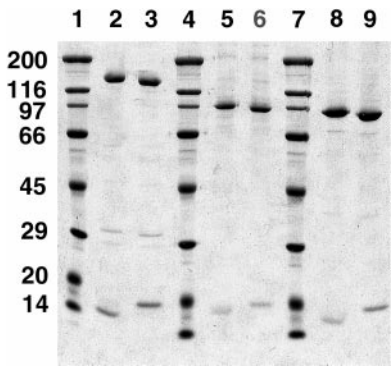

FIG. 2. **SDS-polyacrylamide gel of purified myosin V constructs.** Molecular masses are indicated to the *left.* Apparent molecular mass of each myosin V construct was consistent with the expected molecular mass calculated by the amino acid numbers of each construct. Low molecular mass band shifts its mobility by Ca^{2+}. *Lanes 1, 4,* and *7* are molecular mass standards. *Lanes 2, 5,* and *8* are in 1 mM Ca^{2+}, and *lanes 3, 6,* and *9* are in 1 mM EGTA. *Lanes 2* and *3*, DHM5; *lanes 5* and *6*, SHM5; *lanes 8* and *9*, M5IQ2.

an entire head domain with complete IQ motifs but no coiled-coil domain, thus it is expected to be a single-headed molecule (Fig. 1). On the other hand, M5IQ2 contains a motor domain but only 2 N-terminal IQ motifs out of the six IQ motifs typically present. All constructs contain hexahistidine tags to aid in purification. The histidine tags were introduced at the C-terminal end of the molecule rather than its N-terminal end to avoid possible misfolding of the protein and any effects on motor function. Addition of a hexahistidine tag at the C-terminal end of myosins has been performed with conventional (21) as well as unconventional myosin,[2] and no influence on motor function was observed. The cells were coinfected with myosin V-expressing virus and calmodulin-expressing virus. The ratio of the two viruses to achieve the best myosin V expression was empirically determined. It should be noted that myosin V with bound calmodulin can be obtained without the coinfection of calmodulin-expressing virus but that the fraction of soluble myosin V increases significantly with calmodulin coexpression. This tendency was more prominent for constructs having an entire IQ domain. Fig. 2 shows SDS-polyacrylamide gel electrophoresis of the purified constructs. Each myosin V construct was composed of a high molecular mass band and a low molecular mass band. The molecular mass of each slow mobility band was consistent with the calculated molecular mass of each myosin construct, *i.e.* 130, 100, and 90 kDa for DHM5, SHM5, and M5IQ2, respectively. These bands were recognized by anti-His antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) indicating that these high molecular mass bands are the expressed myosin V heavy chains (not shown). The small sub-

[1] The abbreviation used is: MOPS, 4-morpholinepropanesulfonic acid.

[2] M. Ikebe, unpublished observations.

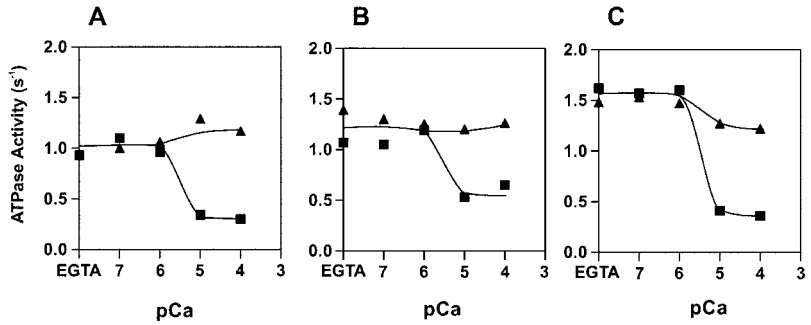

FIG. 3. **Actin-activated ATPase activity of myosin V constructs as a function of free Ca^{2+}.** The actin-activated ATPase activity of myosin V constructs (2 μg/ml) was measured in the buffer containing 30 mM KCl, 1 mM $MgCl_2$, 0.5 mg/ml actin, 0.2 mM [γ-^{32}P]ATP, and 20 mM MOPS, pH 7.0, in the presence (▲) and absence (■) of 12 μM exogenous calmodulin at 25 °C. The activity is indicated per head of myosin V. *A,* DHM5; *B,* SHM5; *C,* M5IQ2.

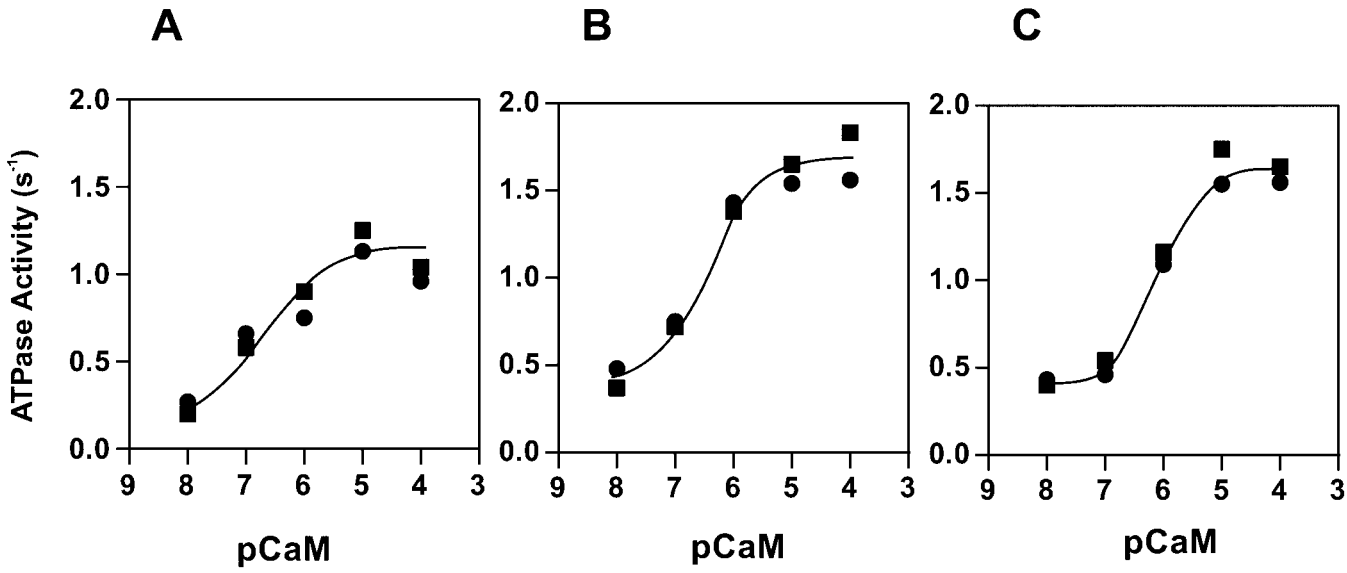

FIG. 4. **Activation of the actin-activated ATPase activity of myosin V constructs by calmodulin.** The actin-activated ATPase activity of myosin V constructs was measured as a function of exogenous calmodulin concentration. The conditions of the assay were the same as described in Fig. 3. *A,* DHM5; *B,* SHM5; *C,* M5IQ2. ■, *p*Ca 5; ●, *p*Ca 4.

units showed a mobility shift with a change in [Ca^{2+}] that is characteristic of calmodulin, suggesting that the small subunits are indeed calmodulin. The identification of the small subunit was also confirmed using anti-calmodulin antibodies (not shown). The stoichiometries of calmodulin *versus* myosin V heavy chain for DHM5, SHM5, and M5IQ2 were 5.7 ± 0.7, 5.3 ± 0.7, and 2.1 ± 0.5, respectively. This is consistent with the number of IQ motifs in each construct.

Actin-activated ATPase Activity—The actin-activated ATPase activity was measured as a function of free Ca^{2+} (Fig. 3). For most constructs, *i.e.* DHM5, SHM5, and M5IQ2, the ATPase activity decreased markedly above *p*Ca 6. In contrast, M5IQ1 showed no *p*Ca dependence (not shown). Interestingly, inhibition of ATPase activity was reversed by addition of 12 μM calmodulin in assay solution (Fig. 3). The effect of exogenous calmodulin on the ATPase activity was common for all three constructs. Fig. 4 shows the calmodulin concentration dependence of the actin-activated ATPase activity of the myosin V constructs. The calmodulin dependence on the activation of the ATPase activity for the three constructs was similar, and a half-maximal activation was observed at 10^{-6} M calmodulin concentration. There was no difference in calmodulin dependence of ATPase activity between *p*Ca 5 and 4, suggesting that the Ca^{2+} effect is saturated at *p*Ca 5. Fig. 5 shows the F-actin dependence of the actin-activated ATPase activities of the three constructs. The actin concentration dependence was similar to each other for the three constructs. K_{actin} estimated from the actin dependence of the ATPase activity was 0.95, 0.80, and 0.60 μM for DHM5, SHM5 and M5IQ2, respectively, in the presence of EGTA. On the other hand, the actin concentration dependence at high [Ca^{2+}] showed two phases. At low F-actin concentration, activity increased with actin concentration, and it was not dramatically different from that in the presence of EGTA. However, the activity decreased at higher actin concentrations. This result suggests that the Ca^{2+}-dependent inhibition of ATPase activity is not due to a change in the affinity for F-actin. The binding of myosin V constructs to F-actin was examined by cosedimentation analysis in the presence of Mg^{2+}-ATP to test further the effect of Ca^{2+} on the affinity between myosin V and F-actin. For all three constructs a significant amount of myosin V cosedimented with F-actin at low ionic strength (more than 50%), but no significant effect of Ca^{2+} on the binding was observed (not shown). The difference in F-actin dependence of the ATPase activities of myosin V at high and low Ca^{2+} conditions is discussed under "Discussion."

Dissociation of Calmodulin from Myosin V—The reversal of the ATPase activity inhibition by high concentrations of exogenous calmodulin suggests that it is due to a decrease in the affinity of calmodulin for myosin V when calmodulin binds Ca^{2+}. To address this notion, the dissociation of calmodulin from myosin V was examined. Myosin V constructs were mixed with F-actin in various free Ca^{2+} concentrations and then

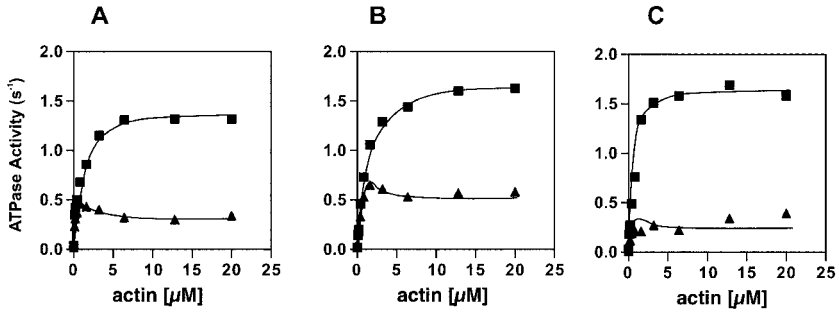

FIG. 5. **F-actin dependence of the actin-activated ATPase activity of myosin V constructs.** The actin-activated ATPase activity of myosin V constructs was measured as described in Fig. 3. No exogenous calmodulin was added. *A,* DHM5; *B,* SHM5; *C,* M5IQ2. ■, EGTA; ▲, *p*Ca 4.

ultracentrifuged to determine bound calmodulin. F-actin coprecipitated myosin V with bound calmodulin was analyzed by SDS-polyacrylamide gel electrophoresis, and the calmodulin band was quantitated by densitometry normalized with myosin V heavy chain. As a control free calmodulin was centrifuged with F-actin, but no calmodulin coprecipitation was detected. Myosin V constructs were also tested for precipitation, but no myosin V was precipitated in the absence of F-actin. As shown in Fig. 6, the calmodulin bound to myosin V decreased significantly between *p*Ca 6 and 5 for all three constructs. Approximately 55% of bound calmodulin was dissociated from M5IQ2 heavy chain, indicating that 1 mol of calmodulin is dissociated from M5IQ2. On the other hand, approximately 35% of the bound calmodulin was dissociated from the DHM5 and SHM5 constructs, indicating that 2 mol of bound calmodulin out of 6 total are dissociated.

Effect of Ca^{2+} on the Motility Activity of Myosin V—Fig. 7 shows the motility activity of DHM5 and M5IQ2 at various calcium ion concentrations. Consistent with the actin-activated ATPase activity, the motility activity was completely inhibited at high Ca^{2+} concentrations. An important finding is that the inhibition of motility was achieved at much lower Ca^{2+} concentrations than the inhibition of the ATPase activity. Furthermore, in contrast to its effects on the inhibition of ATPase activity, addition of exogenous calmodulin did not reverse the motility inhibition (Fig. 7, *C* and *D*). We added exogenous calmodulin up to 59 μM at *p*Ca 4, but the motility activity was not recovered in contrast to the ATPase activity. A similar result was also obtained at *p*Ca 6. The recovery of motility inhibited at high Ca^{2+} was only achieved by reducing Ca^{2+} concentration (Fig. 7, *A* and *B*).

Inhibition of the Actin-activated ATPase Activity by ADP—It was quite recently shown that the ADP release step is the rate-limiting step for the actin-activated ATPase cycle of truncated myosin V (22). Therefore, ADP inhibition of the ATPase activity of myosin V and the effect of Ca^{2+} on this inhibition were studied. As shown in Fig. 8, ADP strongly inhibited the actin-activated ATPase activity both in the presence and absence of Ca^{2+}. By using a K_{ATP} of 1.4 μM (22), a K_i of 1.47 μM was obtained from the ADP dependence of the ATPase activity under EGTA conditions, whereas a K_i of 1.80 μM was found under *p*Ca 4 conditions. This value was significantly lower than for conventional myosins (23–24). Interestingly, Ca^{2+} did not affect the ADP inhibition indicating that the affinity of myosin V for ADP is not influenced by Ca^{2+}.

DISCUSSION

Except for striated muscle myosin II, the biochemically well characterized myosins have self-regulated motor function or contain regulatory components within the myosin molecule itself. The motor function of conventional myosin from vertebrate smooth muscle and non-muscle tissues is regulated by the phosphorylation of its regulatory light chain (25–27). Molluscan conventional myosin is regulated by direct Ca^{2+} binding to the essential light chain as supported by the regulatory light

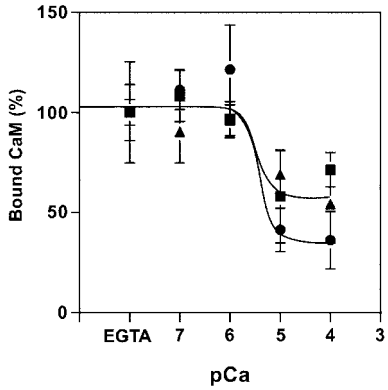

FIG. 6. **Dissociation of bound calmodulin from myosin V constructs at high Ca^{2+}.** Molar ratio of calmodulin to myosin V heavy chain under different *p*Ca conditions is determined as described under "Experimental Procedures." ■, DHM5; ▲, SHM5; ●, M5IQ2.

chain (28). Interestingly, the regulation requires a two-headed structure and the involvement of inter-head interaction in the regulatory mechanism (20, 29–30). Myosin I regulation is the most well studied of the unconventional myosins from vertebrates. These vertebrate myosin Is contain calmodulin as their light chains, and Ca^{2+} binding to this calmodulin light chain regulates their motor activity (8–13). These results raise several questions concerning the regulation of myosin V. Does Ca^{2+} regulate myosin V motor function? If so, does calmodulin play a critical role in the regulation? Is the number of calmodulins in the myosin V molecule important for the regulation? Finally, is the two-headed structure critical for regulation? In this study, we produced three different constructs of myosin V to address these questions concerning regulation, *i.e.* double-headed myosin V, single-headed myosin V with complete IQ motifs, and single-head myosin V with two IQ motifs.

It was reported that the essential light chain of conventional myosin copurified with myosin V from chicken tissue (31) suggesting that it participates as a subunit of myosin V. However, the essential light chain was not found in a myosin V preparation from mouse. Moreover, it was quite recently reported that myosin V expressed in Sf9 cells that were coinfected with calmodulin and essential light chain contains calmodulin exclusively and not the essential light chain (32). Therefore, the myosin V variants produced in the present study that have only calmodulin as light chains represent the likely subunit composition of physiologically relevant myosin V.

Quite recently, double-headed and single-headed myosin V constructs similar to those produced in the present study were reported (32). It was reported that the double-headed myosin V was quite unstable and degraded during cell culture by proteolysis at the IQ domain (32). In contrast, we have not seen any significant degradation of our double-headed myosin V sample. Although the difference is unclear, since the degradation was found at the IQ domain it is likely that the degradation occurred due to a lack of calmodulin binding there. Consistent

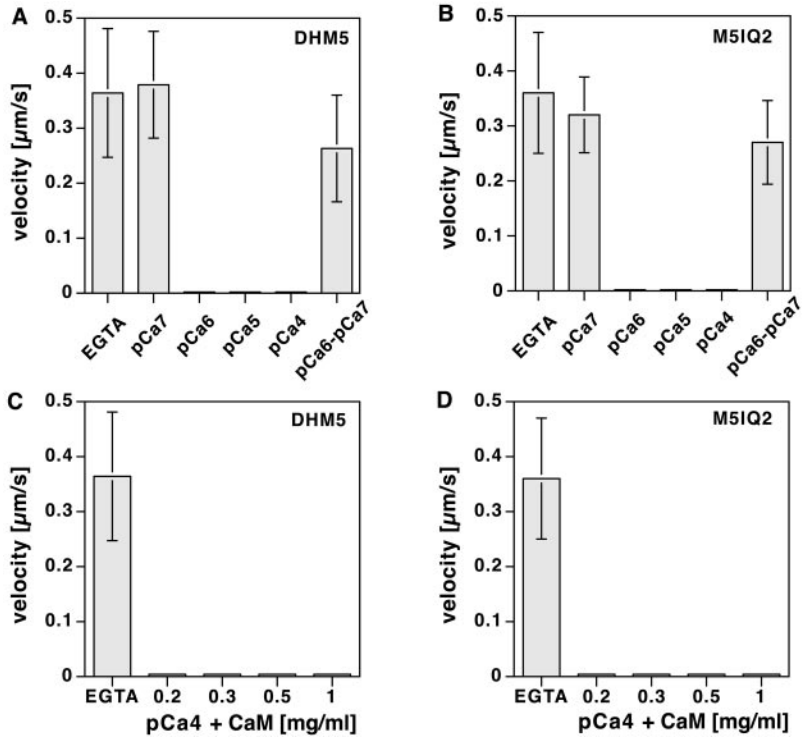

FIG. 7. **Ca^{2+} and calmodulin dependence of actin filament sliding velocity of myosin V constructs.** *A,* Ca^{2+} dependence of motility activity of DHM5; *B,* Ca^{2+} dependence notility activity of M5IQ2. The flow cells with myosin V constructs filled with *p*Ca 6 buffer was refilled with *p*Ca 7 buffer for restoration of the motility. *C* and *D* show the effect of exogenous calmodulin on the motility activity of DHM5 and M5IQ2, respectively. Exogenous calmodulin failed to rescue the motility activity of myosin V at *p*Ca 4. The same result was also obtained at *p*Ca 6 condition. Actin filament motility was observed in 30 mM KCl, 1 mM $MgCl_2$, 12 μM calmodulin, 20 mM MOPS, pH 7.0, 4.5 μg/ml glucose, 216 μg/ml glucose oxidase, 36 μg/ml catalase, and 2 mM ATP at 25 °C. Measurements were made with three independent preparations, and 11–20 actin filaments were measured to obtain an average velocity for preparation. All values are mean velocity ± S.D. In the conditions where actin filaments were moved, nearly all actin filaments observed moved continuously.

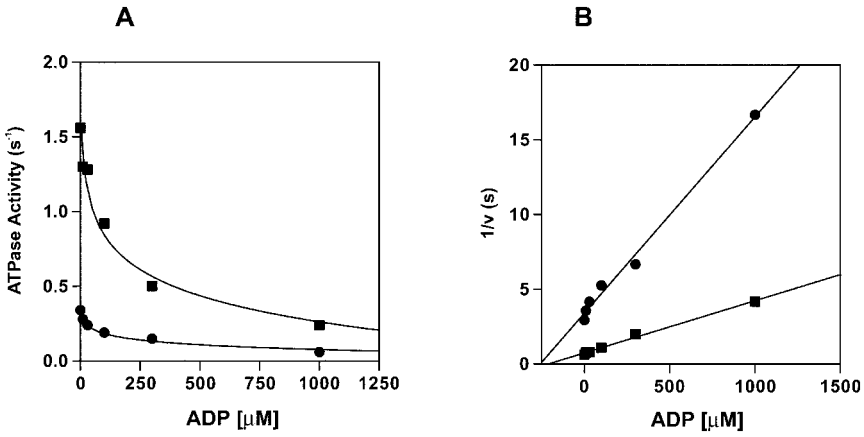

FIG. 8. **Inhibition of the actin-activated ATPase activity by ADP.** The ATPase activity was measured as described in Fig. 3. *A,* ADP concentration dependence of the actin-activated ATPase activity of M5IQ2. *B,* Dixon plots of *A*. ●, *p*Ca 4; ■, 1 mM EGTA.

with this notion, the SHM5 isolated by Wang *et al.* (31) increased in ATPase activity with exogenous calmodulin even while in EGTA, suggesting that their SHM5 was not saturated with calmodulin. The myosin V constructs in this study were not further activated by exogenous calmodulin under EGTA conditions, suggesting that the sites are saturated with bound calmodulin consistent with the determined stoichiometry of the bound calmodulin.

All of these myosin V constructs, *i.e.* DHM5, SHM5, and M5IQ2, showed the same *p*Ca dependence in their actin-activated ATPase activity. The activity was significantly reduced above *p*Ca 6. It can be concluded that this decrease in the ATPase activity is due to the dissociation of calmodulin molecule from the heavy chain because: 1) myosin V-bound calmodulin decreased at *p*Ca 5 and higher, and approximately 1 mol of calmodulin from M5IQ2 or 1–2 mol of calmodulin from DHM5 and SHM5 were dissociated from the heavy chain; and 2) the decrease in ATPase activity observed at *p*Ca 5 and higher was completely reversed by addition of exogenous calmodulin. Since Ca^{2+} binds to calmodulin within this range of free Ca^{2+}, the scenario would be that Ca^{2+} binding to the calmodulin bound to myosin V decreases the affinity of calmodulin for myosin V. However, since only 1 or 2 mol of the bound calmodulin dissociates from myosin V, the apparent decrease in affinity should be the site-specific. The nature of the binding of the IQ peptide with calmodulin has been modeled (33). In the absence of Ca^{2+}, the N-terminal lobe of calmodulin adopts a closed conformation, whereas the C-terminal lobe adopts a semi-open conformation that interacts with the N-terminal side of the IQ peptide via a number of H bonds. Upon Ca^{2+} binding, calmodulin adopts the open conformation, thus the C-terminal lobe cannot bind to the IQ peptide. While the interaction between apocalmodulin and the IQ peptide may diminish, Ca^{2+}/calmodulin may now bind in a distinct manner to the IQ peptide since in general it retains the amphipathic character also found in various calmodulin target peptides (34). It is plausible, therefore, that the dissociation of calmodulin at high Ca^{2+} occurs at specific IQ sites that do not allow calmodulin to rebind at high Ca^{2+} presumably due to a lack of amphipathic character. The present result suggests that 1–2 mol of calmodulin are dissociated from myosin V at high Ca^{2+}. One such Ca^{2+}-dependent site would be the second IQ region, since M5IQ2 loses 1 mol of calmodulin at high Ca^{2+} but M5IQ1 does not. This result is consistent with the recent report (14). Another possible site is the last IQ region, which is least conserved and where the second consensus Arg that interacts via three H bonds with the

N-terminal lobe of calmodulin (according to the model (33)) is replaced by Lys.

It is of interest to identify how Ca^{2+} decreases the actin-activated ATPase activity. Actin binding of myosin V in the presence of ATP was unchanged with Ca^{2+}; therefore, Ca^{2+} does not alter its affinity for F-actin. It was reported quite recently (21) that the rate-limiting step of the unregulated truncated myosin V construct, *i.e.* (M5IQ1), is the ADP off step. We examined the effect of ADP on the inhibition of the ATPase activity, but the apparent K_i value was not influenced by Ca^{2+}. Interestingly, an inhibition of ATPase activity was observed at high, but not low, F-actin concentration in the presence of Ca^{2+}. For conventional myosin it is known that the inhibition of ATPase activity by high F-actin concentrations is due to the inhibition of the ATP hydrolysis step by F-actin (35). Therefore, it is plausible that the inhibition of the ATPase activity by Ca^{2+} for myosin V is due to an inhibition of ATP hydrolysis step.

The inhibition of the actin-activated ATPase activity was completely reversed by the presence of micromolar levels of calmodulin. Since a concentration of free calmodulin of several micromolar is present in cells, the inhibition of ATPase activity may not be operating *in vivo*. An important finding is that the motility of myosin V (DHM5) is completely abolished below *p*Ca 6 where no apparent dissociation of calmodulin takes place. Furthermore, addition of a high concentration of exogenous calmodulin failed to rescue this inhibition of motility. These results indicate that the inhibition of the motility of myosin V is not due to dissociation of calmodulin from the heavy chain. The inhibition occurs between *p*Ca 7 and 6 where cytoplasmic Ca^{2+} concentration is regulated in most cell types; therefore, the observed inhibition is physiologically relevant. Similar results were reported for mammalian myosin Iβ (12, 13). Since this range of Ca^{2+} concentrations corresponds to the high affinity C-terminal sites of calmodulin, it is likely that Ca^{2+} binding to the C-terminal lobe of calmodulin and consequent conformational changes are responsible for the inhibition of motility. Supporting this notion, it was shown that deletion of the C-terminal Ca^{2+}-binding sites abolishes inhibition of the motility of myosin Iβ (13).

Quite recently, it was reported that truncated myosin V containing 2 IQ motifs showed motility activity even at high Ca^{2+} when high exogenous calmodulin was present although the velocity of the motility is reduced (14). At present the reason of this apparent discrepancy between the present result and that by Trybus *et al.* (14) is obscure. The loss of the motility at high Ca^{2+} was not due to the denaturation of myosin V since the perfusion of the flow cell with low Ca^{2+} buffer (*p*Ca 7 or less) completely restored the motility (Fig. 7). We examined various exogenous calmodulin concentrations up to 59 μM that is 5 times higher than that used by Trybus *et al.* (14); therefore, it is unlikely that the loss of the motility is due to the unsaturation of the bound calmodulin. Actually, the inhibition of the motility is observed at *p*Ca 6, where virtually no calmodulin is dissociated from myosin V heavy chain. The present results suggest that true regulation of motility could require two N-terminal IQ domains, in which the conformational change of calmodulin, but not the physical dissociation of calmodulin, would trigger the inhibition of motility. There is an apparent de-coupling between the ATP hydrolysis cycle and mechanical events at higher Ca^{2+}. This is different from the regulation of conventional myosins in which the regulatory domain regulates both ATPase and mechanical activities (25–28). It is plausible that the change in calmodulin conformation alters the rigidity of the "lever arm" and thus decouples the chemical and mechanical events. Alternatively, the conformational change of calmodulin could alter the interaction between calmodulin and the "converter" domain of myosin V, thus inhibiting motility.

The present results clearly indicate that the two-headed structure is not critical for regulation of mechanoenzymatic activity of myosin V. This is distinct from the regulation of conventional myosin, in which an interaction between the two heads is involved in the regulation (20–21, 28–30). Mammalian myosin I, a single-headed unconventional myosin having calmodulin as light chain subunits, has been shown to have Ca^{2+} dependence similar to that shown for myosin V in the present study (8–13). Therefore, it is plausible that there is a common motor activity regulatory mechanism in unconventional myosins carrying calmodulin light chains. However, more detailed biophysical and biochemical information is required for further understanding of the molecular mechanism.

Acknowledgments—We thank Dr. D. J. Schmidt (University of Massachusetts) for reading the manuscript. We also thank Dr. H. D. White (Eastern Virginia Medical School) for comments. We thank Dr. Nancy Jenkins for mouse myosin V cDNA.

REFERENCES

1. Cheney, R. E., and Mooseker, M. S. (1992) *Curr. Opin. Cell Biol.* **4,** 27–35
2. Cheney, R. E., Riley, M. A., and Mooseker, M. S. (1993) *Cell Motil. Cytoskeleton* **24,** 215–223
3. Goodson, H. V., and Spudich, J. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 659–663
4. Mooseker, M. S., and Cheney, R. E. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 633–675
5. Titus, M. A. (1997) *Trends Cell Biol.* **7,** 119–123
6. Espindola, E. M., 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
7. Cheney, R. E., O'Shea, M. K., Heuser, J. E., Coelho, M. V., and Wolenski, J. S., Espreafico, E. M., Forscher, P., Larson, R. E., and Mooseker, M. S. (1993) *Cell* **75,** 13–23
8. Collins, K., Sellers, J. R., and Matsudaira, P. T. (1990) *J. Cell Biol.* **110,** 1137–1147
9. Swanljung-Colling, H., and Colling, J. H. (1991) *J. Biol. Chem.* **266,** 1312–1319
10. Wolenski, J. S., Hayden, S. M., Forscher, P., and Mooseker, M. S. (1993) *J. Cell Biol.* **122,** 613–621
11. Barylko, B., Wagner, M. C., Reizes, O., and Albanesi, J. P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 490–494
12. Zhu, T., and Ikebe, M. (1996) *Biochemistry* **35,** 513–522
13. Zhu, T., Beckingham, K., and Ikebe, M. (1998) *J. Biol. Chem.* **273,** 20481–20486
14. Trybus, K. M., Kremensova, E., and Freyzon, Y. (1999) *J. Biol. Chem.* **274,** 27448–27456
15. Spudich, J. A., and Watt, J. (1971) *J. Biol. Chem.* **246,** 4866–4871
16. Chien, Y., and Dawid, I. (1984) *Mol. Cell. Biol.* **4,** 507–513
17. Ikebe, M., Kambara, T., Stafford, W. F., Sata, M., Katayama, E., and Ikebe, R. (1998) *J. Biol. Chem.* **273,** 17702–17707
18. Laemmli, U. K. (1970) *Nature* **227,** 680–685
19. Ikebe, M., and Hartshorne, D. J. (1985) *J. Biol. Chem.* **260,** 13146–13153
20. Sata, M., Matsuura, M., and Ikebe, M. (1996) *Biochemistry* **35,** 11113–11118
21. Li, X.-D., Saito, J., Ikebe, R., Mabuchi, K., and Ikebe, M. (2000) *Biochemistry* **39,** 2254–2260
22. DeLa Cruz, E. M., Wells, A. L., Rosenfeld, S. S., Ostap, E. M., and Sweeney, H. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 13726–13731
23. White, H. D., Belknap, B., and Jiang, W. (1993) *J. Biol. Chem.* **268,** 10039–10045
24. White, H. D., Belknap, B., and Webb, M. R. (1997) *Biochemistry* **36,** 11828–11836
25. Hartshorne, D. J. (1987) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., ed) 2nd Ed., pp. 423–482, Raven Press, Ltd., New York
26. Sellers, J. R., and Adelstein, R. S. (1987) *Enzymes* **18,** 381–418
27. Tan, J. L., Ravid, S., and Spudich, J. A. (1992) *Annu. Rev. Biochem.* **61,** 721–759
28. Xie, X., Harrison, D. H., Schlichting, I., Sweet, R. M., Kalabokis, V. N., Szent-Gyorgyi, A. G., and Cohen, C. (1995) *Nature* **368,** 306–312
29. Cremo, C. R., Sellers, J. R., and Facemyer, K. C. (1995) *J. Biol. Chem.* **270,** 2171–2175
30. Matsuura, M., and Ikebe, M. (1995) *FEBS Lett.* **363,** 246–250
31. Espindola, F. S., Cheney, R. E., Kings, S. M., Suter, D. M., and Moosekar, M. S. (1996) *Mol. Biol. Cell* **7,** 372
32. Wang, F., Chen, L., Arcucci, O., Harvey, E. V., Bowers, B., Yuhui, X., Hammer, J. A., III, and Sellers, J. R. (2000) *J. Biol. Chem.* **275,** 4329–4335
33. Houdusse, A., Silver, M., and Cohen, C. (1996) *Structure* **4,** 1475–1490
34. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Kle, C. B., and Bax, A. (1992) *Science* **256,** 632–638
35. Meador, W. E., Means, A. R., and Quiochu, F. A. (1992) *Science* **257,** 1251–1254