THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 43, Issue of October 26, pp. 39812–39818, 2001

Printed in U.S.A.

High Affinity Binding of Brain Myosin-Va to F-actin Induced by Calcium in the Presence of ATP*

Received for publication, March 22, 2001, and in revised form, August 8, 2001
Published, JBC Papers in Press, August 21, 2001, DOI 10.1074/jbc.M102583200

Sinji B. F. Tauhata‡§¶, Daniela Vital dos Santos‡‖, Edwin W. Taylor§, Mark S. Mooseker§‡‡, and Roy E. Larson‡§§**

*From the ‡Department of Cellular and Molecular Biology, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil, 14049-900, the §Marine Biological Laboratory, Woods Hole, Massachusetts 02543, the **Department of Cell & Molecular Biology, Northwestern University Medical School, Chicago, Illinois 60611, and the ‡‡Departments of MCD-Biology, Cell Biology, and Pathology, Yale University, New Haven, Connecticut 06511*

Brain myosin-Va consists of two heavy chains, each containing a neck domain with six tandem IQ motifs that bind four to five calmodulins and one to two essential light chains. Previous studies demonstrated that myosin-Va exhibits an unusually high affinity for F-actin in the presence of ATP and that its MgATPase activity is stimulated by micromolar Ca^{2+} in a highly cooperative manner. We demonstrate here that Ca^{2+} also induces myosin-Va binding to and cosedimentation with F-actin in the presence of ATP in a similar cooperative manner and calcium concentration range as that observed for the ATPase activity. Neither hydrolysis of ATP nor buildup of ADP was required for Ca^{2+}-induced cosedimentation. The Ca^{2+}-induced binding was inhibited by low temperature or by 0.6 M NaCl, but not by 1% Triton X-100. Tight binding between myosin-Va and pyrene-labeled F-actin in the presence of ATP and Ca^{2+} was also detected by quenching of the pyrene fluorescence. Negatively stained preparations of actomyosin-Va under Ca^{2+}-induced binding conditions showed tightly packed F-actin bundles cross-linked by myosin-Va. Our data demonstrate that high affinity binding of myosin-Va and F-actin in the presence of ATP or 5′-*O*-(thiotriphosphate) is induced by micromolar concentrations of Ca^{2+}. Since Ca^{2+} regulates both the actin binding properties and actin-activated ATPase of myosin-Va over the same concentration range, we suggest that the calcium signal may regulate the mechanism of processivity of myosin Va.

Class V myosins are widely expressed, actin-based motors that have been implicated in the transport and/or localization of a wide range of organelles as well as mRNA (reviewed in Refs. 1–3). Class V myosins have two motor head domains, typical of myosins, connected to the tail domain via an extended neck domain with six tandem IQ motifs that bind multiple light chains most or all of which, depending on the myosin, consist of calmodulin. Brain myosin-Va (M-Va)[1] has lent itself quite well to biochemical characterization, since the native protein is readily purified from chicks (4), mice (5), and other vertebrates (6), and functional constructs of the mechanochemical head domain and regulatory light chain domain have been expressed in the baculovirus system (5, 7–11). A noteworthy biochemical property of M-Va is its relatively high affinity for F-actin in the presence of ATP (5, 7, 9, 12). K_{ATPase} values of 1.8, 0.8, and 0.15 μM have been reported for native chick M-Va (12), native mouse M-Va (5), and recombinant mouse heavy meromyosin-like fragment of M-Va (11), respectively. Recently, kinetic and visual evidence that M-Va is a processive motor has been obtained (8–10, 13–15). The mechanism of processivity for M-Va seems to include two-headed, linear binding to a single actin filament (10) and delayed ADP release from the myosin, nucleotide-binding site, plus rapid ATP binding and hydrolysis, resulting in a high duty ratio (8) for each head. These properties are consistent with the postulated cellular functions of M-Va in organelle transport (16–19). Thus, high affinity for actin and processive movement would guarantee that M-Va with its cargo maintains contact with actin tracks.

A troublesome difference between properties determined on the native protein *versus* the baculovirus-expressed constructs has been the lack of consistent results on the Ca^{2+} regulation of the mechanochemical events. Whereas the actin-activated ATPase of native M-Va is tightly regulated by Ca^{2+} (5, 6, 12, 20), this property has not been consistently reported for the recombinant proteins. On the other hand, both recombinant and native proteins move actin in the *in vitro* motility assays in the presence of EGTA, whereas calcium inhibits this motility (5, 7, 11, 13, 20). This apparent paradoxical effect of calcium on the ATPase activity of native M-Va in solution *versus* its motility as a surface-adsorbed molecule has not been satisfactorily explained, suggesting that there is much to be learned about the regulatory role of Ca^{2+} on the mechanochemical cycle of M-Va. The likely targets for Ca^{2+} are the calmodulin light chains, each with four potential Ca^{2+} binding sites, bound to the neck domain. There is direct evidence that calmodulin dissociates from M-Va in the presence of micromolar Ca^{2+} (11, 12, 21). Excess calmodulin protects against the irreversible blocking of motility by calcium, although the velocity of movement is still lowered in the presence of calcium (20).

* This work was supported by grants (to R. E. L.) from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Programa de Apoio ao Desenvolvimento Científico e Tecnológico (PADCT), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação Apoio ao Ensino, Pesquisa e Assistência do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto (FAEPA) and by National Institutes of Health Grants DK 25387 and DK 55389 (to M. S. M.). A portion of these studies was conducted in the Physiology Course (1998), Marine Biological Laboratory, Woods Hole, MA. 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.

¶ Predoctoral and Postdoctoral Fellow supported by FAPESP.
‖ Held an undergraduate stipend from FAPESP.
§§ To whom correspondence should be addressed. Tel.: 55-16-602-3319; Fax: 55-16-633-1786; E-mail: relarson@fmrp.usp.br.

[1] The abbreviations used are: M-Va, myosin-Va; ATPγS, adenosine 5′-*O*-(thiotriphosphate); Pipes, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

In the experiments described here, we show that micromolar Ca^{2+} induces high affinity binding of native chick M-Va to actin filaments in the presence of ATP or its nonhydrolyzable derivative, ATPγS. Three distinct methods have been used to characterize the actomyosin-Va complex induced by calcium: (*a*) cosedimentation of M-Va with F-actin, (*b*) quenching of pyrene-labeled F-actin by M-Va, and (*c*) electron microscopy imaging of the negative-stained actomyosin-Va. The results indicate that micromolar Ca^{2+} can be a switch that regulates the actin binding properties of M-Va and, consequently, may participate in the mechanism underlying processivity of this myosin.

EXPERIMENTAL PROCEDURES

Material—EDTA, EGTA, dithiothreitol, aprotinin, benzamidine, electrophoresis chemicals, imidazole, phalloidin, and ATP (grades I and II) were purchased from Sigma. ATPγS was purchased from Calbiochem and ADP from ICN. Chromatography media were purchased from Amersham Pharmacia Biotech and EM Separations Technology. Pefabloc was from Roche Molecular Biochemicals. *N*-(1–1-Pyrenyl)iodoacetamide was purchased from Molecular Probes. Electron microscopy material was obtained from Electron Microscopy Science.

Proteins—M-Va was purified from chick brains essentially as described by Cheney (4). Actin was purified from chicken breast muscle or rabbit dorsal muscle by the method of Spudich and Watt (22). Calmodulin was initially purified from bovine brain by the method of Gopalakrishna and Anderson (23) and further purified by ion exchange chromatography as described previously (24). Fragment S1 from skeletal muscle myosin II was obtained by the method of Margossian and Lowey (25).

Actin Binding Assay—The binding of M-Va to phalloidin-stabilized F-actin was assayed in buffer A (10 mM imidazole, pH 7.4, 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol) in the presence of 2 mM ATP, ADP, or ATPγS and Ca^{2+} concentrations as indicated. When done in the presence of the ATP-regenerating system, the reactions also contained 0.22 mM NADH, 20 units/ml lactic dehydrogenase, 100 units/ml pyruvate kinase, and 0.5 mM phospho(enol)pyruvate, as described by De La Cruz (29). A Ca-EGTA buffer was used in the assays covering a range of free [Ca^{2+}] up to 10 μM. Free [Ca^{2+}] was calculated using the computer program Mcalc (26) with the Schwartzenbach dissociation constants. Unless otherwise stated, samples were incubated at 25 °C for 10 min followed by ultracentrifugation at 4 °C (100,000 × *g* for 30 min) to pellet F-actin. Resulting supernatants, and pellets were analyzed by SDS-PAGE. Coomassie Blue-stained bands were quantified by densitometry and analyzed by using the ImageQuant software (Molecular Dynamics).

Fluorescence Assay of M-Va and F-actin Interaction—Fluorescence assays were performed in quartz microcuvettes using buffer P (20 mM Pipes, pH 7.4, 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol). The concentration of pyrene-actin was fixed at 0.30 μM, and varying concentrations of M-Va or muscle myosin S1 were added as indicated. The mixtures were excited at 365 nm and the emission monitored at 405 nm using a DeltaScan fluorimeter (Photon Technology International, Mammoth Junction, NJ). The data were collected on line using the computer program Felix (Photon Technology International). This instrument was on loan from Photon Technology International to the Marine Biological Laboratory. For the steady state studies, reactions were mixed 1 min before taking a reading, and for the kinetic studies, readings were initiated as quickly as possible after mixing and thereafter taken at 1–30-s intervals. The S1 fragment from skeletal muscle myosin II was used as a control.

Electron Microscopy—For negative staining of actin filaments decorated with M-Va, mixtures of phalloidin-stabilized F-actin (0.48–1.0 μM) and M-Va (0.1–0.48 μM) were incubated for 5–10 s in buffer A in the presence or absence of 2 mM ATP or ATPγS and then immediately applied to nitrocellulose-carbon-coated copper grids for 10–20 s, rinsed, and fixed by application of buffer A containing 2% glutaraldehyde and negatively stained with 0.5% uranyl acetate.

Miscellaneous—Pyrene-actin was obtained by conjugating *N*-(1–1-pyrenyl)iodoacetamide to purified actin as described by Kouyama and Mihashi (27). SDS-PAGE was performed using 4–20% linear gradient mini-gels. The concentrations of purified proteins were calculated based on the following extinction coefficients at 280 nm for 1 mg/ml solutions: 1.04 for M-Va (20) and 1.09 for actin.

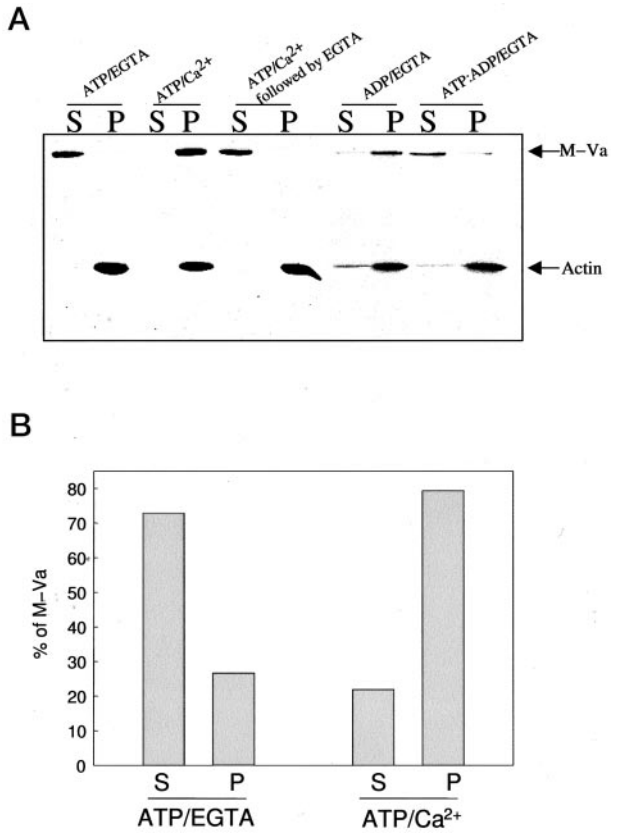

FIG. 1. **Ca^{2+} induced the cosedimentation of M-Va and F-actin in the presence of ATP.** The supernatant (*S*) and pellet (*P*) from cosedimentation assays containing 2 mM ATP, 0.1 μM M-Va, and 1 μM phalloidin-stabilized F-actin were analyzed by SDS-PAGE stained with Coomassie Blue. *A*, reactions contained 2 mM ADP, 4 mM EGTA, 13 μM free Ca^{2+}, or 13 μM free Ca^{2+} followed by 5 mM EGTA, as indicated. The bands representing M-Va heavy chain and actin are indicated by *arrows* to the *right* of the figure. *B*, the reactions contained the ATP-regenerating system described under "Experimental Procedures"and either 4 mM EGTA or 13 μM free Ca^{2+}, as indicated. Samples were analyzed by SDS-PAGE stained with Coomassie Blue, and the band corresponding to M-Va heavy chain was scanned and quantified. Data are expressed as percentage of total M-Va from three experiments.

RESULTS

Ca^{2+} Induces the Cosedimentation of M-Va with F-actin in the Presence of ATP in a Highly Cooperative Manner—Several laboratories have now shown that M-Va has an unusually high affinity for F-actin in the presence of ATP, based on determinations of the activation of the MgATPase by F-actin (K_{ATPase} values ranging from 0.15 to 1.8 μM) and by cosedimentation and light scattering studies of M-Va and low concentrations of F-actin in the presence of ATP (7, 12). Previous studies on native chick M-Va (12) have shown that the ATPase activity and the percentage of M-Va that cosedimented with F-actin was significantly increased by the addition of Ca^{2+} at micromolar concentrations. In the experiments described here (Fig. 1*A*), we obtained very little cosedimentation in the presence of ATP at submicromolar concentrations of Ca^{2+} and nearly 100% cosedimentation of chick M-Va with 1 μM F-actin in the presence of ~10 μM free [Ca^{2+}]. The effect of Ca^{2+} was reversible, since the sequential addition of EGTA, after incubation with Ca^{2+}, liberated M-Va to the supernatant (Fig. 1*A*). Since Ca^{2+} stimulates the ATP hydrolytic activity of M-Va, and since ADP has a relatively high affinity for M-Va, we considered the possibility that the Ca^{2+} effect is mediated by the formation of ADP and displacement of ATP at the nucleotide-binding site. However, the presence of ADP at equimolar concentration with ATP did not induce cosedimentation of M-Va and actin in the

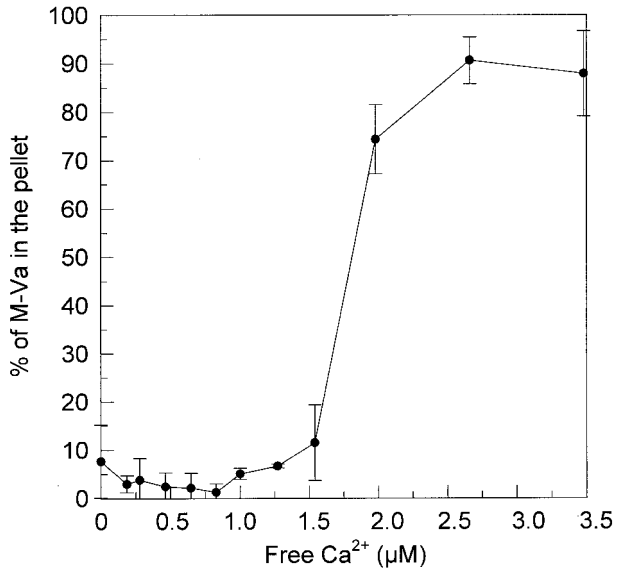

FIG. 2. **The effect of Ca^{2+} on the cosedimentation of M-Va and F-actin is highly cooperative in the micromolar range.** The percentage of M-Va in the pellet was determined by densitometry of the M-Va heavy chain after cosedimentation assays performed as in Fig. 1, except that the free Ca^{2+} concentration was varied from submicromolar concentrations up to 3.5 μM. The cosedimentation of M-Va in the absence of ATP was considered to be 100%. Each data point is the average of three determinations. The *line* was drawn connecting the average values.

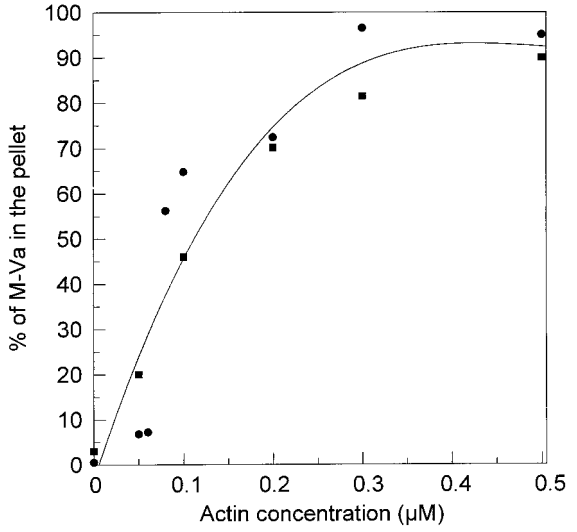

FIG. 3. **M-Va shows high affinity binding to F-actin in the presence of ATP or ATPγS, a nonhydrolyzable derivative.** Cosedimentation assays were performed with 0.3 μM M-Va in the presence of 13 μM free Ca^{2+}, 2 mM ATP (●) or ATPγS (■), and concentrations of F-actin were varied from 0 to 0.5 μM. Each data point is a single determination. The *line* was traced by the SigmaPlot software, including all points.

absence of Ca^{2+}, although ADP alone clearly did (Fig. 1*A*). Also, when done in the presence of an ATP-regenerating system, Ca^{2+} still induced the cosedimentation of M-Va and actin (Fig. 1*B*). Thus, the data indicate that ADP does not mediate the Ca^{2+} effect. The Ca^{2+} concentration dependence of the cosedimentation (Fig. 2) was highly cooperative with a maximum effect at about 3 μM Ca^{2+}. A Hill plot of the data (not shown) suggests the involvement of about eight Ca^{2+}-binding sites per myosin molecule. This result is strikingly similar to the activation curve of the actin-activated, MgATPase of M-Va over the same range of Ca^{2+} concentrations (12) and, thus, suggests that calcium is affecting the ATPase activity and myosin binding to actin via a common effector. Since there are four to five calmodulin molecules bound to each neck domain of M-Va, and each calmodulin has four potential Ca^{2+}-binding sites, the data are consistent with the involvement of at least two or more molecules of calmodulin per M-Va. The present data show that micromolar Ca^{2+} indeed induces reversible binding of M-Va to F-actin in the presence of ATP.

M-Va Shows High Affinity Binding to F-actin in the Presence of Either ATP or ATPγS—Almost complete cosedimentation of M-Va was obtained at submicromolar concentrations of phalloidin-stabilized F-actin in the presence of Ca^{2+} and either ATP or its nonhydrolyzable analog, ATPγS (Fig. 3). Both nucleotides gave similar activation curves, and the joint data indicate that 50% of the M-Va cosedimented with F-actin at concentrations of ~0.1 μM. Note that about 0.3 μM actin was capable of binding 0.3 μM M-Va, indicating that single-headed binding predominated under the conditions of this experiment. Thus, the Ca^{2+}-induced binding shows a very high affinity for actin and does not appear to require the hydrolysis of ATP.

The Ca^{2+}-induced Binding Is Inhibited by Cold or by High Ionic Strength but Not by 1% Triton X-100—To further characterize the Ca^{2+}-induced cosedimentation, we tested the effects of temperature, elevated NaCl concentration, and Triton X-100. Cosedimentation experiments were normally done by mixing M-Va and F-actin from stock solutions on ice into buffered reaction mixtures at room temperature followed by incubation at 25 °C for 10 min in a water bath, followed by centrifugation for 30 min at 4 °C. In the experiments illustrated in Fig. 4*A*, the reaction tubes were maintained on ice for 10 min immediately after the addition of the proteins, and either centrifuged directly or incubated at 25 °C for 1 or 10 min, followed by centrifugation. The results demonstrated that an incubation of about 10 min at 25 °C was necessary for complete cosedimentation of M-Va and actin. In Fig. 4*B*, the addition of 0.6 M NaCl to the reaction mix partially inhibited the cosedimentation induced by Ca^{2+} in the presence of ATP, but not under rigor conditions (Fig. 4*B*). Thus, the binding properties under Ca^{2+}-induced cosedimentation are not equivalent to the rigor state. On the other hand, extraction with 1% Triton X-100 (Fig. 4*C*) did not alter the cosedimentation whether in the presence or absence of ATP, indicating that Ca^{2+} induces a robust, detergent-resistant, binding state.

M-Va Promotes Quenching of the Fluorescence of Pyrene-labeled Actin in the Presence of ATP Only when Ca^{2+} Is Present—A sensitive method for analyzing tight binding of myosin to actin is to monitor the fluorescence of pyrene-labeled F-actin (25). As seen in Fig. 5*A*, increasing concentrations of M-Va promoted a decrease in the pyrene-actin fluorescence under rigor conditions or in the presence of ATP when Ca^{2+} was present. A single determination at 0.33 μM M-Va showed that Ca^{2+} was necessary for the quenching effect when ATP was present. The end point was approximately the same under either rigor or with ATP and Ca^{2+}, although the concentration of M-Va necessary to attain 50% of the maximum quench was three times greater when ATP was present than under rigor conditions. The S1 fragment from skeletal muscle myosin also quenched pyrene-actin under rigor conditions to a similar extent, but with lower affinity, as M-Va, but not when ATP and Ca^{2+} were present (Fig. 5*B*). Thus, M-Va is distinctive in this property.

This method also allowed for a time course analysis of the effects of individual components on the myosin-actin binding. The fluorescence of pyrene-actin was first monitored with M-Va in the rigor state (Fig. 6*A*). The addition of ATP resulted in a rapid increase in fluorescence with a slower second component reaching maximum fluorescence in about 2 min. Then,

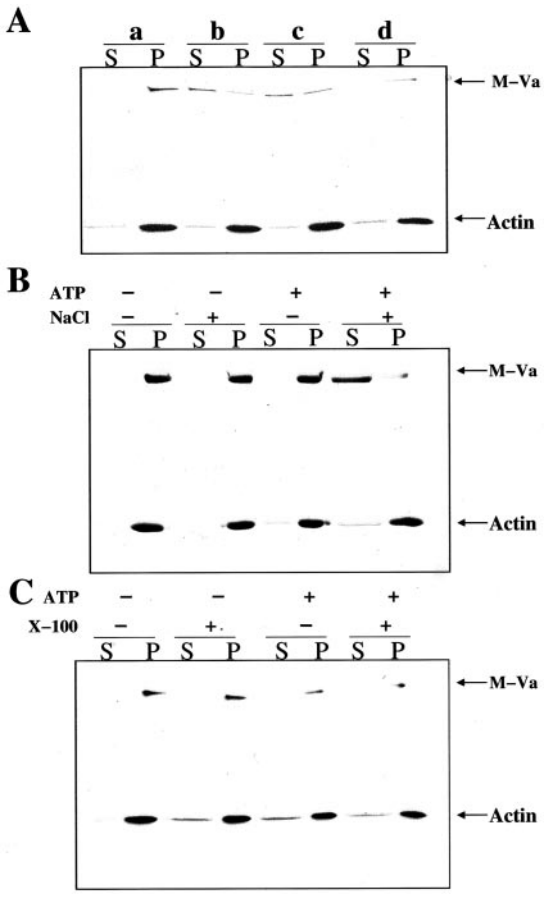

FIG. 4. **The effects of temperature, NaCl, and Triton X-100 on the Ca^{2+}-induced cosedimentation.** The supernatant (*S*) and pellet (*P*) from cosedimentation assays containing 2 mM ATP (unless otherwise indicated), 13 μM free Ca^{2+}, 0.1 μM M-Va, and 1 μM phalloidin-stabilized F-actin were analyzed by SDS-PAGE stained with Coomassie Blue. *A*, immediately before centrifugation the reactions were incubated at 25 °C for 10 min (*a*); left on ice for 10 min (*b*); left on ice for 10 min, followed by incubation at 25 °C for 1 min (*c*); left on ice for 10 min, followed by incubation at 25 °C for 10 min (*d*). *B*, the reaction mixture contained 0.6 M NaCl and/or 2 mM ATP as indicated. *C*, the reaction mixture contained 1% Triton X-100 (*X-100*) and/or 2 mM ATP as indicated.

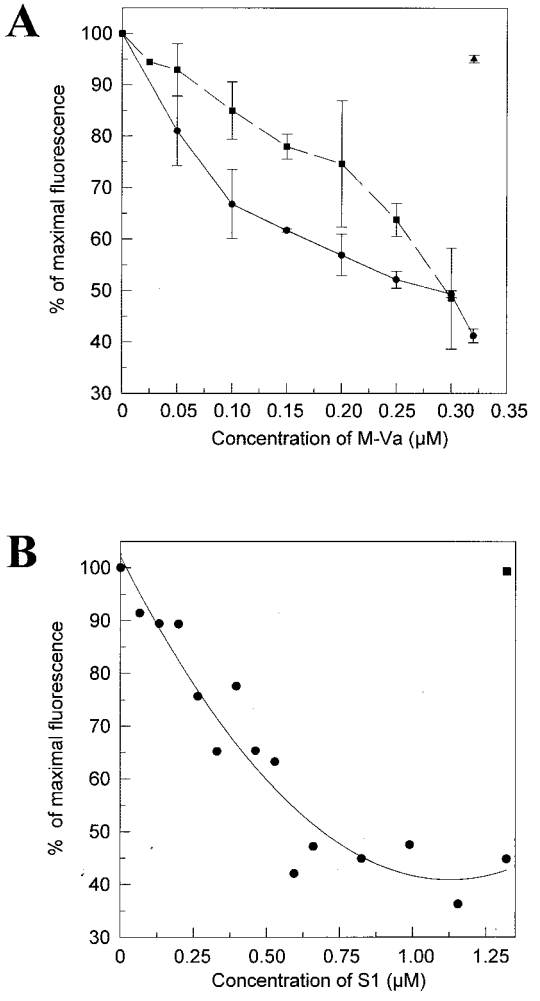

FIG. 5. **The fluorescence of pyrene-labeled F-actin is quenched by M-Va in the presence of ATP and Ca^{2+}.** The steady state fluorescence of 0.3 μM pyrene-actin was monitored at varying concentrations of M-Va in the presence of 2 mM ATP plus 13 μM free Ca^{2+} (*A*, ■), Ca^{2+} but no ATP (*A*, ●), or ATP but no Ca^{2+} (*A*, ▲). Each data point is the average of three determinations. The *lines* were drawn connecting the average values. *B*, S1 fraction of muscle myosin in the presence of 13 μM free Ca^{2+} with (■) or without (●) 2 mM ATP. Each data point represents a single determination. The *line* was traced by the SigmaPlot software, including all points.

the addition of Ca^{2+} resulted in an initial rapid drop in fluorescence, 50% of the total quenching effect occurring within about 40 s, followed by a slower component attaining fluorescent levels near those of the rigor state in about 10 min. (Note that since these experiments involved manual mixing, the exact kinetics was not determined.) Similar experiments with S1 from muscle (Fig. 6*B*) showed that ATP induced its disassociation from actin, but no significant quenching occurred upon Ca^{2+} addition.

Ca^{2+} Induces Actin Bundling Activity of M-Va in the Presence of ATP and ATPγS—The complexes formed by the addition of M-Va to F-actin in the presence of Ca^{2+} and ATP were examined by electron microscopy of negatively stained preparations using conditions identical to those in the cosedimentation assays of Figs. 1 and 2 (M-Va:actin at 1:10). As noted in earlier studies (20), under rigor conditions M-Va induces the formation of tight, actin bundles in both the absence (not shown) and presence of Ca^{2+} (Fig. 7*B*), although "arrowhead" decoration of the filaments is not observed at these low ratios of M-Va to actin. In the presence of ATP, bundles were also seen in the presence (Fig. 7*D*) but not in the absence of Ca^{2+} (Fig. 7*C*). These bundles, seen best at low magnification (*inset* to Fig. 7*D*), are more curved than under rigor conditions. At saturating ratios of M-Va to actin we were unable to adequately visualize the bundles at high magnification because of the formation of uranyl phosphate precipitates. However, in the presence of Ca^{2+} and ATPγS, 1:1 mixtures of M-Va and actin contained tight, often highly curved bundles of actin (forming circles and figure eights) on which the M-Va cross-links could be detected (Fig. 8). In contrast to rigor conditions (20); however, arrowhead decoration under these conditions was not observed. Since the tail domain does not bind actin (12), these bundles are presumably formed by head-head linkages between two actin filaments.

DISCUSSION

We have shown by three distinct methods that Ca^{2+} reversibly induces a high affinity binding between native M-Va and F-actin in the presence of ATP. This effect of Ca^{2+} has not been observed in any other myosin biochemically characterized to date. The similarity in the concentration range and cooperative behavior of Ca^{2+} on the actin-binding properties of M-Va shown here, compared with its effect on the actin-activated MgATPase activity (12), suggests that both effects result from a common site of action, most likely the specific binding of Ca^{2+} to multiple calmodulins associated with the elongated neck

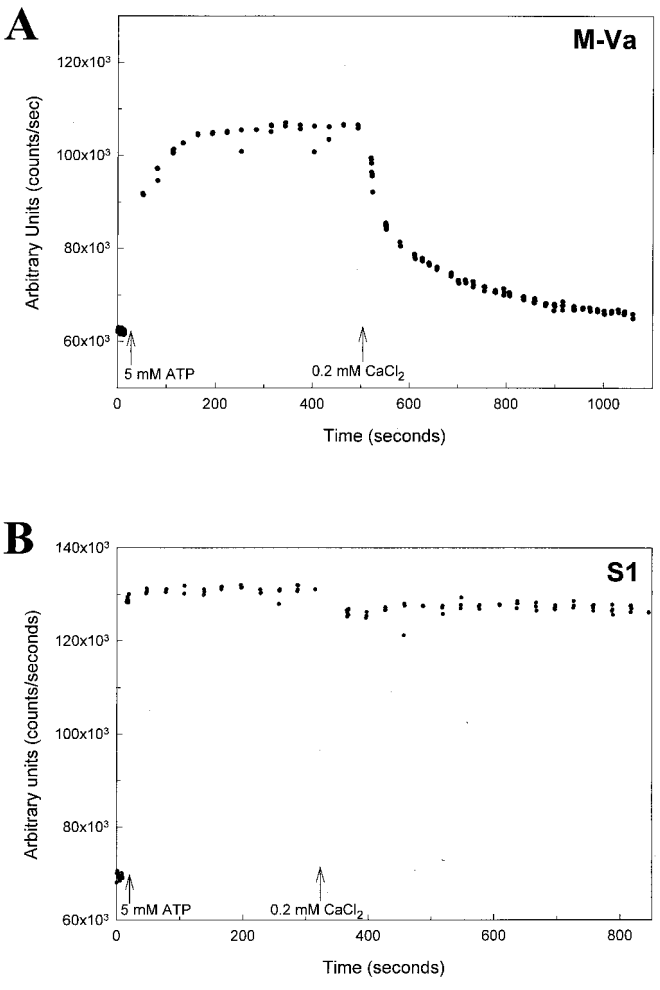

FIG. 6. **Changes in the fluorescence of pyrene-labeled F-actin in the presence of M-Va by ATP and Ca^{2+}.** *A*, the time course of fluorescence change of a mixture of pyrene-actin (0.3 μM) and M-Va (0.32 μM) was followed immediately after the sequential additions of 5 mM ATP and 0.2 mM $CaCl_2$ as indicated in the figure. Readings were taken at 2 s before additions and at every 15–30 s after additions. *B*, a similar experiment was done with the S1 fraction from skeletal muscle myosin.

domain of this myosin. The exact mechanism that links Ca^{2+} binding to these effects is not known, although models for the structural interaction of light chains with the α-helical neck domain have been proposed (28), and recent work has shown that at least two IQ motifs with bound calmodulins are sufficient to obtain Ca^{2+}-regulated ATPase and *in vitro* motility activities (7, 11). Hill plots of the Ca^{2+} effect on ATPase activity (12) and actin binding of native chick M-Va (data not shown) are consistent with the involvement of multiple Ca^{2+}-binding sites per neck domain. It has been demonstrated that Ca^{2+} induces the release of one or more calmodulins from the neck domain at micromolar concentrations (11, 20, 21). Thus, the accumulated evidence clearly indicates that the calmodulins associated with the neck domain have a role in the Ca^{2+} regulation of the mechanochemical events of the motor-head domain.

There is a clear, but unexplained, difference in Ca^{2+} effects on the recombinant *versus* native M-Va samples. All of the recombinant proteins that express the head domain plus varying IQ and coiled-coil regions show actin-activated MgATPase activities and motility on *in vitro* assays more or less equivalent to the native protein, indicating that the basic, mechanochemical properties are well preserved for all of these biochemical fractions. On the other hand, quite unlike native M-Va (5, 6, 12, 20), Ca^{2+} either has no effect or inhibits the actin-activated MgATPase of the recombinant proteins (5, 7, 11). Exogenous calmodulin can either partially or completely overcome the inhibition by Ca^{2+}, but in no case has activation been shown. This difference was most pointedly demonstrated by directly comparing the Ca^{2+} effects on recombinant and native murine M-Va (5). Thus, either the tail domain, present in the native but not in the recombinant fractions, is somehow involved in regulation of the mechanochemical events, or the functional, native light chain arrangement has not been achieved on the recombinant proteins. A third possibility, suggested by Trybus and collaborators (7), is that the purified native protein has become "uncoupled", perhaps due to the extraction procedure. The high sensitivity, cooperative behavior, and the reversibility of the Ca^{2+} effects on M-Va shown in the present study argue against this latter possibility.

Recent evidence suggests that ADP release is the rate-limiting step of the mechanochemical cycle of M-Va (8). Also, the affinity of M-Va for ADP is quite high, thus product inhibition rapidly becomes significant over short reaction times (29). Since Ca^{2+} also stimulates the actin-activated MgATPase of M-Va, we must consider the possibility that the effect of Ca^{2+} on the M-Va/F-actin affinity is indirect, via the formation of ADP and its competition for the nucleotide-binding site. The most direct evidence against this possibility, shown in Fig. 1, is the demonstration that the presence of ADP at equimolar concentration with ATP did not result in cosedimentation in the absence of Ca^{2+} and that even in the presence of an ATP-regenerating system the addition of Ca^{2+} induced the cosedimentation between actin and M-Va. Furthermore, the pyrene-actin experiments, shown in Figs. 5 and 6, also address this question. Under conditions appropriate for low ATPase activity (very low actin concentration, 0.3 μM, well below the K_{ATPase} of chick M-Va), the presence of 5 mM ATP brought the fluorescence up to free pyrene-actin levels, and in turn, the addition of Ca^{2+} caused an immediate, rapid drop in fluorescence, indicating the reformation of a tight actomyosin-Va complex. Finally, equivalent, high affinity binding also occurred with ATPγS, a nonhydrolyzable ATP analog, as with ATP itself (Fig. 3); thus, the hydrolysis of ATP to form ADP was not a necessary step. Together, the evidence indicates that purified, native M-Va forms a tight complex with F-actin in the presence of ATP if Ca^{2+} is also present. This complex has properties unlike the rigor state, is reversibly undone by removing Ca^{2+}, and does not require ATP hydrolysis nor the presence of ADP. The fact that Ca^{2+} has a dual effect on the mechanochemical properties of M-Va, activation of the actin-activated MgATPase activity and induction of high affinity binding between M-Va and F-actin, suggests that Ca^{2+} may participate in the mechanism of processivity of this molecular motor.

There is much evidence that class V myosins associate with organelles in eukaryotes and have a role in their cellular transport (for recent reviews, see Refs. 1, 2, 16, 30, and 31). The phenotype produced by mutations in the *dilute* locus in mice, whose product is M-Va, is illustrative of this role. *Dilute* melanocytes are characterized by an accumulation of melanosomes at the cell center, whereas wild-type melanocytes show a polarized distribution of melanosomes toward the dendritic tips. In the latter case, this process delivers pigment to the keratinocytes and subsequently to hair and skin. Thus, M-Va has a role in the transport and/or peripheral localization of the pigment granules. A recently advanced hypothesis, coined the "cooperative/capture mechanism" (32), is based on evidence that long range transport of pigment granules occurs principally by bidirectional movement along microtubules, but a M-Va- and actin-dependent capture of the melanosomes occurs at the cell

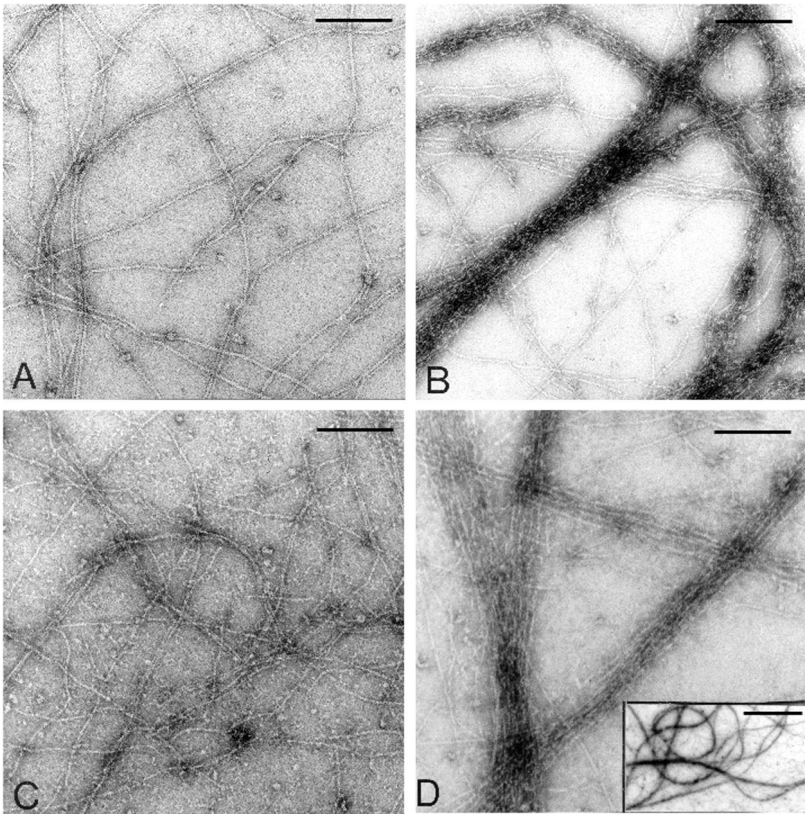

FIG. 7. **Electron micrographs of actomyosin Va in the presence of Ca^{2+} and ATP.** Mixtures of 1 μM phalloidin-stabilized F-actin alone (*A*) or with 0.1 μM M-Va (*B–D*) in the absence (*B*) or presence (*A*, *C*, *D*) of ATP and either 13 μM free Ca^{2+} (*A*, *B*, *D*) or 2 mM EGTA (*C*) were negatively stained immediately after mixing. Samples in *C* and *D* were fixed with 1% glutaraldehyde prior to negative staining. *Inset* in *D*, low magnification showing bundles formed in the presence of Ca^{2+} and ATP. *Bar*: *A–D*, 0.2 μm; *D*, *inset*, 2 μm.

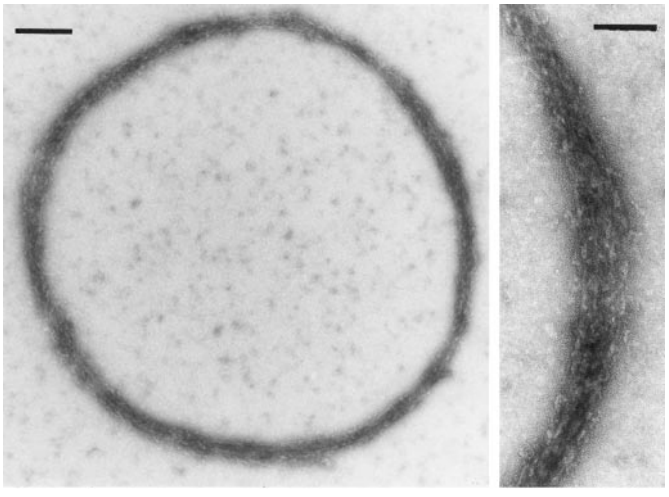

FIG. 8. **Electron micrographs of actomyosin Va in the presence of Ca^{2+} and ATPγS.** Mixtures of 0.24 μM phalloidin-stabilized F-actin with 0.48 μM M-Va in the presence of ATPγS and 13 μM free Ca^{2+} were negatively stained immediately after mixing. *Left panel*, example of the curved bundles formed in the presence of ATPγS. *Right panel*, higher magnification of a portion of this bundle. *Bar*: *left panel*, 0.2 μm; *right panel*, 0.1 μm.

periphery where microtubules terminate and the cortical F-actin network is abundant. The mechanism of capture may involve physical restriction inherent to an isotopic actin network (32), but we also suggest, based on the biochemical properties of M-Va we have described here, that Ca^{2+} may act as a switch that would favor capture by increasing the M-Va and actin interaction. We would hypothesize that, under the normally low Ca^{2+} concentration in the cell, M-Va would be activated as a motor whenever in contact with the actin cytoskeleton. On the other hand, at local regions of higher Ca^{2+} concentration actomyosin Va would switch to a higher affinity, higher ATP turnover state, or "into an extremely low gear" as expressed by Provance and Mercer (1). This would be a dynamic state that would tend to decrease overall motility as well as maintain localization and could be reversed by lowering Ca^{2+} levels. Indeed, Wu *et al.* (32) and Tsakraklides *et al.* (33) have seen just that at the dendritic tips of mouse melanocytes where there is a large accumulation of melanosomes mostly not moving but some of which work their way back to microtubule-rich regions where they then move back toward the cell center by centripetal, microtubule-dependent movement. This dynamic process would confer plasticity to organelle traffic, linking melanosome distribution to signal transduction mechanisms. In fish chromatophores, increases in free Ca^{2+} have been seen to accompany pigment aggregation (34, 35), while a decrease in Ca^{2+} led to dispersion (35). Thus, Ca^{2+} could trigger the accumulation mechanism at the periphery via regulation of M-Va/actin binding and induction of a dynamic anchored state at the cost of ATP cycling.

Acknowledgments—We thank Domingus E. Pitta and Silvia Regina Andrade for expert technical assistance. We also thank Matt Tyska for critical comments on the manuscript.

REFERENCES

1. Provance, D. W., Jr., and Mercer, J. A. (1999) *Cell. Mol. Life Sci.* **56,** 233–242
2. Reck-Peterson, S. L., Provance, D. W., Jr., Mooseker, M. S., and Mercer, J. A. (2000) *Biochim. Biophys. Acta* **1496,** 36–51
3. Eggleston, A. (2000) *Nat. Cell Biol.* **2,** E125
4. Cheney, R. E. (1998) *Methods Enzymol.* **298,** 3–18
5. Wang, F., Chen, L., Arcucci, O., Harvey, E. V., Bowers, B., Xu, Y., Hammer, J. A., III, and Sellers, J. R. (2000) *J. Biol. Chem.* **275,** 4329–4335
6. Espindola, F. S., Espreafico, E. M., Coelho, M. V., Martins, A. R., Costa, F. R., Mooseker, M. S., and Larson, R. E. (1992) *J. Cell Biol.* **118,** 359–368
7. Trybus, K. M., Krementsova, E., and Freyzon, Y. (1999) *J. Biol. Chem.* **274,** 27448–27456
8. De La 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
9. Sakamoto, T., Amitani, I., Yokota, E., and Ando, T. (2000) *Biochem. Biophys. Res. Commun.* **272,** 586–590
10. Walker, M., Burgess, S. A., Sellers, J. R., Wang, F., Hammer, J. A., III, Trinick, J., and Knight, P. A. (2000) *Nature* **405,** 804–807
11. Homma, K., Saito, J., Ikebe, R., and Ikebe, M. (2000) *J. Biol. Chem.* **275,** 34766–34771
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. Mehta, A. D., Rock, R. S., Rief, M., Spudich, J. A., Mooseker, M. S., and Cheney, R. E. (1999) *Nature* **400,** 590–593
14. Rief, M., Rock, R. S., Mehta, A. D., Mooseker, M. S., Cheney, R. E., and Spudich, J. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 9482–9486
15. Reck-Peterson, S. L., Tyska, M. T., Novick, P. J., and Mooseker, M. S. (2001) *J. Cell Biol.* **153,** 1121–1126
16. Mermall, V., Post, P. L., and Mooseker, M. S. (1998) *Science* **279,** 527–532
17. Evans, L. L., Lee, A. J., Bridgman, P. C., and Mooseker, M. S. (1998) *J. Cell Sci.* **111,** 2055–2066
18. Bridgman, P. C. (1999) *J. Cell Biol.* **146,** 1045–1060
19. Rogers, S. L., and Gelfand, V. I. (1998) *Curr. Biol.* **8,** 161–164
20. 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
21. Cameron, L. C., Carvalho, R. N., Araujo, J. R. V., Santos, A. C., Tauhata, S. B. F., Larson, R. E., and Sorenson, M. M. (1998) *Arch. Biochem. Biophys.* **355,** 35–42
22. Spudich, J. A., and Watt. S. (1971) *J. Biol. Chem.* **246,** 4866–4871
23. Gopalakrishna, R., and Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun.* **104,** 830–836
24. Costa, M. C., Mani, F., Santoro Jr., W., Espreafico, E. M., and Larson, R. E. (1999) *J. Biol. Chem.* **274,** 15811–15819
25. Margossian, S. S., and Lowey, S. (1982) *Methods Enzymol.* **85,** part B, 55–71
26. Fabiato, A. (1988) *Methods Enzymol.* **157,** 378–417
27. Kouyama, T., and Mihashi, K. (1981) *Eur. J. Biochem.* **114,** 33–38
28. Houdusse, A., Silver, M., and Cohen, C. (1996) *Structure* (*Lond.*) **4,** 1475–1490
29. De La Cruz, E. M., Sweeney, H. L., and Ostap, E. M. (2000) *Biophys. J.* **79,** 1524–1529
30. Tuma, M. C., and Gelfand, V. I. (1999) *Pigment Cell Res.* **12,** 283–294
31. Brown, S. S. (1999) *Annu. Rev. Cell Dev. Biol.* **15,** 63–80
32. Wu, X., Bowers, B., Rao, K., Wei, Q., and Hammer, J. A., III (1998) *J. Cell Biol.* **143,** 1899–1918
33. Tsakraklides, V., Krogh, K., Wang, L., Bizario, J. C., Larson, R. E., Espreafico, E. M., and Wolenski, J. S. (1999) *J. Cell Sci.* **112,** 2853–2865
34. de Lauro Castrucci, A. M., Visconti, M. A., Hadley, M. E., Hruby, V. J., Oshima, N., and Fujii, R. (1988) *Prog. Clin. Biol. Res.* **256,** 547–557
35. McNiven, M. A., and Ward, J. B. (1988) *J. Cell Biol.* **106,** 111–125