# Calcium-Calmodulin and Regulation of Brush Border Myosin-I MgATPase and Mechanochemistry

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*Abstract.* We examined the $Ca^{2+}$-dependent regulation of brush border (BB) myosin-I by probing the possible roles of the calmodulin (CM) light chains. BB myosin-I MgATPase activity, sensitivity to chymotryptic digestion, and mechanochemical properties were assessed using 1–10 $\mu M$ $Ca^{2+}$ and in the presence of exogenously added CM since it has been proposed that this myosin is regulated by calcium-induced CM dissociation from the 119-kD heavy chain. Each of these BB myosin-I properties were dramatically altered by the same threshold of 2–3 $\mu M$ $Ca^{2+}$. Enzymatically active $NH_2$-terminal proteolytic fragments of BB myosin-I which lack the CM binding domains (the 78-kD peptide) differ from CM-containing peptides in that the former is completely insensitive to $Ca^{2+}$. Furthermore, the 78-kD peptide exhibits high levels of MgATPase activity which are comparable to that observed for BB myosin–I in the presence of $Ca^{2+}$. This suggests that $Ca^{2+}$ regulates BB myosin-I MgATPase by binding directly to the CM light chains, and that CM acts to repress endogenous MgATPase activity. $Ca^{2+}$-induced CM dissociation from BB myosin-I can be prevented by the addition of exogenous CM. Under these conditions $Ca^{2+}$ causes a reversible slowing of motility. In contrast, in the absence of exogenous CM, motility is stopped by $Ca^{2+}$. We demonstrate this reversible slowing is not due to the presence of inactive BB myosin-I molecules exerting a "braking" effect on motile filaments. However, we did observe $Ca^{2+}$-independent slowing of motility by acidic phospholipids, suggesting that factors other than $Ca^{2+}$ and CM content can affect the mechanochemical properties of BB myosin-I.

CHICKEN intestinal brush border (BB)[1] myosin-I is the best characterized of a growing number of unconventional myosins recently characterized which use multiple calmodulins (CMs) as light chains (Pollard et al., 1991; for reviews on the myosin superfamily see Cheney et al., 1993; Mooseker, 1993; Titus, 1993). This class I myosin consists of a single 119-kD heavy chain and 3–4 CM light chains bound to a "neck" domain positioned between its head and tail domains. The neck consists of multiple tandomly repeated ~24–amino acid repeats termed IQ motifs (Cheney and Mooseker, 1992). All myosins whose primary structure has been determined have at least one similarly positioned IQ motif (Cheney and Mooseker, 1992). Other unconventional CM-containing myosins include myosins-I from kidney (Coluccio, 1992), liver (Coluccio and Conaty, 1993), and adrenal medulla (Barylko et al., 1992), as well as the two headed chick brain myosin-V (Espreafico et al., 1992). Like BB myosin-I, the neck domain of brain myosin-V, which consists of six IQ motifs, has been shown to be the site for CM binding (Espreafico et al., 1992).

A number of studies have demonstrated that $Ca^{2+}$ has profound effects on BB myosin-I. These include the promotion of differential proteolytic cleavage by chymotrypsin (Coluccio and Bretscher, 1988, 1990), dissociation of one or more CMs (Collins et al., 1990; Swanljung-Collins and Collins, 1991), activation of actin-independent MgATPase (Coluccio and Bretscher, 1987; Conzelman and Mooseker, 1987; Mooseker and Coleman, 1989; Swanljung-Collins and Collins, 1991), and variable activation of actin-dependent MgATPase (Conzelman and Mooseker, 1987; Collins et al., 1990; Swanljung-Collins and Collins 1991). One reason for this variation may be CM light chain content. Swanljung-Collins and Collins (1991) have reported that preparations with three (of four total) but not two CM light chains retain actin-activated MgATPase in the presence of $Ca^{2+}$. Moreover, several studies have demonstrated a role for $Ca^{2+}$ in regulating the mechanochemical activity of BB myosin-I, although markedly different effects of $Ca^{2+}$ have been observed. Mooseker and Coleman (1989) reported that BB myosin-I-coated beads, when assessed using the *Nitella* assay, exhibited slow (~10 nm/s) motility in the presence but not absence of 10 $\mu M$ $Ca^{2+}$. It has subsequently been shown that these slow motility rates are a consequence of the *Nitella* assay (Wolenski et al., 1993). However, we have not been able to obtain movement in $Ca^{2+}$ using our more stable preparations of purified BB myosin-I. In contrast, microvillar membrane-associated BB myosin-I preparations exhib-

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1. *Abbreviations used in this paper:* BB, brush border; CM, calmodulin; PG, phosphatidylglycerol.

ited faster (average rate 33 nm/s) motility that occurred in the absence but not presence of $Ca^{2+}$ (Mooseker et al., 1989). Using the sliding filament assay of Kron and Spudich (1986), Collins et al., (1990) have demonstrated that BB myosin-I translocates actin ~37–40 nm/s at 25°C, and up to ~56–65 nm/s at 37°C. Higher concentrations of $Ca^{2+}$ (100 $\mu$M) were required to inhibit motility, although this inactivation could be reversed by addition of exogenous CM (Collins et al., 1990).

The rationale of the present study was to further investigate the molecular basis for calcium regulation of BB myosin-I. This was done through detailed analysis of the effects of $Ca^{2+}$ on the enzymatic and mechanochemical properties of BB myosin-I. Our results indicate that both CM content and CM light chain–heavy chain interaction can contribute to the regulation of this myosin. Based on the results presented here, possible mechanisms for regulating BB myosin-I mechanochemistry in vitro versus in vivo are discussed.

## Materials and Methods

### Purification and Chymotryptic Digestion of Brush Border Myosin-I

BB myosin-I was purified from preparations of chicken small intestinal BBs by the method of Coluccio and Bretscher (1987) using modifications described in Hayden et al., (1990). After purification, BB myosin-I was dialyzed against Storage Buffer consisting of 75 mM KCl, 2 mM MgCl$_2$, 0.1 mM EGTA, 0.5 mM DTT, and 10 mM imidazole-Cl, pH 7.4, and then stored at 4°C. By SDS gel densitometry (Hayden et al., 1990; see below), these preparations had a CM light chain:BB myosin-I heavy chain molar ratio of ~3.9:1 to 5.2:1. BB myosin-I was used within 1 wk of this final step. The 78 and 90-kD proteolytic fragments were prepared by limited chymotryptic (Sigma Chemical Co., St. Louis, MO) digestion in the presence (for the 78-kD peptide) or absence of 0.2 mM CaCl$_2$ as described by Coluccio and Bretscher (1987). Phosphatidylglycerol (PG) liposomes,

prepared as described by Hayden et al., (1990) were added to terminated digests to a final concentration of 1 mM and used as a vehicle for removal of intact BB myosin-I. Liposomes, with bound BB myosin-I, were pelleted by sedimentation at 100,000 $g$ for 30 min, and discarded. The supernatant fractions, containing the chymotryptic head fragments of BB myosin-I, were dialyzed into Storage Buffer before use in the ATPase assays. We verified that the chymotryptic cleavage sites which generated the CM-free 78-kD and CM-retaining 90-kD peptides were in the COOH-terminal "tail" domain by performing immunoblot analysis with mAbs reactive with epitopes in the head (mAb CX-1; Carboni et al., 1988), and tail (mAb CX-7; Garcia et al., 1989) domain of BB myosin-I heavy chain.

### ATPase Assays

The dependence of F-actin (0–40 $\mu$M) on BB myosin-I MgATPase was assessed in the absence and presence of $Ca^{2+}$. Low actin concentrations (0–1 $\mu$M) were stabilized by equimolar concentrations of phalloidin (Boehringer Mannheim Corp., Indianapolis, IN). Assay conditions were as follows: ~5 mM KCl, 5 mM MgCl$_2$, 0.5 mM DTT, 2 mM ATP, 20 mM imidazole-Cl, pH 7.2, 0.3 $\mu$M BB myosin-I and either 1 mM EGTA or 4 mM calcium/EGTA buffers containing ~10 $\mu$M free $Ca^{2+}$ (37°C). For comparisons to the activities reported in Mooseker et al., (1989), several MgATPase assays were also performed in the above buffer using 75 mM KCl. The effect of $Ca^{2+}$ on the MgATPase of BB myosin-I and its 78 and 90-kD subfragments was assayed under the same conditions as listed above but using calcium/EGTA mixtures (4 mM EGTA; 0–4 mM CaCl$_2$) to vary the free $Ca^{2+}$ between the subnanomolar and 10 $\mu$M range (Portzehl et al., 1964). These assays were done in either the absence or presence of 23.3 $\mu$M F-actin.

### Analysis of BB Myosin-I Light Chain Content After $Ca^{2+}$ Treatment in the Presence and Absence of Exogenous CM

The CM light chain content of BB myosin-I preparations exposed to elevated $Ca^{2+}$ in the absence and presence of exogenously added CM was measured under solution conditions similar to that used for the MgATPase and motility assays performed in this study (20 mM KCl, 10 mM Imidazole-Cl, 5 mM MgCl$_2$, 1 mM DTT with 4 mM calcium/EGTA buffers; free $Ca^{2+}$ ~10 $\mu$M). Aliquots (0.1 ml) containing 0.5 $\mu$M BB myosin-I were incubated at room temperature for 10 min in the absence or presence of 25 $\mu$M CM with 4.6 $\mu$M F-actin that had been prestabilized with equimolar concentrations of phalloidin. As controls, identical concentrations of BB myosin-I or
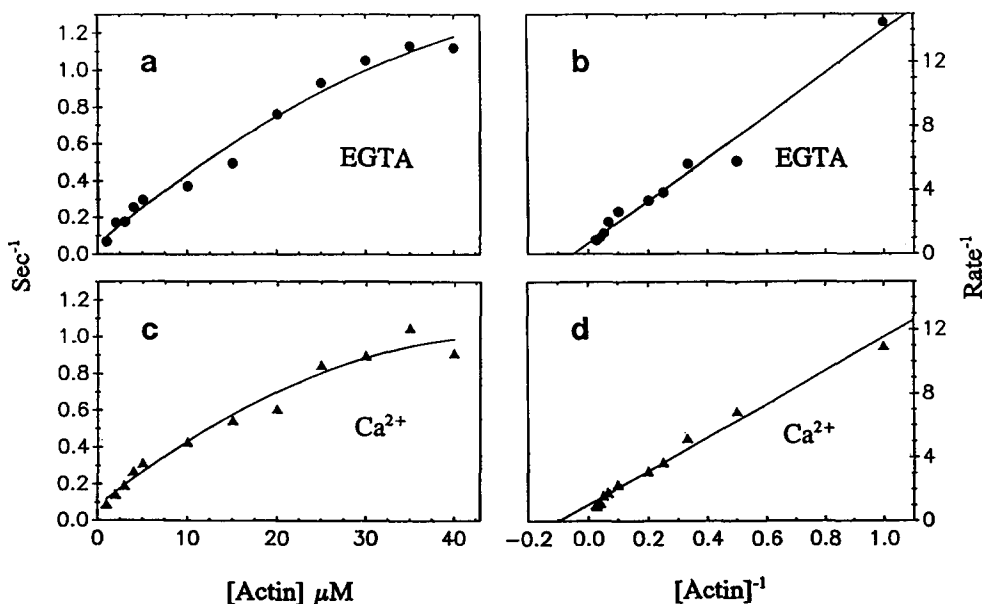


Figure 1. Characterization of the actin-dependent MgATPase activity of BB myosin-I (0.3 $\mu$M) in assay buffers containing either 1 mM EGTA ($a$ and $b$) or 10 $\mu$M $Ca^{2+}$ ($c$ and $d$). The values plotted were determined by subtracting the activities of actin alone, and the basal activity of BB myosin-I in the absence of actin (0.048 s$^{-1}$ in EGTA and 0.69 s$^{-1}$ in 10 $\mu$M $Ca^{2+}$). Results are from the same preparation of BB myosin-I. ($b$ and $d$) Double reciprocal plots of the data in ($a$ and $c$) showing the $V_{max}$ and $K_{ATPase}$ of BB myosin-I are reduced in the presence of $Ca^{2+}$ (see text for details). The slight sigmoidal nature of the data points in $a$ and $c$ at intermediate concentrations of actin was not reproducible in identical experiments using two additional BB myosin-I preparations.

F-actin alone, were incubated in the absence or presence of 25 μM CM. Each sample was then layered onto a 12% (wt/vol) sucrose cushion and centrifuged at 100,000 g for 1 h at 5°C in 5 × 41 mm ultracentrifuge tubes (Beckman Instruments Inc., Palo Alto, CA) using a Beckman SW-50 swinging bucket rotor. The sucrose cushion was made using the same assay buffer containing 1 mM EGTA and no added CaCl₂ so as to prevent any additional loss of CM from BB myosin-I. In most experiments, 15 μM BSA was included in the overlay mixture as a marker for nonspecific trapping of protein in the F-actin pellet. After centrifugation and removal of the supernatants, the tubes were gently washed with assay buffer containing 1 mM EGTA and any resulting pellet was resuspended and analyzed by SDS-PAGE. All samples from a single experiment were run on the same 12% acrylamide gel and stained with Coomassie blue (Fairbanks et al., 1973). The integrated optical density of the CM band relative to the BB myosin-I heavy chain band was determined using a Kodak Bio Image digital gel scanner (Eastman Kodak Co., Rochester, NY) equipped with Millipore Visage (version 4) software (Millipore Corp., Bedford, MA). The molar ratios were estimated using molecular weight values of 16.7-kD for CM and 119-kD for the BB myosin-I heavy chain using known concentrations of CM and phosphorylase a as dye-binding standards (Conzelman and Mooseker, 1987).

## In Vitro Motility Assays

Visualization of the movement of rhodamine-phalloidin-labeled actin filaments on the surface of BB myosin-I-coated coverslips was performed using modifications of the Kron and Spudich (1986) assay described in Collins et al. (1990) and Wolenski et al. (1993). To test the effect of BB myosin-I molecules inactivated by Ca²⁺ on the motility of active BB myosin-I, motility chambers were constructed containing varied ratios of Ca²⁺-inactivated motors and mechanochemically active motors. This was done by first applying 50-μl aliquots of BB myosin-I of the following concentrations: 0 μM (0% inactive); 1.4 μM (50% inactive); 2.1 μM (75% inactive); 2.52 μM (90% inactive), and 2.8 μM (100% inactive). This aliquot was allowed to adsorb to the coverslip for 5 min and then flushed several times for an additional 5-min period with 0.5 ml of motility buffer containing 10 μM free Ca²⁺ to inactivate the adsorbed BB myosin-I and to remove unbound motors. After a 1 ml wash with motility buffer containing 1 mM EGTA to remove residual Ca²⁺, a second 50-μl aliquot of BB myosin-I (the active fraction) was added using a concentration to yield a total of 2.8 μM for the sum of concentrations of inactive and active BB myosin-I solutions added to each chamber. While the exact percentage of BB myosin-I which binds to the coverglass in each step cannot be precisely determined, a greater percentage of protein is likely to remain bound during the first application. Thus, the percentage of inactivated motors present in each condition represent the minimum values. All assays were performed within an 8-h time period using the same BB myosin-I preparation. The mechanochemistry of BB myosin-I in these chambers was then assayed using low Ca²⁺ motility buffers.

Motility of BB myosin-I bound to PG was performed essentially as described above for assays using nitrocellulose-coated coverslips except for minor modifications. The PG was mixed with 1% (wt/wt) FITC-phosphatidylethanolamine and made into fluorescent phospholipid vesicles according to the methods described in Hayden et al. (1990). Vesicles at 5-10 mM were added to poly-L-lysine-coated coverslips and allowed to adhere for no less than 10 min. The coverslips were then rinsed with motility buffer and examined using fluorescence microscopy to assess the extent of phospholipid adhesion. Using this protocol, the coverslips were typically observed to be coated with a dense and homogeneous layer of phospholipid. BB myosin-I was then added to the motility chamber, allowed to adhere to the anionic phospholipid substrate for 5 min, and the unbound protein washed out with 2-3 chamber volumes of motility buffer. The addition of labeled actin filaments and velocity measurements were as described above. Control experiments in the absence of phospholipids were performed using BB myosin-I bound to nitrocellulose-coated coverslips since motility could not be consistently reproduced using poly-L-lysine as a substrate.

## Other Methods

Actin was purified from chicken skeletal muscle by the method of Spudich and Watt, (1971). For most studies, G-actin was purified by gel filtration over a Sephadex G-150 column (MacLean-Fletcher and Pollard, 1980). CM was purified from bovine brain according to the procedure of Burgess et al., (1980). Some CM was a generous gift of Dr. A. Harris (Department of Pathology, Yale University, New Haven, CT) or was purchased from Calbiochem Corp., (La Jolla, CA). Protein concentrations were determined using the BCA assay following the vendor's protocol (Pierce, Rockford, IL).

Gel electrophoresis and immunoblots were performed as described in Carboni et al., (1988).

# Results

## Calcium Reduces the $V_{max}$ and $K_{ATPase}$ of the Actin-dependent MgATPase and Activates the Actin-independent MgATPase of BB Myosin-I

The MgATPase of BB myosin-I depends on the concentrations of both Ca²⁺ and actin (Figs. 1 and 2). At low [Ca²⁺], the basal MgATPase activity of BB myosin-I (0.048 s⁻¹) was activated 25-38-fold by F-actin ($V_{max}$ of 1.26-1.58 s⁻¹;
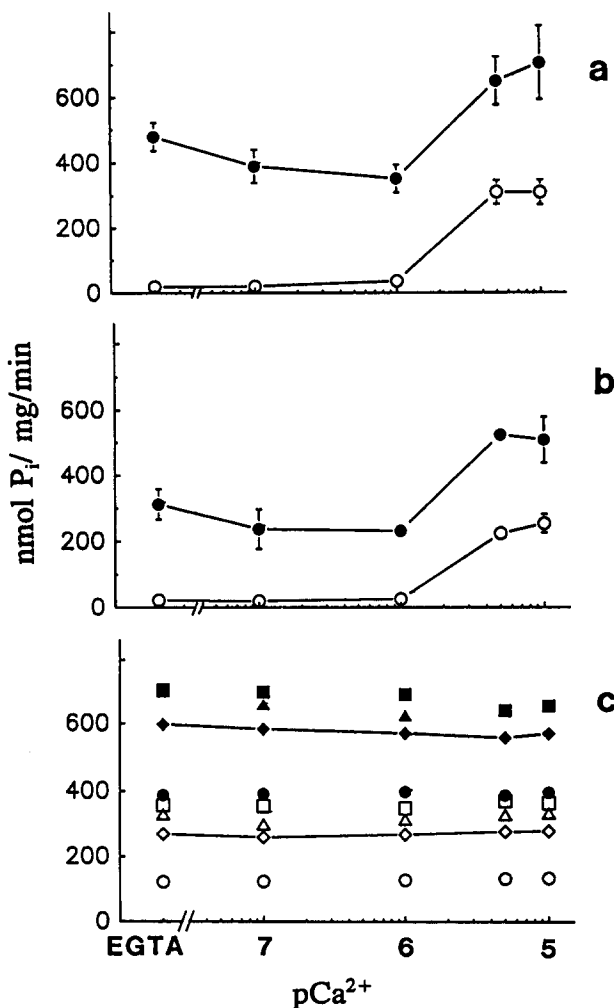


*Figure 2.* Effects of Ca²⁺ on the MgATPase activity of BB myosin-I and its chymotryptic peptides. (*a*) Three different preparations of intact BB myosin-I were assayed for enzymatic activity in the absence (O) or presence (●) of F-actin. The average values for each condition were then plotted. (*b*) The MgATPase activities for the CM-containing 90-kD peptide in the absence (o) and presence of actin (●). Average rates for two experiments are shown. (*c*) Effects of Ca²⁺ on the MgATPase activity of the CM-free 78-kD peptide. Data from three separate experiments are plotted to demonstrate the 78-kD peptide exhibits high levels of activity and is insensitive to Ca²⁺. Open symbols: activity in the absence of actin. Closed symbols: activity in the presence of actin. The connected plots represent the average MgATPase activities of the 78-kD peptide in the absence (◇) and presence (◆) of actin.
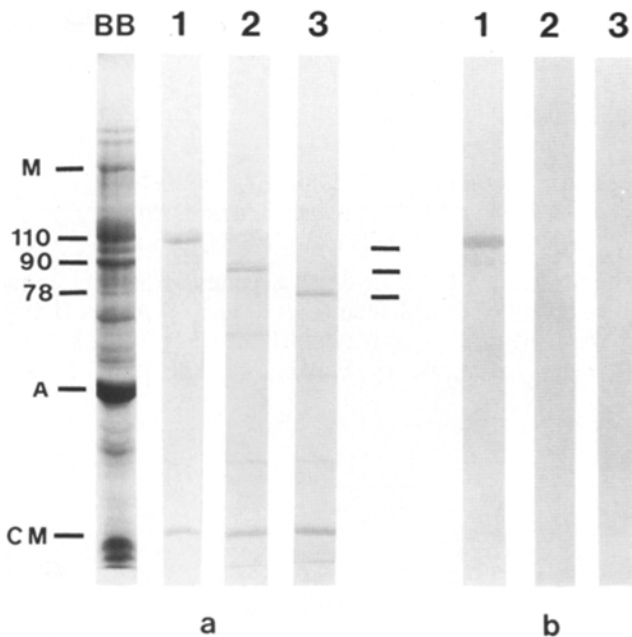
$K_{ATPase}$ of 17–48 $\mu$M) for three different protein preparations (Fig. 1, $a$ and $b$). These values are comparable with those measured by Collins et al., (1990) under similar assay conditions using BB myosin-I preparations isolated by different methods. At 10 $\mu$M $Ca^{2+}$ (Figs. 1 $c$ and $d$), the basal activity of BB myosin-I increased (to 0.69 $s^{-1}$) and actin activated the rate to a $V_{max}$ of 0.94 $s^{-1}$ with a $K_{ATPase}$ of 10 $\mu$M. The threshold concentration of $Ca^{2+}$ required for actin-independent MgATPase activation of these preparations was between ~1 and 5 $\mu$M (Fig. 2 $a$). These MgATPase activities were substantially higher than those in Mooseker et al. (1989) due to the lower salt concentrations in the current study (Table I).

## Calcium Effects on BB Myosin-I MgATPase Are Mediated by Its CM Light Chains

To assess whether the $Ca^{2+}$ effects on BB myosin-I MgATPase are mediated by the CM light chains, or by $Ca^{2+}$ binding sites on the heavy chain, we compared the $Ca^{2+}$-sensitive activities of BB myosin-I to that of the 90-kD CM-containing and 78-kD CM-free chymotryptic fragments derived from the $NH_2$ terminus of native BB myosin-I heavy chain (Fig. 3, *upper panel*). Chymotryptic cleavage of BB myosin-I in the presence of < ~2 $\mu$M $Ca^{2+}$ resulted primarily in the formation of the 90-kD peptide, whereas higher concentrations favored the formation of the CM-free 78-kD peptide with a threshold of 2–3 $\mu$M $Ca^{2+}$ (Fig. 3, *lower panel*). Preparations of the 90-kD peptide exhibited MgATPase activity that was qualitatively similar to BB myosin-I (Fig. 2 $b$). However, the activity of the 78-kD preparation was unique (Fig. 2 $c$). This fragment, lacking the COOH terminal tail and the CM-neck domain, exhibited relatively high levels of $Ca^{2+}$-insensitive ATPase activity in the absence of actin (Fig. 2 $c$). The 78-kD fragment MgATPase was markedly activated by actin, but the activity was still insensitive to changes in $[Ca^{2+}]$ (Fig. 2 $c$). At all concentrations of $Ca^{2+}$ examined, the 78-kD preparation exhibited high MgATPase activity that was comparable to intact BB myosin-I in the presence of 5–10 $\mu$M $Ca^{2+}$.
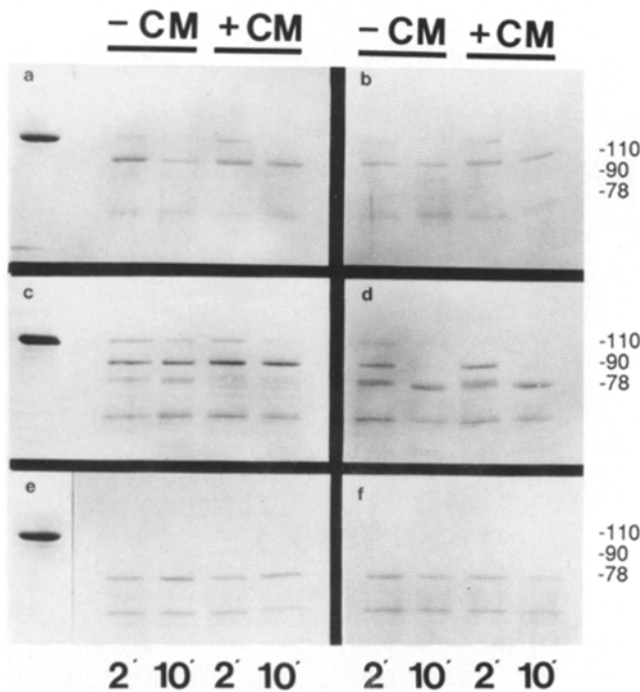
*Figure 3.* (*Upper panel*) SDS-PAGE and immunoblot analysis of chymotryptic fragments of BB myosin-I. Digestion of native BB myosin-I was performed as described in Coluccio and Bretscher (1988). (*a*) Coomassie blue–stained gel of the samples used for immunoblot analysis. (*b*) Immunoblot stained using a mAb (CX-7) specific for the COOH-terminal ~20-kD tail of BB myosin-I heavy chain. *BB*, chicken brush border cytoskeletal proteins; lanes *1*, intact BB myosin-I; lanes *2*, 90-kD peptides derived by digestion of BB myosin-I in the presence of EGTA; and lanes *3*, 78-kD peptide mixture obtained by digestion of BB myosin-I in the presence of micromolar $Ca^{2+}$. *M*, conventional BB myosin heavy chain; *110*, BB myosin-I heavy chain; *A*, actin; *CM*, calmodulin. Note that the 90- and 78-kD peptides are not recognized by the BB myosin-I tail antibody. (*Lower panel*) Calcium dependence of chymotryptic digestion in the absence and presence of exogenous CM. Shown (*a–f*) are protein-stained acrylamide gels of intact BB myosin-I (*left* lanes in *a*, *c*, and *e*) and chymotryptic fragments generated by digestion of BB myosin-I for 2 and 10 min. Digestion of BB myosin-I was performed in the absence (−CM) or presence (+CM) of exogenously added CM (25 $\mu$M) in either 1 mM EGTA (*a*), 0.4 (*b*), 1 (*c*), 2 (*d*), 5 (*e*) or 10 (*f*) $\mu$M $Ca^{2+}$.

## The Ca²⁺-induced Dissociation of CM from BB Myosin-I Can Be Prevented by the Presence of Free CM

Since Ca²⁺ promotes CM light chain dissociation, BB myosin-I may vary with respect to its CM composition in vivo. If so, then Ca²⁺ could have differential regulatory effects on BB myosin-I depending on its CM light chain content. To assess this possibility, we first determined whether the reduction in BB myosin-I light chain content could be prevented by addition of exogenous CM. Consistent with previous studies (Collins et al., 1990; Swanljung-Collins and Collins, 1991) treatment of BB myosin-I with 10 μM Ca²⁺ in the absence of exogenous CM resulted in a loss of ~1–2 CM light chains as estimated by comparing the heavy chain:CM ratio of the complex before and after sedimentation through a sucrose cushion. In contrast, analysis of the pellet fractions containing BB myosin-I aliquots treated with 10 μM Ca²⁺ in the presence of 25 μM exogenous CM showed these samples to be CM replete (Table II). In six separate experiments the number of CM molecules bound to the heavy chain after recovery of the pellet fractions were 3.4 ± 0.6 in the absence of exogenous CM, and 4.4 ± 0.5 in the presence of exogenous CM (mean ± S.D.). Control samples revealed that under these conditions, no pelleting of CM or BB myosin-I occurred unless both F-actin and BB myosin-I were present in the mixture (results not shown).

## Exogenously Added CM Does Not Alter Ca²⁺ Effects on BB Myosin-I Protease Sensitivity or MgATPase Activity, but Does Differentially Affect the Mechanochemistry

We then assayed whether the light chain content could differentially effect BB myosin-I activity in the presence of Ca²⁺. A comparison of the chymotryptic digestion profiles of BB myosin-I in the absence and presence of threshold
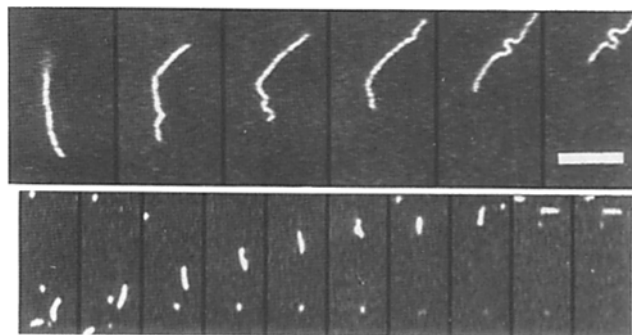


Figure 4. Fluorescence microscopy images of rhodamine-phalloidin-labeled actin filaments being translocated by native BB myosin-I attached to a nitrocellulose-coated coverslip. Two examples of movement are shown (upper and lower panels) in 1 mM EGTA. Successive frames, from left to right, represent 30-s intervals. Bar, 5 μm.

[Ca²⁺] (i.e., ~2 μM) required for generation of the 78-kD fragment revealed no significant effects of exogenously added 25 μM CM (Fig. 3, lower panel). Two separate assays of the actin- and Ca²⁺-dependent MgATPase of BB myosin-I were also performed in the absence and presence of 25 μM CM. BB myosin-I MgATPase activities were unaffected by the addition of exogenous CM under these conditions (data not shown).

Using the sliding actin filament motility assay (see Fig. 4), we measured filament translocation rates for BB myosin-I as a function of free [Ca²⁺] at 24 ± 1°C in both the absence (Fig. 5 a) and presence (Fig. 5, b and c) of exogenous 25 μM CM. Two different experimental strategies were used. We either used single motility chambers in which rate measurements were made at different [Ca²⁺] (for example, see Fig. 5, a and b), or a new motility chamber was used for each Ca²⁺ condition (Fig. 5 c) to insure that any decrease in filament velocities was not due to gradual deterioration of BB myosin-I over time. In both the absence and presence of exogenous CM, fastest rates of actin filament translocation (average rate >50 nm/s) were observed in buffers containing <1 μM Ca²⁺.

Elevated calcium (>1–2 μM) had a pronounced inhibitory effect on motility, the nature of which differed depending on whether exogenous CM was present. In the absence of exogenous CM, Ca²⁺ caused a complete arrest of motility which was not reversible by Ca²⁺ removal (Fig. 5 a). Consistent with the results of Collins et al., (1990) some restoration of motility to such Ca²⁺-inactivated preparations occurred upon addition of exogenous CM. For example, in the experiment depicted in Fig. 5 a, addition of 25 μM CM partially restored motility in the presence of ~5 μM Ca²⁺, but to velocities significantly slower than those observed in the absence of Ca²⁺ (11–23 nm/s; point No. 5 on the graph). Similarly, we assayed the Ca²⁺-dependent motility of BB myosin-I under conditions where the motility chambers were continuously exposed to buffers containing 25 μM exogenous CM (Fig. 5, b and c). Under these conditions, instead of arresting motility, elevated Ca²⁺ caused a slowing of motility that was reversed to almost maximal rates upon Ca²⁺ removal (Fig. 5 b, point No. 5 on the graph).
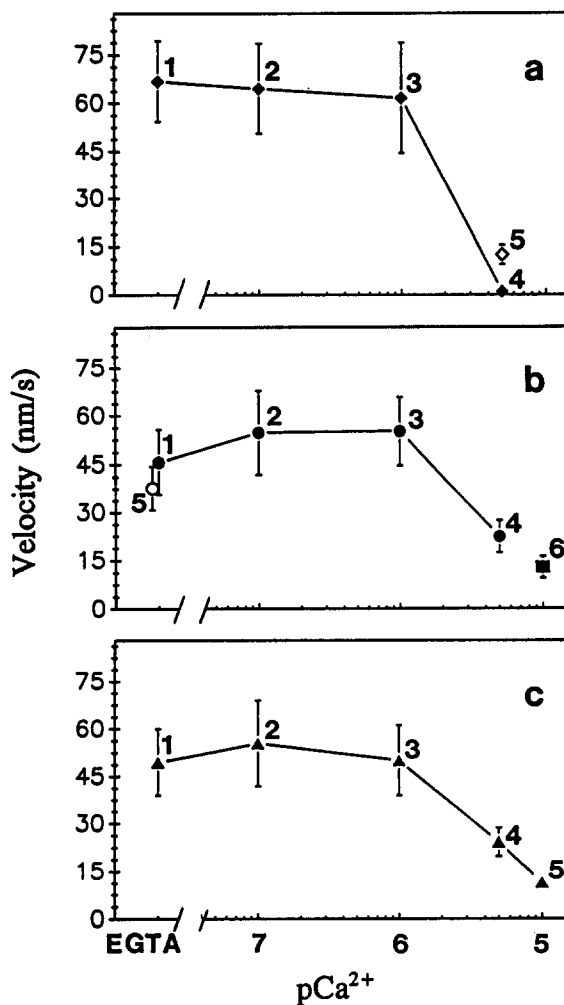
The slower motility rates observed in the presence of >1

Table II. Cosedimentation of BB Myosin-I with F-actin in the Absence and Presence of Exogenous Calmodulin in 10 μM Buffered Calcium

| BB myosin-I preparation | Experiment | Conditions ±CM | Molar ratio CM:BB myosin-I hc in pellet fraction |
|---|---|---|---|
| 1 | 1 | −CM | 2.9:1 |
|   |   | +CM | 3.9:1 |
|   | 2 | −CM | 2.7:1 |
|   |   | +CM | 3.7:1 |
|   | 3 | −CM | 3.9:1 |
|   |   | +CM | 4.7:1 |
|   | 4 | −CM | 4.2:1 |
|   |   | +CM | 5.0:1 |
| 2 | 5 | −CM | 3.1:1 |
|   |   | +CM | 4.7:1 |
|   | 6 | −CM | 3.5:1 |
|   |   | +CM | 4.3:1 |

BB myosin-I was incubated ±25 μM exogenous CM for 10 min in buffers containing ~10 μM free Ca²⁺ and centrifuged in the presence of F-actin. Estimated CM:heavy chain ratios are listed (see Materials and Methods). The molar ratios of CM:BB myosin-I hc for preparations 1 and 2 before centrifugation were 3.9:1 and 5.2:1, respectively.

Figure 5. The effects of $Ca^{2+}$ and CM on BB myosin-I mechano-chemistry. (a–c) Plots of average filament translocation velocities (n = 25–50) as a function of [$Ca^{2+}$]. For each experiment, the numbers next to the points on the graphs indicate the order of addition of $Ca^{2+}$-buffers to the motility chamber. All experiments were performed at 24 ± 1°C. (a) A single motility chamber was exchanged with increasing concentrations of $Ca^{2+}$ (♦). Motility was inhibited by ~5 μM $Ca^{2+}$ (4), but could be partially restored upon the addition of exogenous 25 μM CM (5). (b) In the maintained presence of exogenous 25 μM CM, BB myosin-I mechanochemistry is slowed (4) but not completely inhibited by ~5 μM $Ca^{2+}$. Addition of 1 mM EGTA to the chamber after exposure to $Ca^{2+}$ increases the velocity of translocation (5). Subsequent readdition of $Ca^{2+}$ results in a decrease in motor velocity (6). (c) BB myosin-I mechanochemistry in the maintained presence of exogenous 25 μM CM assayed using a separate motility chamber for each $Ca^{2+}$ concentration.

μM $Ca^{2+}$ and exogenous CM could result from the presence of inactive BB myosin-I motors which exert a braking effect on filaments being translocated by active motors. To address this issue we prepared a series of motility chambers containing mixtures of active and $Ca^{2+}$-inactivated BB myosin-I molecules (see Materials and Methods). Subsequent to the addition of ATP, the velocity of filament translocation was then examined using chambers containing either 50, 75, 90, or 100% of the total BB myosin-I inactivated by washing

with $Ca^{2+}$. No difference was observed in the velocity of filaments being translocated by mixed populations of inactive and active motors compared to control experiments containing only active motors. Mixtures of both $Ca^{2+}$-inactivated- and active BB myosin-I molecules translocated actin filaments at velocities (39 ± 7 nm/s; mean ± SD) and were comparable to those observed in motility chambers in which none of the adsorbed motors were inactivated by exposure to $Ca^{2+}$ (41 ± 6 nm/s; mean ± SD). By contrast, actin filaments were nonmotile in motility chambers containing only $Ca^{2+}$-inactivated BB myosin-I. This strongly suggests that the slowed velocities observed in the presence of $Ca^{2+}$ and exogenous CM (see Fig. 5) were not due to a heterogeneous population of active and inactive motors.

## Acidic Phospholipids Slow the In Vitro Motility of BB Myosin-I in the Absence of $Ca^{2+}$

We also performed studies to examine the effects of phospholipid binding on the in vitro motility of BB myosin-I. BB myosin-I binds to acidic phospholipids via its C-terminal tail domain (Hayden et al., 1990). We reasoned that a phospholipid-coated surface might provide an optimal substrate for motility studies by properly orienting molecules in the "heads up" position. The motility of phospholipid-bound BB myosin-I was examined using coverslips coated with PG liposomes. Results from three different protein preparations indicate that phospholipid-associated BB myosin-I assayed in the presence of 1 mM EGTA exhibits slower velocities compared to BB myosin-I bound to nitrocellulose (Table III).

## Discussion

### Calcium and Regulation of the MgATPase of BB Myosin-I and Other Myosins with Multiple CM Light Chains

The results presented here provide a number of novel insights into the effects of $Ca^{2+}$ on BB myosin-I. The diverse effects of $Ca^{2+}$ on protease sensitivity, CM light chain dissociation, MgATPase and in vitro motility all occur at the same [$Ca^{2+}$], suggesting that all these effects are mediated by CM-$Ca^{2+}$ interactions rather than direct interactions of this ion with the heavy chain. The studies on the MgATPase of BB myosin-I and its 78 and 90-kD head fragments strongly support this conclusion (Fig. 2).

At low [$Ca^{2+}$], BB myosin-I exhibits MgATPase activities that are typical of most myosins characterized to date; low

Table III. BB Myosin-I Bound to Phosphatidylglycerol Translocates Actin Filaments at Slowed Velocities

| BB myosin-I preparation | Rate | |
| --- | --- | --- |
| | BB myosin-I bound to nitrocellulose | BB myosin-I bound to phosphatidylglycerol |
| | nm/s | |
| 1 | 65.3 ± 10.4 | 27.9 ± 5.5 |
| 2 | 60.2 ± 10.6 | 10.8 ± 4.7 |
| 3 | 63.9 ± 7.5 | 15.0 ± 4.9 |

Velocities (±S.D.) were calculated from measurements made on not ≥25 filaments.

basal activity that is robustly activated by F-actin (for example see Figs. 1 and 2). In contrast, the actin-independent activation of BB myosin-I by 2–3 $\mu$M $Ca^{2+}$ is unusual, and is a property that has been observed using different preparations of BB myosin-I (Conzelman and Mooseker, 1987; Coluccio and Bretscher, 1987; Swanljung-Collins and Collins, 1991; for an exception see Collins et al., 1990). A key finding in the present study is that the CM-free 78-kD head fragment of BB myosin-I exhibits high basal levels of MgATPase throughout the range of [$Ca^{2+}$] tested that are comparable to the intact molecule in the presence of $Ca^{2+}$. This suggests that one or more of the CM light chains serve as suppressors of the head domain's intrinsic ATPase activity. Upon $Ca^{2+}$ binding to the CM light chains, this suppression is released. A similar suppressor model has been proposed for light chain regulation of scallop muscle myosin MgATPase (Wells and Bagshaw, 1985). The activation of BB myosin-I by $Ca^{2+}$ does not require CM release, since exogenously added CM, which prevents CM light chain loss (Table II), has no effect. On the other hand, BB myosin-I partially depleted in CM (Conzelman and Mooseker, 1987; Swanljung-Collins and Collins, 1991) still exhibits $Ca^{2+}$ activation, indicating that a full complement of light chains is not required for this effect.

In contrast to $Ca^{2+}$ effects on BB myosin-I MgATPase in the absence of actin, CM light chain content does profoundly affect the activation of this myosin by actin. Original preparations were quite low in CM content (0.1–1.0 CM per heavy chain) and were not activated by actin in either the absence or presence of $Ca^{2+}$ (Howe and Mooseker, 1983; Collins and Borysenko, 1984). The preparations of Conzelman and Mooseker (1987), which had CM:hc ratios of ~2:1, exhibited actin-activated MgATPase in the absence but not presence of $Ca^{2+}$; although some actin activation in the presence of $Ca^{2+}$ could be attained by addition of exogenous CM. Consistent with these earlier studies, a quantitation of MgATPase as a function of CM light chain content performed by Swanljung-Collins and Collins (1991) indicates that actin activation in the presence of $Ca^{2+}$ is lost for BB myosin-I preparations containing less than three CM light chains. In the present study we have analyzed in detail the effects of actin and $Ca^{2+}$ on the MgATPase of BB myosin-I preparations which as isolated, contain at least four CM light chains per heavy chain. Although the MgATPase of these preparations is activated by actin in the presence of $Ca^{2+}$, both the $V_{max}$ and $K_{ATPase}$ are reduced. $Ca^{2+}$ apparently increases the affinity of BB myosin-I for actin, while at the same time reducing the rate at which it can hydrolyze ATP. Based on these steady state results, one envisions an ATPase cycle in the presence of $Ca^{2+}$ and actin in which hydrolysis can proceed with similar rates along two pathways, one involving actomyosin intermediates and the other by BB myosin-I alone. Obviously, kinetic analysis of the BB myosin-I ATPase cycle is a critical next step.

Recently, the effects of $Ca^{2+}$ on the MgATPase of two other unconventional myosins with multiple CM light chains have been described. The best characterized of these is a myosin-I purified from bovine adrenal medulla (Barylko et al., 1992). Like BB myosin-I, this myosin exhibits actin-activated MgATPase with maximal total activity in the presence of $Ca^{2+}$ and actin. However, the basal MgATPase of this myosin-I is only slightly activated by $Ca^{2+}$ and both the $V_{max}$ and $K_{ATPase}$ of actin-dependent MgATPase are in-

creased by this ion. The second example, is p190-calmodulin (or chick myosin-V; Espindola et al., 1992; Espreafico et al., 1992). This myosin is analogous to scallop muscle myosin in that it required both $Ca^{2+}$ and actin for maximal activation of its MgATPase. Thus, all three of these CM-containing myosins exhibit $Ca^{2+}$-regulated MgATPase activities, but vary considerably in the nature of that regulation.

## Calcium and the Inhibition of BB Myosin-I Motility In Vitro: Possible Mechanisms

BB myosin-I exhibits optimal motility in vitro at [$Ca^{2+}$] below 1–2 $\mu$M, a result consistent with its enzymatic properties. The inhibition of motility by $Ca^{2+}$ is also consistent with the $Ca^{2+}$-dependent reduction in the $V_{max}$. The decrease in $K_{ATPase}$ could result in reduced velocity if the higher affinity of BB myosin-I for actin resulted in a longer dwell time on the filament at a rate limiting step in the mechanochemical cycle. The apparent discrepancy in comparing the enzymatic and motile properties of BB myosin-I arises from the effects of exogenously added CM on these two assays. Two critical questions raised by the findings presented here will be addressed. First, why does $Ca^{2+}$ irreversibly inactivate BB myosin-I motility in the absence of exogenous CM under solution conditions where exogenous CM has no effect on MgATPase? Second, what is the molecular basis for and significance of the reversible slowing of BB myosin-I in the presence of $Ca^{2+}$ and exogenous CM?

An obvious answer to the first question is that $Ca^{2+}$-dependent loss of CM light chains occurs more readily and/or to a greater extent from BB myosin-I molecules adsorbed to nitrocellulose than from molecules in solution. Reduction in CM light chain content to less than two CMs/heavy chain would result in loss of actin-activated MgATPase-and presumably motility. For example, surface-bound BB myosin-I may have reduced affinity for CM, or the nitrocellulose substrate might promote CM dissociation through competitive binding interactions with the highly basic neck domain. The protection of BB myosin-I motility from $Ca^{2+}$ inactivation by exogenously added CM provides firm support for this notion.

There are several plausible explanations for the $Ca^{2+}$-dependent slowing of motility in the presence of exogenous CM. First, as noted above, the reduced velocity could be a direct effect of $Ca^{2+}$ on the mechanochemical properties of CM-replete BB myosin-I, as reflected in changes in its enzymatic properties. On the other hand, reduced velocity could be due to the presence of a mixed population of active and inactive BB myosin-I molecules in the motility chamber, the latter imparting load on actin filaments moved by the former (Warshaw et al., 1990). This possibility seems unlikely given the results presented here using motility chambers containing mixtures of active and $Ca^{2+}$-inactivated BB myosin-I. Similarly, recent studies have shown that certain actin binding proteins (Janson et al., 1992; Shirinsky, 1992) can, at even quite low concentrations, dramatically reduce sliding filament velocities by imparting load. If such contaminating proteins were present in our BB myosin-I preparations, they would have to bind actin preferentially in the presence but not absence of $Ca^{2+}$ (e.g., like calpactin-I; Gerke and Weber, 1984). The presence of such $Ca^{2+}$-dependent actin binding proteins in our preparations seems un-

likely since BB isolation and all subsequent purification steps are performed under low $Ca^{2+}$ conditions.

A final explanation which merits discussion is that the $Ca^{2+}$-dependent inhibition of motility results from a change in how BB myosin-I is adsorbed to the coverslip surface after treatment with $Ca^{2+}$. This possibility is derived from emerging ideas regarding probable structural features of the neck domain of myosins including BB myosin-I and brain myosin-V. As depicted elsewhere in a speculative model (Mooseker, 1993) the neck of BB myosin-I may be a highly extended ~10-nm rod with CMs clamped in series along its length in a fashion analogous to that recently revealed by structural studies of the complex formed between CM and a binding peptide obtained from a target enzyme (Ikura et al., 1992; Meador et al., 1992). This model suggests that each CM might play discrete roles in controlling the flexural rigidity of the neck domain. Such control could be imparted through a change in CM "clamp pressure" along the neck or more dramatically by dissociation, exposing a now unsupported region of the neck domain. On the other hand, if this model is reflective of BB myosin-I structure, then one can envision that loss of a CM would expose a segment of the highly charged neck domain which might then stick down to the coverslip surface. As a result, the head domain would be moved closer to the surface with greatest proximity resulting from loss of the CM at the head–neck junction. Assessment of the motile properties of the 78- and 90-kD head fragments would directly address this possibility. Unfortunately, repeated attempts to obtain motility with these head fragments have not been successful. Studies on the motility of skeletal muscle HMM and S-1 using the sliding filament assay indicate that the proximity of the head domain to the coverslip surface may dramatically affect myosin velocity in this assay. For example, HMM exhibits velocities which are ~4–8-fold greater than the tail-less S-1 (Toyoshima et al., 1987). Thus, the changes in velocity observed for BB myosin-I in the presence of $Ca^{2+}$ in the absence or presence of exogenous CM may be a consequence of differences in head distance from the nitrocellulose surface rather than a direct effect of $Ca^{2+}$ on the molecule. The studies reported here on the inhibitory effect of phospholipid binding on motility provide evidence that substrate association state can affect motor velocity independently of $Ca^{2+}$.

Given the reservations raised above about currently available motility assays, as well as the documented differences in CM content among preparations of BB myosin-I characterized to date, the variabilities in the motile behavior of BB myosin-I, as summarized in the Introduction, are not surprising. Future studies examining the motility of native membranes containing BB myosin-I along actin bundles may be required to definitively address the effects of $Ca^{2+}$ on this myosin (e.g., as performed by Adams and Pollard [1986] for *Acanthamoeba* membrane vesicles using the *Nitella* assay). Unfortunately, there are no known methods for producing inside-out vesicles from BB membranes—a requisite to analyze microvillar membrane-associated BB myosin-I. Perhaps the recently described Golgi vesicle fractions containing BB myosin-I (Fath and Burgess, 1993) might be ideal for such studies.

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