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# Motility of myosin V regulated by the dissociation of single calmodulin

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**Myosin V is a calmodulin-binding motor protein. The dissociation of single calmodulin molecules from individual myosin V molecules at 1 μM $Ca^{2+}$ correlates with a reduction in sliding velocity in an *in vitro* motility assay. The dissociation of two calmodulin molecules at 5 μM $Ca^{2+}$ correlates with a detachment of actin filaments from myosin V. To mimic the regulation of myosin V motility by $Ca^{2+}$ in a cell, caged $Ca^{2+}$ coupled with a UV flash system was used to produce $Ca^{2+}$ transients. During the $Ca^{2+}$ transient, myosin V goes through the functional cycle of reduced sliding velocity, actin detachment and reattachment followed by the recovery of the sliding velocity. These results indicate that myosin V motility is regulated by $Ca^{2+}$ through a reduction in actin-binding affinity resulting from the dissociation of single calmodulin molecules.**

Calcium regulates various enzyme reactions of cellular processes through direct interaction to modify the function of a protein or through indirect interaction with a $Ca^{2+}$-sensing protein. Among the $Ca^{2+}$-sensing proteins expressed in eukaryotic cells, calmodulin (CaM) is a member of the EF-hand family, and has been known to participate in many signaling pathways that affect crucial processes such as cell growth, proliferation and cell movement[1,2].

Myosin V is an unconventional double-headed myosin that transports synaptic and endoplasmic reticulum vesicles in neurons[3–5], pigment granules in melanocytes[6–8], and vacuoles in yeast[9,10]. Myosin V is a processive motor capable of taking 36-nm steps along actin filaments[11–13]. Structurally, myosin V is a homodimer. Each monomer has one N-terminal head domain, one extended neck domain that contains six IQ motifs, and a tail domain containing a coiled coil region attached to a C-terminal globular region[14–16]. The IQ motifs form binding sites for CaM and CaM-related light chains[14,16,17]. Recent studies have investigated whether CaM bound to the IQ motifs can regulate the function of myosin V in response to $Ca^{2+}$ (refs. 16,18–21). Two of those studies have reported that myosin V stops moving but remains bound to actin filaments at high $Ca^{2+}$ concentrations (>1 μM) in *in vitro* motility assays using nitrocellulose coated glass[19,20]. In this study, using an *in vitro* motility assay on uncoated glass slides, we observed the detachment of actin filaments from myosin V at high $Ca^{2+}$ concentrations. This finding suggests that high $Ca^{2+}$ concentrations are a novel way of inactivating the motility of myosin V. Furthermore, upon transient release of $Ca^{2+}$, myosin V reversibly detaches from and slides along actin filaments in the presence of free CaM. To provide insights into the underlying mechanism of this observation, we used single-molecule imaging to monitor the dissociation of individual CaM molecules from myosin V at various $Ca^{2+}$ concentrations. Based on these observations, we propose a model explaining the regulation of the myosin V motor function resulting from the dissociation of single CaM molecules. This model shows how a motor protein detaches and then reattaches to their moving track. This may also explain how a multimotor-binding vesicle could be switched between different tracks for targeting in a cell.

## RESULTS

### Effect of $Ca^{2+}$ on myosin V motility

To estimate the effect of $Ca^{2+}$ on myosin V motility, we first developed a suitable glass surface for observing reversible motility in the *in vitro* assay (**Supplementary Methods** online). Using nitrocellulose-coated glass, movement was restored for only 56 ± 16% (mean values ± s.d., $n = 15$) of the actin filaments after removing $Ca^{2+}$ in the solution. Using an uncoated glass surface, 96 ± 3% ($n = 15$) of the actin filaments showed movement. Thus, the remainder of our experiments were done using uncoated glass slides. The irreversible inactivation of myosin V on nitrocellulose-coated glass was most likely due to irreversible binding of myosin V molecules to the nitrocellulose at the hydrophobic site of IQ domains (containing ~40% hydrophobic residues)[15], which is exposed after the dissociation of CaM.

The *in vitro* motility assays for myosin V were done in the presence of various $Ca^{2+}$ concentrations. At pCa 6.0, the number of sliding actin filaments was less than that in the absence of $Ca^{2+}$ (pCa > 7.0). At pCa 5.6, all actin filaments detached from myosin (**Fig. 1a**). To analyze the effect of $Ca^{2+}$ in detail, we quantified the number and velocity of actin filaments (**Fig. 1b**,**c**). In the absence of $Ca^{2+}$ or at pCa 6.4, the density of sliding actin filaments was ~0.17 filaments $\mu m^{-2}$, and most of them (>95%) moved smoothly. However, at pCa 6.0 there was a large reduction in both the density of sliding filaments and velocity of sliding. At pCa 5.8, the velocity of a few actin filaments could still be observed. It was similar to that recorded at pCa 6.0 (~230 nm $s^{-1}$). Finally, at

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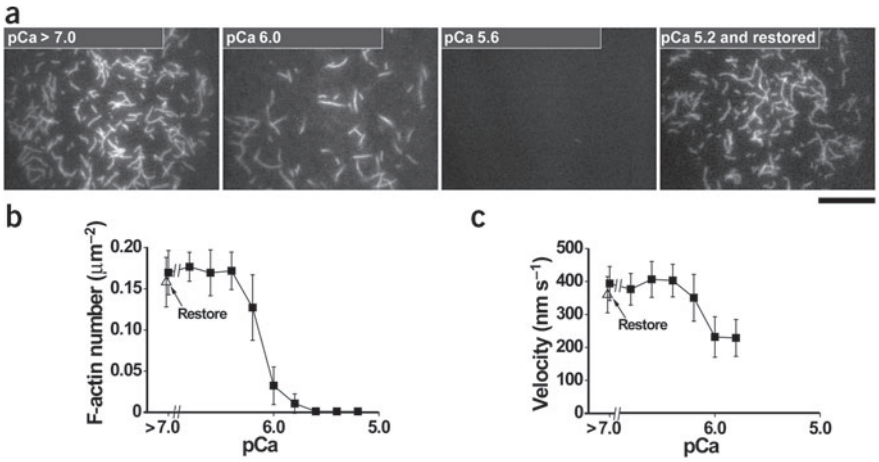


**Figure 1** Effects of $Ca^{2+}$ on the *in vitro* motility assay of myosin V. (**a**) *In vitro* motility assays carried out in the absence of $Ca^{2+}$ (pCa > 7.0), and at pCa 6.0 and 5.6. At pCa 5.2, movement was restored by washing out $Ca^{2+}$ in the presence of 10 μM CaM. Bar, 10 μm. (**b**,**c**) The number (**b**) and velocity (**c**) of sliding actin filaments (F-actin) at different pCa values in the *in vitro* motility assay. Data in **b** and **c** are mean values ± s.d. from three independent experiments.

pCa ≤ 5.6, all actin filaments were detached from myosin V, and thus sliding velocity could not be measured. Both the number and the velocity of sliding filaments could be restored to original levels by removing $Ca^{2+}$ from the solution in the presence of 10 μM CaM (**Fig. 1**) but not in the absence of CaM (data not shown).

To determine the reason for the reduction in both the motility and the actin-binding affinity of myosin V, the number of CaM dissociated from myosin V in the presence of $Ca^{2+}$ was estimated using single-molecule analysis. The dissociated CaM was substituted with Cy3-calmodulin (*CaM) and the number and intensity of fluorescent spots of *CaM were observed at single-molecule level (**Fig. 2a** and **Supplementary Methods** online). At pCa values of 6.0 and 5.8 (**Fig. 2b**), at least one CaM dissociated from each myosin V as the number of fluorescent spots (~0.19 spots $\mu m^{-2}$) of the substituted *CaM inside the chamber was almost equal to the calculated number of myosin V molecules initially applied to the chamber (binding of 0.025 nM myosin to the total contact area of $47 \times 10^7$ $\mu m^2$ in a chamber of 6 μl would give a value of ~0.19 molecules $\mu m^{-2}$). This number did not change after several washes with the $Ca^{2+}$ solution followed by substitution with *CaM. In the absence of $Ca^{2+}$ (pCa > 7.0) (**Fig. 2b**), only very few fluorescent spots could be detected (~10 spots) indicating that most of myosin V (~95%) was saturated with CaM before treatment with $Ca^{2+}$.

To quantify the effect of $Ca^{2+}$ on the dissociation of CaM from myosin V, the dissociated CaM was substituted with *CaM and the population of single, double and triple substitutions of *CaM molecules (**Fig. 2c** and **Supplementary Methods** online) on the myosin V were estimated as a function of $Ca^{2+}$ concentration (**Fig. 2d**). At pCa 6.2, ~50% of the myosin V molecules dissociated a single CaM molecule. At pCa 6.0, ~70% of myosin V dissociated a single CaM molecule, and ~30% of the myosin V dissociated two molecules. The dissociation of two CaM from one myosin V reached saturating levels at pCa 5.6. The triple bleaching steps were negligibly small (<5%) at all $Ca^{2+}$ concentrations, whereas four or more bleaching steps were not observed. This suggests that each myosin V could lose a maximum of two CaM. To ensure that two was the maximum number, a sample was treated three times at pCa 5.2 after *CaM substitution and the number of spots having triple bleaching steps was still <5%.

Because it is well known that one CaM has four calcium-binding sites[3], the number of $Ca^{2+}$ that binds to a CaM and triggers the dissociation of that CaM was estimated. The data for the dissociation of CaM from a myosin V in the presence of $Ca^{2+}$ could be well fitted to the Hill equation giving a best fit value of 3.6 (**Fig. 2e**), suggesting that 3–4 $Ca^{2+}$ are required to trigger the dissociation of one CaM.

### $Ca^{2+}$-dependent dissociation rate of CaM

To determine the kinetic effect of $Ca^{2+}$ on the regulation of myosin V motility, the dissociation rate of CaM from myosin V in the presence of $Ca^{2+}$ was estimated by substituting the dissociated CaM with 100 nM *CaM. To answer whether the association rate of 100 nM *CaM to myosin V affected the estimation of dissociation rate or not, the association rate of 100 nM *CaM to myosin V had to be determined first (**Supplementary Methods** online). The time course of *CaM substitu-

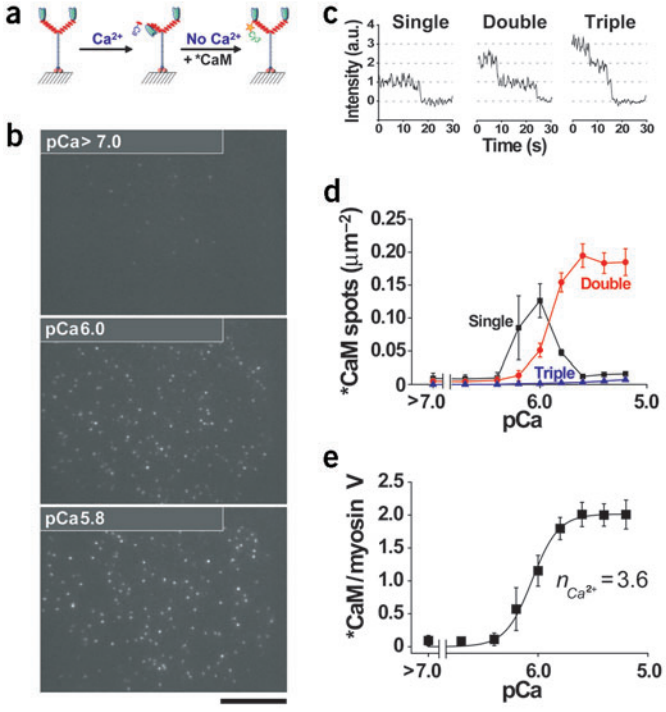


**Figure 2** Effect of $Ca^{2+}$ on the dissociation of CaM from myosin V. (**a**) Myosin V was treated with $Ca^{2+}$ at various concentrations. The free $Ca^{2+}$ was washed out and the dissociated CaM was substituted with *CaM. (**b**) Fluorescent spots of the substituted *CaM bound to individual myosin V on the glass surface were observed under TIRFM without $Ca^{2+}$ treatment (pCa > 7.0) or after $Ca^{2+}$ treatments at pCa 6.0 and 5.8. The density of myosin V was ~0.19 molecules $\mu m^{-2}$ on the observation area (1,000 $\mu m^2$). Bar, 10 μm. (**c**) The bleaching steps of fluorescent intensity of single, double and triple *CaM molecules. (**d**) Number of *CaM substituted for CaM that dissociated from individual myosin V at different $Ca^{2+}$ concentrations. (**e**) Dissociation of CaM (replaced with *CaM) from myosin V. Data were collected from D and fitted with the Hill equation[36] to determine the number of $Ca^{2+}$ bound to CaM when CaM dissociates from myosin V. Data in **d** and **e** are mean values ± s.d. from three independent experiments.

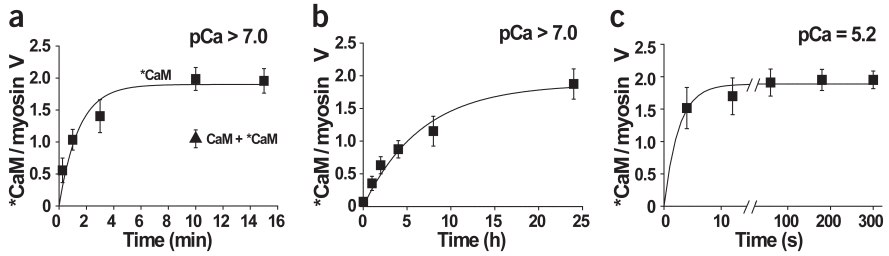


**Figure 3** $Ca^{2+}$-dependent dissociation rate of CaM from myosin V (**Supplementary Methods** online). (**a**) The time course at 100 nM of *CaM substitution on myosin V that had lost a CaM in the absence of $Ca^{2+}$. Data were fitted with an exponential curve to obtain the association constant of *CaM to myosin V in the absence of $Ca^{2+}$. (**b**) The time course of *CaM exchange for CaM dissociated from myosin V in the absence of $Ca^{2+}$. Data were fitted with an exponential curve to obtain the dissociation constant of CaM from myosin V in the absence of $Ca^{2+}$. (**c**) The time course of CaM dissociated from myosin V at pCa 5.2, which was determined from the substitution of *CaM. Data were fitted with an exponential curve to obtain the dissociation constant of CaM from myosin V at pCa 5.2. Data are mean values ± s.d. from three independent experiments.



tion on myosin V was fitted to an exponential curve (**Fig. 3a**) to determine the pseudo first-order association rate constant ([*CaM]$k_{7.0+}$) at 100 nM *CaM in the absence of $Ca^{2+}$. To estimate the second-order association rate constant ($k_{7.0+}$), two similar experiments were done at 20 and 50 nM *CaM. The best-fit line on the graph of *CaM concentrations versus first-order association rate constants showed that $k_{7.0+}$ was ~$1 \times 10^5$ $M^{-1}$ $s^{-1}$ (data not shown). To confirm that labeling with Cy3 does not affect the association rate of *CaM, a 1:1 mixture of nonlabeled CaM and *CaM (100 nM each) was used in a similar experiment as described above. After a 10-min incubation, the number of re-bound *CaM was reduced to ~50% (**Fig. 3a**), indicating that Cy3 labeling had no substantial effect on the association rate of *CaM.

To measure the dissociation rate of CaM from myosin V in the absence of $Ca^{2+}$, myosin V bound to a glass surface was incubated with 100 nM *CaM for various periods of time up to 24 h (**Supplementary Methods** online). The data were fitted to an exponential curve (**Fig. 3b**) and the dissociation rate constant ($k_{7.0-}$) in the absence of $Ca^{2+}$ was ~$0.4 \times 10^{-5}$ $s^{-1}$. The dissociation constant ($K_d$) of CaM from myosin V in the absence of $Ca^{2+}$ was calculated as $k_{7.0-}$ / $k_{7.0+}$, giving a value of ~0.4 nM. To determine the dissociation rate of CaM in the presence of $Ca^{2+}$ (**Supplementary Methods** online), the time course of CaM dissociated from myosin V at pCa 5.2 was fitted to an exponential curve (**Fig. 3c**). The dissociation rate constant ($k_{5.2-}$) at pCa 5.2 was ~0.4 $s^{-1}$, which is $10^5$ times faster than that in the absence of $Ca^{2+}$.

### Regulation of myosin V by caged $Ca^{2+}$

The conventional *in vitro* motility assay does not show the transient effect of $Ca^{2+}$ on myosin V that normally occurs in cells. To overcome this limitation, the sliding of the filaments was regulated by incorporating an *in vitro* motility assay with caged $Ca^{2+}$ that can generate Ca transients using a UV flash (**Supplementary Methods** online). The duration of the UV flash is 4 s. Before the UV flash, actin filaments moved smoothly on the glass surface (**Fig. 4a**(i)). At the end of the UV flash, most of actin filaments had detached (**Fig. 4a**(ii)), and 40 s after the completion of the UV flash most of them had reattached (**Fig. 4a**(iii)) (**Supplementary Video 1** online).

The time course of the $Ca^{2+}$ concentration was measured using Rhod-2 (ref. 22), a $Ca^{2+}$-sensitive fluorophore (**Fig. 4b**). Quantification of the number of sliding actin filaments and velocity at which these filaments were moving under various concentrations of CaM show that, before the UV flash, there were ~0.15 actin filaments $\mu m^{-2}$ sliding at ~400 nm $s^{-1}$ (**Fig. 4c**,**d**). Within a second after starting the UV flash, actin filaments became detached in the illumination area (**Fig. 4c**). In the absence of free CaM, the number of sliding actin filaments did not increase, even after several minutes (data not shown). In contrast, in the sample containing free CaM, actin filaments were able to gradually reattach and the rate of reattachment was dependent on the free CaM concentration. During the UV flash and before detachment of the actin filaments, the velocity slowed to ~230 nm $s^{-1}$. When the UV illumination was completed, the velocity was gradually increased and the acceleration was dependent on the free CaM concentration (**Fig. 4d**). The velocity of sliding in the samples could not be measured without adding free CaM, as otherwise the actin filaments did not attach. The number and velocity of sliding actin filaments remained unchanged using UV flash in the absence of caged $Ca^{2+}$ (data not shown). In conclusion, using photolysis of caged $Ca^{2+}$, we observed the transient regulation of the number of actin filaments and their sliding velocity in the *in vitro* motility assay of myosin V. The movement commenced with detachment and then slowed within a few seconds. Finally, the filaments reattachment and movement recommenced in the presence of free CaM.

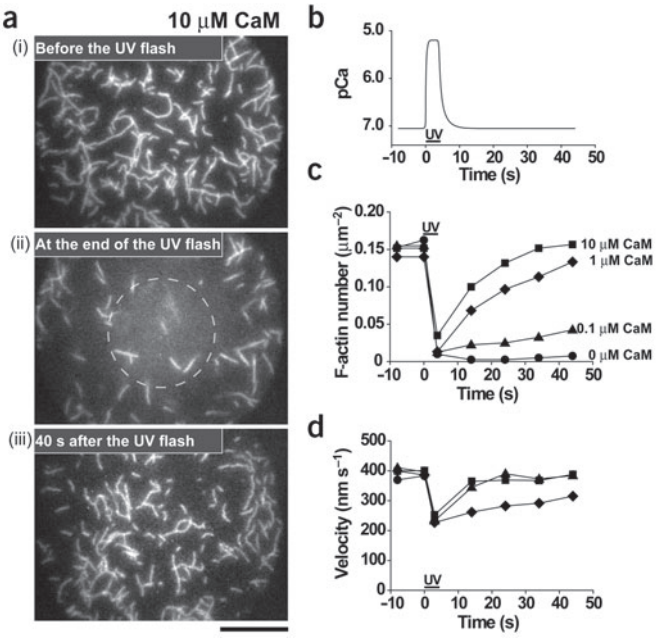


**Figure 4** Reversible regulation of myosin V function with transient $Ca^{2+}$ generated by UV photolysis of caged $Ca^{2+}$. The *in vitro* motility assay was carried out in the presence of transient $Ca^{2+}$ concentrations, which were generated locally by UV photolysis of caged $Ca^{2+}$ within a time frame of 4 s. (**a**) Experiments in the presence of 10 μM CaM at the moment just before (i), at the end of the flash (ii), and 40 s after the 4-s UV photolysis of caged $Ca^{2+}$ (iii). Bar, 10 μm; circle, UV illumination area (**Supplementary Video 1** online). (**b**) The transient $Ca^{2+}$ concentration generated by a 4-s flash of UV photolysis of caged $Ca^{2+}$ was measured using Rhod-2 as the signal. (**c**,**d**) Effects of the transient $Ca^{2+}$ concentration on the number (**c**) and velocity (**d**) of sliding actin filaments (F-actin) at various CaM concentrations. Data in **c** and **d** are mean values from three independent experiments.

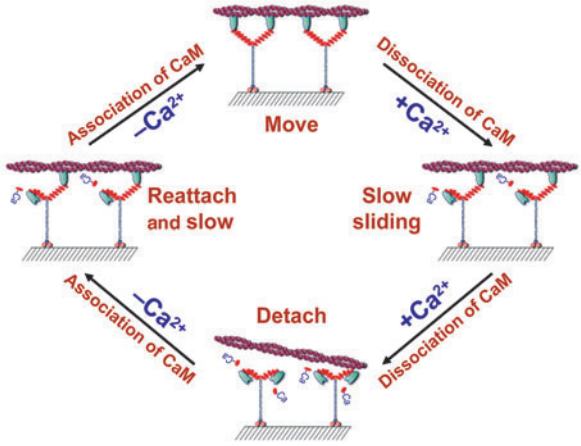


**Figure 5** Model for the reversible regulation of myosin V motor functions by $Ca^{2+}$ in the presence of free CaM. In the absence of $Ca^{2+}$ (pCa > 7.0) (move) the actin filaments slide on myosin V with both heads intact. When the $Ca^{2+}$ concentration increased to pCa ≅ 6.0 (slow sliding), one CaM was lost from one of the myosin heads. The single intact head was still able to move the actin filaments by multiple molecules of myosin V but at a velocity ~60% slower than that of native myosin V. When the $Ca^{2+}$ concentration increased to pCa ≤ 5.6 (detach), myosin V lost both CaM from each of the two heads and the actin filaments became detached from myosin V. When the $Ca^{2+}$ concentration returned to the normal low level, free CaM quickly bound to the vacant IQ motif and myosin V quickly recovered to the single-headed active stage (reattach and slow) and the stage of two heads regained their normal function (move). The structure of the motor domain folding back to the neck domain was adapted from a reported structure[27].

## DISCUSSION

In the *in vitro* motility assay, actin filaments exhibited a slow sliding velocity at pCa 6.0 and detached at pCa ≤ 5.6. The sliding velocity and number of attached actin filaments could be restored after washing out $Ca^{2+}$ in the presence of free CaM (**Fig. 1a–c**), but could not be restored after washing out $Ca^{2+}$ in the absence of free CaM. This indicates that the slowing of movement and detachment of actin filaments are due to the dissociation of CaM.

### Regulation of myosin V by single CaM

In previous studies, the number of CaM that dissociated from myosin V at certain $Ca^{2+}$ concentrations was estimated by measuring the change in stoichiometry of CaM per heavy chain using a gel scanning method[20,23]. However, the method does not show the distribution of the number of CaM molecules dissociated from an individual myosin molecule. The exact number of CaM that dissociated also could not be estimated owing to the limitations in resolution using this gel scanning method, because the dissociation of a single CaM molecule from a total of 12 bound[24] to myosin V needed to be observed. These limitations were overcome by using single-molecule imaging techniques to evaluate the exact number of single CaM molecules dissociated from individual molecules of a native myosin V at various pCa values. The results showed that the process of CaM dissociation at gradient $Ca^{2+}$ concentrations includes several distinct dissociation events. In a 3-min incubation at pCa 6.0, at least one or two CaM molecules dissociated from each myosin, and at pCa 5.6, two CaM dissociated from every myosin; this is thought to be the maximum number (**Fig. 2c**). This suggests that one CaM originally dissociated from one heavy chain at a single specific IQ motif, because the myosin V is a homodimer[16]. If this were not the case, the number would be >2. We observed the dissociation of two CaM when myosin V was treated at physiological concentrations of $Ca^{2+}$ (pCa > 5.0)[25], as well as at very high concentrations of $Ca^{2+}$ (pCa 4.5) for 10 s. However, ~15% of the myosin V molecules dissociated three CaM molecules or more when the myosin V was treated at pCa 4.5 for 5 min (data not shown). This agrees with the data from a previous study[26] in which ~2.1 CaM dissociated when CaM was exchanged with *CaM by treatment of myosin V with a solution containing 1 mM EGTA and 1.01 mM $CaCl_2$ (pCa ≅ 4.6) for 5 min. Using the same method, another group of researchers[13] obtained a myosin V mixture containing ~76% of the labeled CaM exchanged at the IQ motif at the end of neck domain connecting to the head of myosin V. Their findings also suggested that a single CaM molecule dissociated from a specific site on the myosin V heavy chain.

The good correlation between the number of sliding actin filaments and the dissociation of single CaM molecules from myosin V suggests that actin-binding affinity is regulated by the dissociation of single CaM molecules. The dissociation of CaM from heavy chains results in a conformational change to the head and neck region of the myosin V molecule[27]. The head-neck domain of myosin V is extended in the absence of $Ca^{2+}$, whereas at high $Ca^{2+}$ concentrations, the head domain folds back onto the neck domain; this may reduce the actin-binding affinity of the myosin head. The reduction of actin-binding affinity of the myosin head at high $Ca^{2+}$ concentration was also supported by the biochemical results of the actin-activated ATPase activity measured under similar conditions to our motility assay (100 mM KCl; 25 °C). At high $Ca^{2+}$ concentrations, the $K_{m,actin}$ of single-headed myosin V (19.9 μM) is about two-fold higher compared with that in the absence of $Ca^{2+}$ (9.2 μM)[27]. The increase of $K_{m,actin}$ suggests that the actin-binding affinity of the myosin head was reduced at high $Ca^{2+}$ concentrations. This may disrupt the overlap between the two heads of a myosin V, which would reduce the processivity of double-headed myosin V, causing the detachment of sliding actin filament. The high $Ca^{2+}$ concentrations (>1 μM) reduce the actin-binding affinity of the myosin V head through the dissociation of single CaM molecules by binding $Ca^{2+}$ and folding back of the head domain onto the neck. In contrast to glass binding myosin V, in solution the intact myosin V strongly binds to actin filament in the presence of $Ca^{2+}$ (ref. 28); thus the ATPase activity remains high.

We characterized the kinetics of the $Ca^{2+}$-dependent dissociation of CaM from myosin V at a single-molecule level. In the absence of $Ca^{2+}$, CaM binds very rapidly and with a high affinity to myosin V, maintaining a stable association of CaM with myosin V for a functional motor at normal low cellular $Ca^{2+}$ concentrations. In the presence of $Ca^{2+}$, the rapid dissociation of CaM (within seconds) allows myosin V to be regulated by transient $Ca^{2+}$ bursts in cells.

### Transient regulation of myosin V

Myosin V is abundantly expressed in nerve cells[15]. It has also been reported that the tips of filopodia (the finger-like projections on the tips of developing nerve cells) generate tiny bursts of $Ca^{2+}$ that travel back to the growth cone to stimulate movement in the correct direction[29]. We simulated the cellular $Ca^{2+}$ bursts by using caged $Ca^{2+}$ coupled with a UV flash system to generate $Ca^{2+}$ transients locally within a time range of a few seconds in an *in vitro* motility assay. We observed a quick response of myosin V motor functions as demonstrated by the detachment of filaments and a reduction in velocity of actin filament sliding during the UV flash. Compared with samples containing 10 or 1 μM CaM, the slower recovery in the case of 0.1 μM CaM and the fact that no recovery occurred in the absence of free CaM support our conclusion that CaM dissociation is involved in the regulation of actin-binding affinity and motility of myosin V. Although the association rate of CaM is very quick in the absence of $Ca^{2+}$ (<1 s at 10 μM CaM) the reattachment of the actin filaments was slower, probably because of the low concentration of actin filaments in the medium.

### Model for the regulation of myosin V

We propose a model for the transient regulation of various myosin V functions by $Ca^{2+}$ (**Fig. 5**). In the absence of $Ca^{2+}$, the native myosin V has a full complement of bound CaM and moves the actin filaments with both heads. When the $Ca^{2+}$ concentration increased to pCa ≅ 6.0, one of the two heads of myosin V lost one CaM and myosin was in the single-headed active stage. At this stage, the actin-binding affinity was partially reduced and the velocity was slow. Increasing the $Ca^{2+}$ concentration further (pCa 5.6), caused the other head of myosin V to also lose one CaM. Myosin V then moved to the double-headed inactive stage, causing the actin filaments to detach. When $Ca^{2+}$ was chelated, the free CaM quickly rebound to one of the heads that had lost a CaM and myosin V once again was in the single-headed active stage. Finally, the second CaM associated with myosin V and both heads bound a CaM, in what is known as the double-headed active stage of myosin V. In conclusion, the binding of 3–4 $Ca^{2+}$ to one CaM (**Fig. 2e**) triggers the dissociation of a single CaM molecule from each myosin head, causing a reduction in actin-binding affinity and in the sliding velocity of myosin V. This model of the regulation of myosin V by $Ca^{2+}$ involving various intermediate stages could be useful in understanding how CaM-binding motor proteins bind, move, detach, rebind and move again on actin filament tracks in cells.

## METHODS

**Chemicals and proteins.** Bovine brain CaM was obtained from Sigma. Alexa-647-phalloidin, *o*-nitrophenyl EGTA tetrapotassium salt[30] and $Ca^{2+}$-sensitive bright Rhod-2 tripotassium salt were obtained from Molecular Probes. Myosin V was purified from chick brains[24]. G-actin was purified from chicken breast muscle[31]. Actin filaments were prepared by polymerization of G-actin in high-salt solution and labeled with Alexa-647-phalloidin. *CaM was prepared as described[26] except that the buffer for labeling was adjusted to pH 7.2 for specific labeling at the N terminus. The obtained *CaM has a Cy3/CaM mole ratio of 1.04:1. *CaM bound to a glass surface in 20 mM HEPES, pH 7.2, was observed as single spots for ~20 s and photobleached in one step, proving that purified *CaM were indeed single molecules.

**Buffers and solutions.** Buffer A contained 25 mM K-acetate, 100 mM KCl, 4 mM $MgCl_2$, 10 mg $ml^{-1}$ BSA, 1 mM EGTA and 20 mM HEPES, pH 7.2 (pCa > 7.0). EGTA-$Ca^{2+}$ solutions with different free $Ca^{2+}$ concentrations were adjusted by mixing various ratios of buffer A with solution B (buffer A supplemented with 1.05 mM $CaCl_2$). The free $Ca^{2+}$ concentration in the mixture[32] was calculated based on the $K_d$ of EGTA-$Ca^{2+}$. An oxygen scavenger system[33] was added to all buffers for all imaging experiments.

**Single-molecule microscopy. The** sealed chambers of all assays were observed under total internal reflection fluorescence microscopy (TIRFM)[33,34]. *CaM and Rhod-2 were excited by a Nd:YAG laser (TIM-6222, Transverse) at 532 nm. Alexa-647-labeled actin filaments were excited by a diode laser (F44-30M, Coherent) at 635 nm. The image was recorded on a DVD recorder (DVR-7000; Pioneer). UV flashes for caged $Ca^{2+}$ activation[35] were created by a 100-W high-pressure mercury lamp, passed through a band-pass filter of 340–370 nm, controlled with a shutter, passed through the objective lens and at last focused into the chamber solution.

Slide quartz glasses, cover glasses and spacers (25-μm thick) were sonicated with 0.1 M KOH and then with 5 M HCl and rinsed with distilled water before use to reduce the background interference for single-molecule imaging and to reduce the effect on the motility activity of myosin V in an *in vitro* motility assay. The chamber was assembled using a slide quartz glass, a cover glass and two spacers. The chamber volume was ~6 μl.

***In vitro* motility assay of myosin V.** The *in vitro* motility assays of myosin V were carried out in the absence and presence of $Ca^{2+}$. In the presence of $Ca^{2+}$, the $Ca^{2+}$ concentration was changed in a stepwise or transient manner, depending on the purpose of the experiment (**Supplementary Methods** online).

**Substitution of CaM with Cy3-CaM.** A flow glass chamber was rinsed with BSA in 10 μl buffer A, and exchanged with 6 μl of 0.025 nM myosin V in buffer A (~0.19 molecules $\mu m^{-2}$) for 2 min to allow the myosin to bind to the glass surface. The slide was then treated with 10 μl of the $Ca^{2+}$ solution for 3 min. The free $Ca^{2+}$ was washed out and the dissociated CaM was substituted with 20 μl of 100 nM *CaM in buffer A for 10 min (10 min was sufficient for most of the dissociated CaM to be substituted (**Fig. 3a**) and was also enough time for restoration of myosin V motility back to almost 'normal' levels; data not shown). Finally, the free *CaM was washed by incubation with 20 μl buffer A for 3 min. *CaM bound on myosin V was observed under TIRFM after exchange of the buffer with buffer A supplemented with an oxygen scavenger system.

The majority of the dissociated CaM was substituted with *CaM within 5 min (**Fig. 3a**) and observations could be made for at least 1 h under TIRFM without affecting the number or the intensity of the substituted *CaM fluorescent spots. This indicates that in the presence of 100 nM *CaM and in the absence of $Ca^{2+}$, the dissociation rate of CaM from myosin V is negligibly slow compared with the association rate (at least ten times slower). The number of substituted *CaM fluorescent spots indicates the number of CaM-dissociated myosin V molecules because almost no spots (0.6% compared with sample in the presence of myosin V) were observed in the absence of myosin V.

For determination of the association and dissociation rate of CaM from myosin V, see **Supplementary Methods** online.

All mentioned assays were done at 25 °C.

*Note: Supplementary information is available on the Nature Structural & Molecular Biology website.*

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Ikura, M. Calcium binding and conformational response in EF-hand proteins. *Trends Biochem. Sci.* **21**, 14–17 (1996).
2. Chin, D. & Means, A.R. Calmodulin: a prototypical calcium sensor. *Trends Cell Biol.* **10**, 322–328 (2000).
3. Prekeris, R. & Terrian, D.M. Brain myosin V is a synaptic vesicle–associated motor protein: evidence for a $Ca^{2+}$-dependent interaction with the synaptobrevin–synaptophysin complex. *J. Cell Biol.* **137**, 1589–1601 (1997).
4. Evans, L.L., Lee, A.J., Bridgman, P.C. & Mooseker, M.S. Vesicle-associated brain myosin V can be activated to catalyze actin-based transport. *J. Cell Sci.* **111**, 2055–2066 (1998).
5. Tabb, J.S., Molyneaux, B.J., Cohen, D.L., Kuznetsov, S.A. & Langford, G.M. Transport of ER vesicles on actin filaments in neurons by myosin V. *J. Cell Sci.* **111**, 3221–3234 (1998).
6. Provance, D.W. Jr., Wei, M., Ipe, V. & Mercer, J.A. Cultured melanocytes from dilute mutant mice exhibit dendritic morphology and altered melanosome distribution. *Proc. Natl. Acad. Sci. USA* **93**, 14554–14558 (1996).
7. Wu, X., Bowers, B., Wei, Q., Kocher, B. & Hammer, J.A. 3rd. Myosin V associates with melanosomes in mouse melanocytes: evidence that myosin V is an organelle motor. *J. Cell Sci.* **110**, 847–859 (1997).
8. Karcher, R.L. *et al.* Cell cycle regulation of myosin V by calcium/calmodulin-dependent protein kinase II. *Science* **293**, 1317–1320 (2001).
9. Hill, K.L., Catlett, N.L. & Weisman, L.S. Actin and myosin function in directed vacuole movement during cell division in *Saccharomyces cerevisiae*. *J. Cell Biol.* **135**, 1535–1549 (1996).
10. Catlett, N.L. & Weisman, L.S. The terminal tail region of a yeast myosin V mediates its attachment to vacuole membranes and sites of polarized growth. *Proc. Natl. Acad. Sci. USA* **95**, 14799–14804 (1998).
11. Mehta, A.D. *et al.* Myosin V is a processive actin-based motor. *Nature* **400**, 590–593 (1999).
12. Uemura, S., Higuchi, H., Olivares, A.O., De La Cruz E.M. & Ishiwata, S. Mechano-chemical coupling of 12- and 24-nm substeps in a single myosin V motor. *Nat. Struct Mol. Biol.* **11**, 877–883 (2004).
13. Yildiz, A. *et al.* Myosin V walks hand-over-hand: single fluorophore imaging with 1.5-nm localization. *Science* **300**, 2061–2065 (2003).
14. Espindola, F.S. *et al.* Biochemical and immunological characterization of p190–calmodulin complex from vertebrate brain: a novel calmodulin binding myosin. *J. Cell Biol.* **118**, 359–368 (1992).

15. Espreafico, E.M. *et al.* Primary structure and cellular localization of chicken brain myosin V (p190), an unconventional myosin with calmodulin light chains. *J. Cell Biol.* **119**, 1541–1557 (1992).
16. Cheney, R.E. *et al.* Brain myosin V is a two-headed unconventional myosin with motor activity. *Cell* **75**, 13–23 (1993).
17. Cameron, L.C. *et al.* Calcium-induced quenching of intrinsic fluorescence in brain myosin V is linked to dissociation of calmodulin light chains. *Arch. Biochem. Biophys.* **355**, 35–42 (1998).
18. Nascimento, A.A., Cheney, R.E., Tauhata, S.B., Larson, R.E. & Mooseker, M.S. Enzymatic characterization and functional domain mapping of brain myosin V. *J. Biol. Chem.* **271**, 17561–17569 (1996).
19. Trybus, K.M., Krementsova, E. & Freyzon, Y. Kinetic characterization of a monomeric unconventional myosin V construct. *J. Biol. Chem.* **274**, 27448–27456 (1999).
20. Homma, K., Saito, J., Ikebe, R. & Ikebe, M. $Ca^{2+}$-dependent regulation of the motor activity of myosin V. *J. Biol. Chem.* **275**, 34766–34771 (2000).
21. Martin, S.R. & Bayley, P.M. Regulatory implications of a novel mode of interaction of calmodulin with a double IQ motif target sequence from murine dilute myosin V. *Protein Sci.* **11**, 2909–2923 (2002).
22. Nguyen, V.T., Higuchi, H. & Kamio, Y. Controlling pore assembly of staphylococcal γ-haemolysin by low temperature and by disulphide bond formation in double-cysteine LukF mutants. *Mol. Microbiol.* **45**, 1485–1498 (2002).
23. Krementsov, D.N., Krementsova, E.B. & Trybus, K.M. Myosin V: regulation by calcium, calmodulin, and the tail domain. *J. Cell Biol.* **164**, 877–886 (2004).
24. Cheney, R.E. Purification and assay of myosin V. *Methods Enzymol.* **298**, 3–18 (1998).
25. Poenie, M., Alderton, J., Tsien, R.Y. & Steinhardt, R.A. Changes of free calcium levels with stages of the cell division cycle. *Nature* **315**, 147–149 (1985).
26. Sakamoto, T., Amitani, I. Yokota, E. & Ando, T. Direct observation of processive movement by individual myosin V molecules. *Biochem. Biophys. Res. Commun.* **272**, 586–590 (2000).
27. Li, X.D., Mabuchi, K., Ikebe, R. & Ikebe, M. $Ca^{2+}$-induced activation of ATPase activity of myosin Va is accompanied with a large conformational change. *Biochem. Biophys. Res. Commun.* **12**, 538–545 (2004).
28. Tauhata, S.B., dos Santos, D.V., Taylor, E.W., Mooseker, M.S. & Larson, R.E. High affinity binding of brain myosin Va to F-actin induced by calcium in the presence of ATP. *J. Biol. Chem.* **276**, 39812–39818 (2001).
29. Gomez, T.M., Robles E., Poo M. & Spitzer N.C. Filopodial calcium transients promote substrate-dependent growth cone turning. *Science* **291**, 1983–1987 (2001).
30. Ellis-Davies, G.C. & Kaplan, J.H. Nitrophenyl-EGTA, a photolabile chelator that selectively binds $Ca^{2+}$ with high affinity and releases it rapidly upon photolysis. *Proc. Natl. Acad. Sci. USA* **91**, 187–191 (1994).
31. Spudich, J.A. & Watt, S. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **246**, 4866–4871 (1971).
32. Horiuti, K. *et al.* Mechanism of action of 2,3-butanedione 2-monoxime on contraction of frog skeletal muscle fibres. *J. Muscle. Res. Cell Motil.* **9**, 156–164 (1988).
33. Nguyen, V.T., Kamio, Y. & Higuchi, H. Single molecule imaging of cooperative assembly of γ-hemolysin on erythrocyte membranes. *EMBO J.* **22**, 4968–4979 (2003).
34. Funatsu, T., Harada, Y., Tokunaga, M., Saito, K. & Yanagida, T. Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. *Nature* **374**, 555–559 (1995).
35. Dantzig, J.A., Higuchi, H. & Goldman, Y.E. Studies of molecular motors using caged compounds. *Methods Enzymol.* **291**, 307–348 (1998).
36. West, J.M., Higuchi, H., Ishijima, A. & Yanagida, T. Modification of the bi-directional sliding movement of actin filaments along native thick filaments isolated from a clam. *J. Muscle Res. Cell Motil.* **17**, 637–646 (1996).