

# Myosin Mg-ATPase of molluscan muscles is slightly activated by F-actin under catch state in vitro

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**Abstract** Molluscan muscle twitchin, a titin/connectin-related giant protein, regulates interactions between actin and myosin filaments at low  $\text{Ca}^{2+}$  concentrations. When it is dephosphorylated, actin filaments tightly bind to myosin filaments, resulting in the catch state known as the state of high passive tension with very low energy consumption. Yet when twitchin is phosphorylated actin filaments detach from the myosin filaments, resulting in relaxation of the catch. Here, steady-state Mg-ATPase activities of purified myosin were measured under various conditions: without twitchin, with dephosphorylated twitchin, or with phosphorylated twitchin; with or without phalloidin-stabilized F-actin; and at various  $\text{Ca}^{2+}$  concentrations. At low  $\text{Ca}^{2+}$  concentration, Mg-ATPase was activated by F-actin only in the presence of dephosphorylated twitchin (catch state). The activation was about two orders lower than that fully activated by  $\text{Ca}^{2+}$  and F-actin. In the absence of F-actin, twitchin and its phosphorylation state did not affect Mg-ATPase activities in any of the conditions we tested. Based on these results, we propose a molecular mechanism for the catch, where twitchin alone does not interact with the myosin catalytic motor domain but its complex with F-actin does, forming the bridge between actin and myosin filaments and the myosin slowly hydrolyzes Mg-ATP in the catch state.

**Keywords** Catch muscle · Catch state · Myosin · Mg-ATPase · Twitchin · Phosphorylation

## Introduction

Some bivalve smooth muscles exhibit a characteristic feature called catch, wherein they can maintain high tension after active contractions. The high tension of catch decays when a neurotransmitter, 5-hydroxytryptamine, is added to the muscle (Twarog 1954). This neurotransmitter increases intracellular cAMP concentration resulting in the activation of cAMP-dependent protein kinase, which phosphorylates twitchin causing the relaxation of the catch (Siegman et al. 1997, 1998). Twitchin, originally found in a nematode, *Caenorhabditis elegans* (Benian et al. 1989), is structurally related to a giant protein called titin or connectin, found first in vertebrate striated muscles (Maruyama et al. 1977; Wang et al. 1979), and to less extent, to vertebrate cardiac myosin binding protein C (Funabara et al. 2003). Smooth muscle twitchin of the mussel *Mytilus galloprovincialis* is about 540 kDa in size and its full primary sequence and phosphorylatable serine residues have already been clarified (Funabara et al. 2003). Bivalve twitchin, also called mini-titin, is associated with thick filaments in not only smooth but also striated muscles (Vibert et al. 1993).

Many studies have shown that there is much less energy consumption of the muscles during the catch state than during the active contractions.  $\text{O}_2$  consumption, for instance, was found to be much lower during the catch state than during the active contractions (Baguet and Gillis 1968) and intracellular concentrations of phosphorus compounds related to energy consumption such as arginine phosphate and inorganic phosphate during the catch state

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were found to be more similar to those in the resting state than in the active contracting state (Ishii et al. 1991). Intracellular  $\text{Ca}^{2+}$  concentration during the catch state is low (Ishii et al. 1989) and this state can be reproduced with permeabilized catch muscle fibers at low free  $\text{Ca}^{2+}$  concentrations (Pfitzer and Rüegg 1982). Since molluscan muscle myosin is inactive at low  $\text{Ca}^{2+}$  concentrations (Kendrick-Jones et al. 1970), contractile proteins are not supposed to consume much energy under the catch state. Indeed, the Mg-ATPase activity of permeabilized catch muscle fibers is much lower in the catch state than in the actively contracting state (Güth et al. 1984; Butler et al. 1998, 2001). Since all of these studies were performed with intact or permeabilized muscle fibers or cells, the measured energy consumption must have included that of some systems other than contractile proteins that could be a significant background for the ATPase activity measurements.

In contrast to these studies with muscle fibers and cells, we have developed in vitro reconstitution study system to investigate interactions between thick and thin filaments isolated from catch muscles. Furthermore, the study can be conducted solely with myofilaments synthesized from purified proteins. Using this in vitro system we have found actin filaments bind to myosin filaments associated with dephosphorylated twitchin in the presence of Mg-ATP at low free  $\text{Ca}^{2+}$  concentrations, but they do not bind when twitchin is phosphorylated under the same solution condition. We concluded that the former and the latter states respectively correspond to the catch and the relaxed states of intact muscle fibers (Yamada et al. 2001, 2004). The molecular mechanisms of the binding of thin filaments to thick filaments in catch, however, are not yet fully understood.

In this report, we measured steady-state Mg-ATPase activity in these in vitro states reconstituted with purified proteins where the effects of energy consumption other than the contractile system were excluded, and found that ATPase was activated slightly by F-actin in the catch state condition, i.e., in the presence of dephosphorylated twitchin at low free  $\text{Ca}^{2+}$  concentration. In the absence of F-actin, myosin Mg-ATPase was not affected by the existence and phosphorylation state of twitchin in any of the free  $\text{Ca}^{2+}$  concentration conditions we tested, suggesting that twitchin does not directly affect the myosin catalytic motor domain.

## Materials and methods

### Proteins

Myosin and twitchin were purified from the obliquely striated and smooth adductor muscles of the Japanese

oyster *Crassostrea gigas* (Tsutsui et al. 2007). Myosin filaments were obtained by dialysis of purified myosin against a low-salt buffer solution (Yamada et al. 2001). Twitchin was dephosphorylated with bovine calcineurin (Sigma, C-1907) and calmodulin (Sigma, P-2277) as described (Tsutsui et al. 2005). Actin was obtained from rabbit fast skeletal muscles and purified (Spudich and Watt 1971). For the Mg-ATPase assays,  $\text{MgCl}_2$ , instead of  $\text{CaCl}_2$ , was used for the actin purification for accurate determination of free  $\text{Ca}^{2+}$  concentrations in the reaction mixture. Actin was polymerized by adding 0.1 M NaCl and a slightly excess molar amount of either non-fluorescent phalloidin (Sigma, P-2141) for Mg-ATPase activity assays or tetramethylrhodamine phalloidin (Sigma, P-1951) for in vitro light microscopic reconstitution assays. The latter assays were performed as described elsewhere (Tsutsui et al. 2005). Protein concentration was determined spectrophotometrically based on the Bradford method by using a protein assay kit (Bio-Rad Laboratories), using bovine serum albumin as a standard.

### Mg-ATPase activity assay

Steady-state Mg-ATPase activity was measured spectrophotometrically by determining the amount of liberated inorganic phosphate using an EnzChek Phosphate Assay Kit (Molecular Probes) at 25 °C. The reaction solution contained 90–100 mM NaCl, 1 mM ATP, 2 mM  $\text{MgCl}_2$ , 0.5 mM EGTA, 2 mM dithiothreitol, 0–1.9 mM  $\text{CaCl}_2$ , 20 mM piperazine-*N,N'*-bis(2-ethane sulfonic acid)-NaOH (pH 7.0), 0.05–0.1 mM 2-amino-6-mercapto-7-methylpurine riboside, 0.25–0.5 U  $\text{ml}^{-1}$  purine nucleoside phosphorylase. Absorbance at 360 nm was measured with a DU 800 Spectrophotometer (Beckman Coulter) equipped with a Peltier-temperature controlled 6-Position Cell Holder and a High Performance Peltier Controller. Concentrations of free  $\text{Ca}^{2+}$  were calculated with a computer program, WINMAXC (Bers et al. 1994). In some cases, 20  $\mu\text{g ml}^{-1}$  cAMP-dependent protein kinase (Sigma, P-5511) and  $10^{-5}$  M cAMP (Sigma, A-4137) were added and incubated for 30 min to phosphorylate twitchin and then 4  $\mu\text{g ml}^{-1}$  protein kinase inhibitor (Sigma, P-0300) was added before the assays.

### Equations for fitting obtained data

A simple binding model of twitchin to myosin filaments was applied to explain the results of Fig. 3 and Fig. S2 in the Supplementary Material. We assume binding sites for twitchin on myosin filaments. If there is no cooperativity in binding of twitchin to the binding sites, the following relationships will be present:

$$(M - [Tw]_B)[Tw]_F = K_D [Tw]_B$$

$$[Tw]_T = [Tw]_F + [Tw]_B$$

where  $M$  is the total concentration of the binding sites in the assay solution containing myosin filaments,  $K_D$  is the apparent dissociation constant, and  $[Tw]_T$ ,  $[Tw]_F$ , and  $[Tw]_B$  are total, free, and bound twitchin concentrations, respectively. From these relationships,  $[Tw]_B$  is expressed by a function of  $[Tw]_T$  as the following equation:

$$[Tw]_B = \frac{[Tw]_T + M + K_D}{2} - \left\{ \left( \frac{[Tw]_T + M + K_D}{2} \right)^2 - M [Tw]_T \right\}^{1/2} \quad (1)$$

This equation was fitted to the data in Fig. S2. Assuming that bound twitchin molecules are engaged in the formation of the ‘catch bridges’ and their amount is proportional to the activation of the Mg-ATPase under the catch condition, the following relationship will be present:

$$[Tw]_B / M = (V - V_{0,Tw}) / (V_{max,Tw} - V_{0,Tw})$$

where  $V_{0,Tw}$  and  $V_{max,Tw}$  are the Mg-ATPase activity in the absence of twitchin and the maximum activity in the presence of saturated twitchin, respectively. Together with Eq. (1),  $V$  is expressed by a function of  $[Tw]_T$  as the following equation:

$$V = \frac{V_{max,Tw} - V_{0,Tw}}{2} \left[ \frac{[Tw]_T + K_D}{M} + 1 - \left\{ \left( \frac{[Tw]_T + K_D}{M} + 1 \right)^2 - \frac{4[Tw]_T}{M} \right\}^{1/2} \right] + V_{0,Tw} \quad (2)$$

This equation was fitted to the data in Fig. 3.

## Results

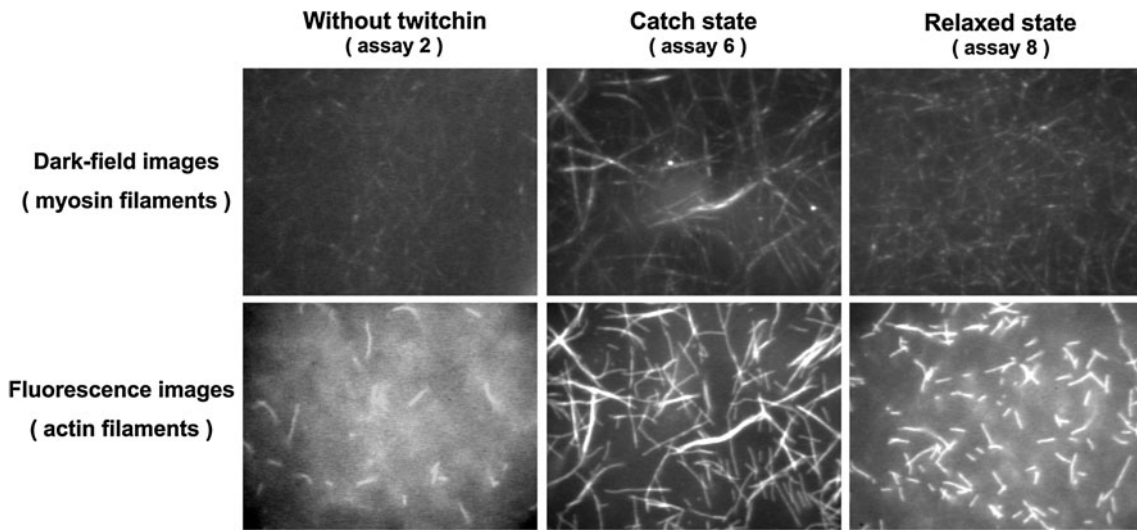
### Light microscopic observations

To clarify the molecular mechanism of the catch, it is important to study the effects of twitchin and its phosphorylation state on steady-state myosin Mg-ATPase activities at low free  $Ca^{2+}$  concentration ( $pCa \approx 8$ ) in detail. We first confirmed the *in vitro* states of the sample mixtures by light microscopic observations (Fig. 1). In the previous studies, we found F-actin binds to myosin filaments associated with dephosphorylated twitchin in the catch state, but it does not bind when twitchin is phosphorylated in the relaxed state (Yamada et al. 2001, 2004). In the present study, to investigate the effect of F-actin on

the Mg-ATPase activities it was stabilized with non-fluorescent phalloidin (see below). For light microscopic observations as shown in Fig. 1, F-actin was stabilized with tetramethylrhodamine-labeled phalloidin to be visualized as in our previous *in vitro* reconstitution studies (Yamada et al. 2001, 2004), and myosin and twitchin were purified from smooth adductor muscle. In the absence of twitchin, F-actin did not bind to myosin filaments (Fig. 1, *left*). Note that this state was the same as that of assay 2 in Fig. 2a (see below) except that phalloidin was conjugated with tetramethylrhodamine. In the presence of dephosphorylated twitchin, a large amount of F-actin bound to myosin filaments and they seemed to form bundles since much thicker filaments were observed under both dark-field and fluorescence observations (Fig. 1, *center*). This state corresponds to the catch state of muscle fibers and assay 6 in Fig. 2a. After the treatment with cAMP-dependent protein kinase, only a small amount of F-actin bound to myosin filaments (Fig. 1, *right*). This state corresponds to the relaxed state of muscle fibers and assay 8 in Fig. 2a. Essentially the same results were obtained when obliquely striated muscle myosin and twitchin were used (data not shown).

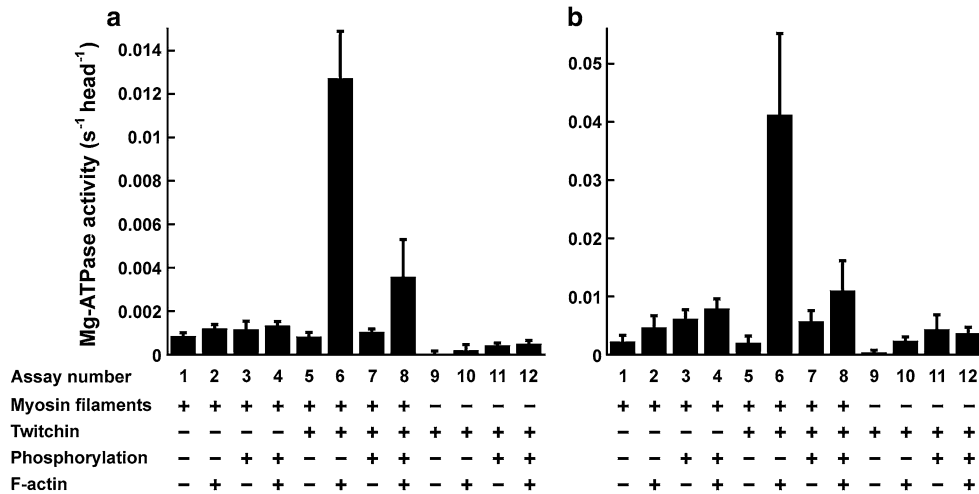
### Mg-ATPase activities at low free $Ca^{2+}$ concentration

Myosin and twitchin of not only smooth but also obliquely striated adductor muscle of the oyster showed the catch state *in vitro* (Tsutsui et al. 2007). We used both muscles to study the steady-state myosin Mg-ATPase activities in detail under various conditions including catch and relaxed state conditions. Each datum was obtained with a spectrophotometric measurement for an hour so that very low Mg-ATPase activity could be detected. The assays were performed under 12 different types of compositions (assay number 1–12 in Fig. 2) for smooth muscle (Fig. 2a) and obliquely striated muscle (Fig. 2b) preparations. Conditions related to myosin filaments (presence [assays 1–8] or absence [assays 9–12]), twitchin (absence [assays 1–4], presence in dephosphorylated form [assays 5, 6, 9, and 10], or presence in phosphorylated form [assays 7, 8, 11, and 12]), and phalloidin-stabilized F-actin (absence [assays in odd numbers] or presence of 2.4  $\mu M$  [assays in even numbers]) were varied among these assays. As shown in Fig. 1, we confirmed by light microscopic observations that dephosphorylated and phosphorylated twitchin used in the present study formed the catch and the relaxed states *in vitro*, respectively. Though the absolute activities were different between the two types of muscles, essentially the same trends were found in the results. (i) F-actin did not seem to activate the myosin Mg-ATPase in the absence of twitchin (compare assay 1 with 2, and assay 3 with 4). Though very small increases in measured activities by the



**Fig. 1** Light microscopic observations at low free  $Ca^{2+}$  concentration showing *catch* and *relaxed* states reconstituted with myosin and twitchin purified from smooth adductor muscle. In the absence of twitchin, F-actin did not bind to myosin filaments (*left*). In the presence of dephosphorylated twitchin, a large amount of F-actin bound to myosin filaments indicating the *catch* state (*center*). After the treatment with cAMP-dependent protein kinase, only a small amount of F-actin bound to myosin filaments (*right*). The solution

conditions shown in the *left*, *center*, and *right* photographs were the same as those of *assays* 2, 6, and 8 in Fig. 2a, respectively, except that tetramethylrhodamine-labeled phalloidin, instead of non-labeled phalloidin, was associated with F-actin. Each observation consists of a dark-field image showing myosin filaments (*upper*) and a fluorescence image showing F-actin (*lower*) in the same field of view. The bar 20  $\mu m$  in all photographs



**Fig. 2** Steady-state Mg-ATPase activities under low free  $Ca^{2+}$  concentration condition ( $pCa \approx 8$ ). Myosin filaments and twitchin were prepared from the smooth adductor muscle in (a), and from the obliquely striated adductor muscle in (b). Myosin filaments were present in assays 1–8 (200 nM double-headed myosin in (a) and 20 nM double-headed myosin in (b)) and absent in assays 9–12. Twitchin was absent in assays 1–4 and present in assays 5–12 (90 nM

in (a) and 9 nM in (b)). Phosphorylation treatment with cAMP-dependent protein kinase was performed as described in the “Materials and Methods” section just before assays 3, 4, 7, 8, 11, and 12. Phalloidin-stabilized F-actin prepared from rabbit skeletal muscles was absent in assays 1, 3, 5, 7, 9, and 11, and present (2.4  $\mu M$ ) in assays 2, 4, 6, 8, 10, and 12. The bar at each assay shows the SE of 5–8 measurements

addition of F-actin were found (assays 1–4), there seemed to be similar increases also in the absence of myosin filaments (assays 9–12). Thus, these apparent increases might have been due to liberation of inorganic phosphate from F-actin. (ii) In the absence of F-actin, twitchin and its

phosphorylation state did not affect the Mg-ATPase activities (compare assay 1 with 5, and assay 3 with 7). (iii) In the presence of dephosphorylated twitchin (catch state), F-actin activated Mg-ATPase (compare assay 5 with 6). When twitchin was phosphorylated, this activation seemed

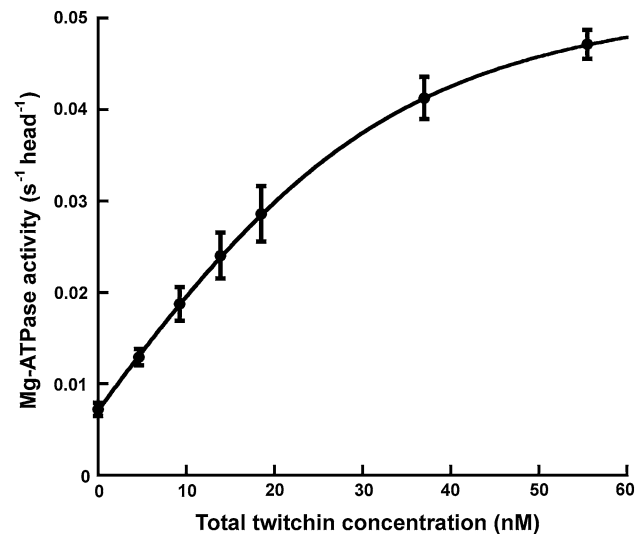
to disappear (assay 8). Though the results shown in Fig. 2 indicate activation by F-actin to some extent in the presence of phosphorylated twitchin (assay 8), this could be because of the incomplete phosphorylation of twitchin in the assay experiments. Indeed, light microscopic observations revealed that in some cases a few actin filaments bound to myosin filaments in the presence of phosphorylated twitchin (see Fig. 1, right). The extent of activation varied among measurements of the assay 8, and almost no activation was found in some measurement cases (data not shown).

We evaluated the full activities of both smooth muscle and obliquely striated muscle myosins at high free  $\text{Ca}^{2+}$  concentration in the presence of F-actin. They were roughly 1 and  $10 \text{ s}^{-1} \text{ head}^{-1}$ , respectively (Fig. S1). Thus, the activations by F-actin in the presence of dephosphorylated twitchin at low free  $\text{Ca}^{2+}$  concentration in the catch state (assay 6) was smaller by roughly two orders than their full activities.

Our finding that the myosin Mg-ATPase is slightly activated by F-actin in the presence of dephosphorylated twitchin at low free  $\text{Ca}^{2+}$  concentration (catch state) is remarkable. We measured Mg-ATPase activities in the presence of a varying amount of dephosphorylated twitchin under this catch condition using obliquely striated muscle preparations (Fig. 3). As total amount of added twitchin was increased, the activity increased and seemed to approach a saturated value. Though the molecular basis of the binding of actin to myosin filaments in catch is not yet clear, we assume the binding occurs by the ‘catch bridges’ between the filaments. Each catch bridge can be either a dephosphorylated twitchin molecule or a myosin molecule affected by a dephosphorylated twitchin bound to a myosin filament. Assuming that the activation of Mg-ATPase in catch is proportional to the number of the catch bridges or dephosphorylated twitchin molecules bound to myosin filaments, and assuming no cooperativity among twitchin molecules, the Mg-ATPase activity can be expressed by Eq. (2) (see ‘‘Materials and Methods’’). The equation was well fitted to the data and the best fit curve (shown in Fig. 3) was obtained with the following values  $\pm$  SE of constants:  $V_{\text{max},\text{Tw}} = 0.056 \pm 0.015 \text{ s}^{-1} \text{ head}^{-1}$ ,  $V_{0,\text{Tw}} = 0.0071 \pm 0.0017 \text{ s}^{-1} \text{ head}^{-1}$ ,  $M = 30 \pm 11 \text{ nM}$ , and  $K_D = 7 \pm 14 \text{ nM}$ . It should be noted that the myosin concentration (20 nM) or myosin head concentration (40 nM) was comparable to  $M$ .

#### $\text{Ca}^{2+}$ -dependence of the Mg-ATPase activities

Effects of free  $\text{Ca}^{2+}$  concentration on the myosin Mg-ATPase activity in the absence of twitchin, in the presence of dephosphorylated twitchin, and in the presence of phosphorylated twitchin were examined with obliquely

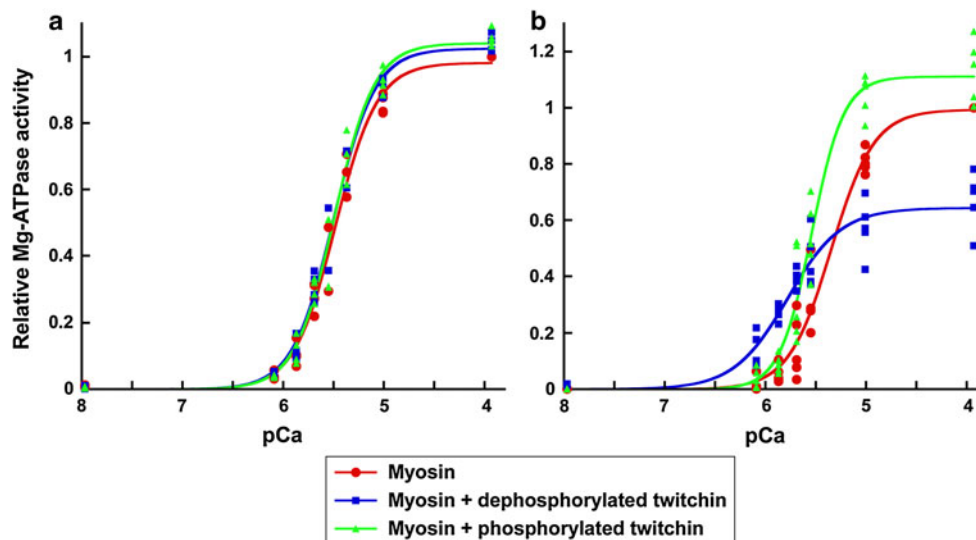


**Fig. 3** Steady-state Mg-ATPase activity of obliquely striated adductor muscle myosin filaments (20 nM double-headed myosin) in the presence of phalloidin-stabilized F-actin (2.4  $\mu\text{M}$ ) at low free  $\text{Ca}^{2+}$  concentration in the presence of various amounts of dephosphorylated twitchin prepared from obliquely striated adductor muscle. The curve the best fit with Eq. (2) and the bar at each point shows the SE of 10–12 measurements

striated muscle preparations (Fig. 4). The assays were performed either in the absence (Fig. 4a) or presence (Fig. 4b) of 2.4  $\mu\text{M}$  phalloidin-stabilized F-actin. The measured activities were normalized to those in the absence of twitchin at  $\text{pCa} = 3.9$ , which were  $0.37 \pm 0.05 \text{ s}^{-1} \text{ head}^{-1}$  (mean  $\pm$  SD,  $n = 8$ ) and  $3.34 \pm 0.95 \text{ s}^{-1} \text{ head}^{-1}$  ( $n = 10$ ) in the absence and presence of F-actin, respectively. Activation by  $\text{Ca}^{2+}$  was found and the activity ( $V$ ) can be approximated by the following Hill equation in all cases:

$$V = \frac{V_{\text{max},\text{Ca}}}{1 + 10^{n_H(\text{pCa} - \text{pCa}_{1/2})}} \quad (3)$$

where  $V_{\text{max},\text{Ca}}$ ,  $\text{pCa}_{1/2}$ , and  $n_H$  are the activity extrapolated to the infinite value of  $\text{Ca}^{2+}$  concentration, the value of  $\text{pCa}$  at which the activity is  $V_{\text{max},\text{Ca}}/2$ , and the Hill coefficient, respectively. Curves in Fig. 4 are the best fits with the equation, and Table 1 shows the values  $\pm$  SE of the constants. It should be noted that in the absence of F-actin neither phosphorylated nor dephosphorylated twitchin affected the Mg-ATPase activities at all  $\text{Ca}^{2+}$  concentrations (Fig. 4a; Table 1). The effects of twitchin and its phosphorylation state were complicated in the presence of F-actin. Dephosphorylated twitchin increased the activity at intermediate  $\text{Ca}^{2+}$  concentrations ( $\text{pCa} \approx 6$ ), whereas it decreased at relatively high free  $\text{Ca}^{2+}$  concentrations ( $\text{pCa} = 5 \sim 4$ ). In contrast, phosphorylated twitchin seemed to increase the activity to some extent at intermediate and high free  $\text{Ca}^{2+}$  concentrations.



**Fig. 4**  $\text{Ca}^{2+}$ -dependence of the Mg-ATPase activities of obliquely striated muscle myosin filaments (20 nM double-headed myosin) in the absence (**a**) and presence (**b**) of phalloidin-stabilized F-actin (2.4  $\mu\text{M}$ ). They were measured without twitchin (red), with dephosphorylated twitchin (blue), and with phosphorylated twitchin (green). The activities were normalized to the activity at  $\text{pCa} = 3.9$  in the

absence of twitchin, in either the absence (**a**) or presence (**b**) of F-actin, and curves the best fits with the Hill equation Eq. (3). Note that in the absence of F-actin neither phosphorylated nor dephosphorylated twitchin affected the Mg-ATPase activities at all  $\text{Ca}^{2+}$  concentrations

## Discussion

### Actin-activated Mg-ATPase under the catch state

Energy consumption of molluscan catch muscle is known to be kept low during the catch state. This knowledge is, however, based on studies with intact or permeabilized muscle fibers or cells, and effects of energy consumption other than the contractile system might have not been excluded in these studies. Therefore, the amount of energy consumed by the contractile system itself during the catch state has not been accurately measured. Our present *in vitro* study excluded these effects because energy consumption was measured with only purified contractile proteins essential for the formation of the catch state. In addition, some conditions that can hardly be controlled in muscle fiber experiments can be controlled in the *in vitro* experiments. For example, we can compare the differences in energy consumption between that in the absence and presence of F-actin (Figs. 2, 4). In addition to the catch state, active and relaxed states could be obtained by controlling the phosphorylation state of twitchin and free  $\text{Ca}^{2+}$  concentration of the assay buffer solution, and the states were confirmed by light microscopic observations (Fig. 1). In some cases, the measurements were continued for up to an hour (e.g., Fig. 2) to detect very low steady-state Mg-ATPase activity. These measurements were possible because of the absence of other ATPase activities that could have had a substantial influence if they had been present.

The most remarkable findings in our present study are that neither phosphorylated nor dephosphorylated twitchin significantly affects the myosin Mg-ATPase activity in the absence of F-actin and that the Mg-ATPase is slightly activated by F-actin in the presence of dephosphorylated twitchin at low free  $\text{Ca}^{2+}$  concentration (catch state). Steady-state ATPase activities of permeabilized catch muscle fibers were previously measured, but no significant difference was found between those in the catch and relaxed states (Butler et al. 1998). Subsequent results of single-turnover experiments with permeabilized muscle fibers suggested very slow ATPase activity in the catch state (Butler et al. 2001). The results could be evidence for the hypothesis that catch tension is maintained by slowly cycling myosin cross-bridges. The fact revealed in the present study that the steady-state Mg-ATPase was slightly but significantly activated by F-actin under the catch condition is consistent with the latter results, and strongly supports involvement of the myosin cross-bridges in the formation of the binding of thin filaments to thick filaments under the catch state.

Smooth muscles are more economical than striated muscles in the catch state

Generally, bivalve adductor muscles consist of two main parts—striated and smooth. The former shorten much faster than the latter. The slower smooth parts have been known as catch muscles and thought to be involved in keeping shells closed for a long time. In the previous work,

**Table 1** Values ± SE of the constants with which the best fit for the Hill equation Eq. (3) was obtained for the results shown in Fig. 4

	–F-actin			+F-actin		
	–Tw	deP-Tw	P-Tw	–Tw	deP-Tw	P-Tw
Relative $V_{max,Ca}$	0.98 ± 0.02	1.02 ± 0.02	1.04 ± 0.02	0.99 ± 0.03	0.64 ± 0.03	1.11 ± 0.03
pCa <sub>1/2</sub>	5.48 ± 0.02	5.50 ± 0.01	5.49 ± 0.02	5.35 ± 0.03	5.78 ± 0.04	5.54 ± 0.03
$n_H$	2.1 ± 0.1	2.1 ± 0.1	2.2 ± 0.2	2.0 ± 0.2	1.6 ± 0.3	2.7 ± 0.4

–Tw myosin (without twitchin), deP-Tw myosin + dephosphorylated twitchin, P-Tw myosin + phosphorylated twitchin

we demonstrated that myosin and twitchin of bivalve fast striated adductor muscles can form a complex with actin filaments similarly to those of smooth muscles under the catch condition. The amounts of twitchin relative to myosin are much lower in striated muscles than in smooth muscles (Tsutsui et al. 2007). The results of the cosedimentation experiments (Fig. S2) suggested that twitchin molecules can bind to every three myosin molecules in a saturating condition. The amounts of twitchin relative to myosin in living molluscan muscles seem lower. The molar ratio of twitchin to myosin in the anterior byssus retractor muscle of the mussel *Mytilus edulis* was estimated to be around 1/15 (Siegman et al. 1997, 1998). From our previous work (Tsutsui et al. 2007), those in the smooth and obliquely striated adductor muscles of the oyster *C. gigas* were estimated to be about 1/4 and 1/10, respectively. Thus, thick filaments do not seem saturated with twitchin even in smooth catch muscles. These facts mean that striated adductor muscles could maintain higher passive tension and keep the shells closed if they contained more twitchin. Bivalves, however, have evolved smooth parts in addition to the striated ones. Our present studies revealed that fast striated muscle myosin consumes much more energy than slow smooth muscle myosin, not only in the active contracting state (Fig. S1) but also in the catch state (Fig. 2). This means that catch muscles with slow myosin are more economical than those with fast myosin when they maintain passive tension, which could be why the amount of twitchin in fast striated parts is lower and slow smooth parts containing a higher amount of twitchin have evolved separately.

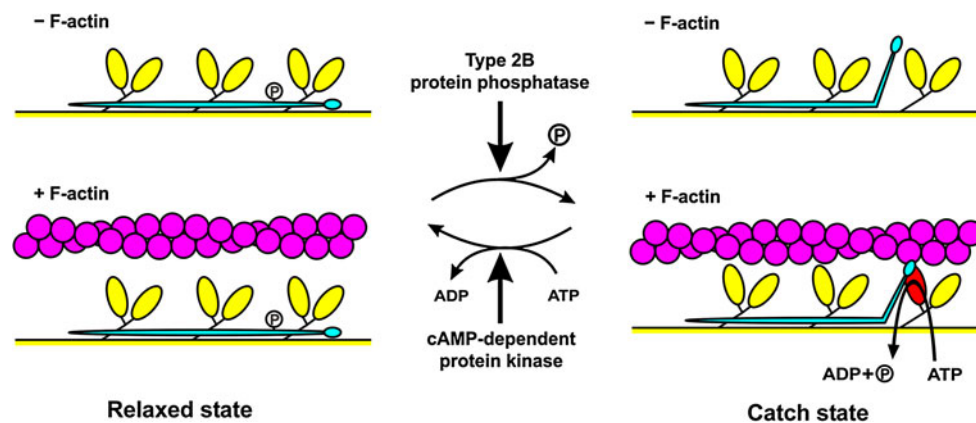
**Molecular mechanism of the regulation by twitchin**

Here, we propose a model of the molecular mechanism of regulation by twitchin (Fig. 5). Our previous work clarified that type 2B protein phosphatase dephosphorylates twitchin, producing the catch state, and cAMP-dependent protein kinase phosphorylates it, producing the relaxed state (Yamada et al. 2004). In the absence of F-actin, either dephosphorylated or phosphorylated twitchin did not affect the myosin Mg-ATPase activity at low free Ca<sup>2+</sup> concentration (Fig. 2). Therefore, it is reasonable to consider that

twitchin alone does not bind to the myosin catalytic motor domain directly irrespective of its phosphorylation states (Fig. 5, upper drawings). Otherwise, even if it binds as suggested elsewhere (Funabara et al. 2007, 2009; Butler et al. 2010), it does not affect on the Mg-ATPase cycle. This could be true even at higher free Ca<sup>2+</sup> concentrations because twitchin did not change the myosin Mg-ATPase activities at any free Ca<sup>2+</sup> concentrations (Fig. 4a).

Some research groups have suggested that twitchin has some affinity to F-actin in a phosphorylation-dependent manner (Shelud’ko et al. 2004; Funabara et al. 2007, 2009; Butler et al. 2010). Moreover, it was demonstrated that dephosphorylated twitchin has affinity for myosin as well and formation of the trimeric complex that can sustain high passive tension of the catch state was suggested (Funabara et al. 2007, 2009). Based on these findings and our present results, we propose a molecular mechanism of the catch as shown in Fig. 5. Since twitchin does not bind to F-actin when it is phosphorylated, F-actin does not bind to myosin filaments (Fig. 5, lower left), and therefore does not activate the myosin Mg-ATPase under the relaxed state. When twitchin is dephosphorylated, a part of the twitchin molecule binds to F-actin and the resulting complex has some affinity for the myosin motor domain forming the trimeric complex or the ‘catch bridge’ (Fig. 5, lower right). We assume that myosin ATPase is activated to some extent because of the interaction between F-actin and the myosin catalytic motor domain in this complex under the catch state (Fig. 2). It should be noted that the amount of binding sites for twitchin on myosin filaments estimated from the myosin Mg-ATPase under the catch state (Fig. 3) was close to the amount of myosin molecules whereas that estimated from the cosedimentation experiment was about 1/3 of myosin molecules (Fig. S2). This discrepancy could be explained by assuming that even twitchin molecules that do not bind to the backbone of myosin filaments could form the catch bridges in the presence of F-actin (Fig. S3).

Effects of twitchin and its phosphorylation state on the myosin Mg-ATPase activity in the presence of F-actin were complicated at higher free Ca<sup>2+</sup> concentrations (Fig. 4b). This may be because the F-actin concentration was 2.4 μM, just below the K<sub>1/2</sub> for actin (Fig. S1). Dephosphorylated twitchin could increase the affinity of



**Fig. 5** Schematic drawing showing the interactions between F-actin (purple), myosin (yellow), and twitchin (light blue) at low free  $\text{Ca}^{2+}$  concentration speculated from the present study. Twitchin molecules associate with the backbone of myosin filaments. *Type 2B protein phosphatase* dephosphorylates twitchin producing the catch state (right), and *cAMP-dependent protein kinase* phosphorylates it, producing the relaxed state (left). P phosphate. In the absence of F-actin (upper), either dephosphorylated or phosphorylated twitchin molecules do not affect the myosin catalytic motor domain. In the presence of F-actin, phosphorylated twitchin molecules do not bind to

F-actin for myosin increasing the Mg-ATPase activity at intermediate free  $\text{Ca}^{2+}$  concentrations. At higher free  $\text{Ca}^{2+}$  concentrations, however, the activity was less than that in the absence of twitchin. Some of myosin heads actively produce forces and actin filaments would move against myosin filaments in these conditions. The interaction between dephosphorylated twitchin and actin may act as an internal load for myosin motility to some extent, decreasing myosin Mg-ATPase activity. In contrast phosphorylated twitchin increased the Mg-ATPase activity at intermediate and high free  $\text{Ca}^{2+}$  concentrations. In this case, phosphorylation of twitchin might have been incomplete and some unphosphorylated twitchin might increase the affinity of F-actin to myosin. It is also possible that phosphorylated twitchin has the effect of strengthening interaction between F-actin and  $\text{Ca}^{2+}$ -activated myosin motor domains.

It should be mentioned that there are also many recent reports arguing that myosin heads might not be involved in the maintenance of the passive force in the catch state (Sugi et al. 1999; Mukou et al. 2004; Galler et al. 2005; Andruchova et al. 2005; Andruchov et al. 2006; Butler et al. 2006; Höpflinger et al. 2006). These arguments are apparently inconsistent with the present results and our model proposed here. The discrepancies should be clarified in future studies. It should also be mentioned that bivalve twitchin has some structural similarities to cardiac myosin binding protein C (Funabara et al. 2003). This protein seems to bind to both thick and thin filaments and regulate interactions between them in a phosphorylation-dependent manner (James and Robbins 2011; Ackermann and

either actin or the myosin catalytic motor domain, and thus, myosin Mg-ATPase is not activated by F-actin (lower left). When twitchin is dephosphorylated, a part of the twitchin molecule binds to F-actin and the resulting complex has some affinity for the myosin catalytic motor domain (red), forming a trimeric complex (lower right). This complex could be the catch bridge that can sustain high passive tension of the catch state. Because of the interaction between F-actin and the myosin catalytic motor domain in this complex, myosin Mg-ATPase is to some extent activated

Kontrogianni-Konstantopoulos 2011). Similarities and differences in functions of these regulatory proteins should also be clarified in future studies.

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