Protein Phosphatase 2B Dephosphorylates Twitchin, Initiating the Catch State of Invertebrate Smooth Muscle*

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“Catch” is the state where some invertebrate muscles sustain high tension for long periods at low ATP hydrolysis rates. Physiological studies using muscle fibers have not yet fully provided the details of the initiation process of the catch state. The process was extensively studied by using an in vitro reconstitution assay with several phosphatase inhibitors. Actin filaments bound to thick filaments pretreated with the soluble protein fraction of muscle homogenate and Ca²⁺ (catch treatment) in the presence of MgATP at a low free Ca²⁺ concentration (the catch state). Catch treatment with >50 μM okadaic acid, >1 μM microcystin LR, 1 μM cyclosporin A, 1 μM FK506, or 0.2 μM calcineurin autoinhibitory peptide fragment produced almost no binding of the actin filaments, indicating protein phosphatase 2B (PP2B) was involved. Use of bovine calcineurin (PP2B) and its activator calmodulin instead of the soluble protein fraction initiated the catch state, indicating that only PP2B and calmodulin in the soluble protein fraction are essential for the initiation process. The initiation was reproduced with purified actin, myosin, twitchin, PP2B, and calmodulin. ³²P Autoradiography showed that only twitchin was dephosphorylated during the catch treatment with either the soluble protein fraction or bovine calcineurin and calmodulin. These results indicate that PP2B directly dephosphorylates twitchin and initiates the catch state and that no other component is required for the initiation process of the catch state.

Invertebrate muscles such as the smooth adductor and byssus retractor muscles of bivalves maintain high tension with little energy expenditure after active contractions (1, 2). This high tension state, called the “catch state,” is terminated by a neurotransmitter, 5-hydroxytryptamine (3), activating cAMP-dependent protein kinase (PKA) through an increase in the intracellular concentration of cAMP. When a catalytic subunit of PKA was added to chemically demembranated catch muscles (7, 8), studies with the anterior byssus retractor muscle of the mussel suggested that its phosphorylation by PKA was involved in the relaxation of the catch state (9). The catalytic subunit of PKA phosphorylates 2–3 serine residues of the mussel catch muscle twitchin (10, 11).

In contrast to the relaxation of the catch state, the regulation of the reverse reaction, i.e. initiation of the catch state, has not been well characterized yet, since the reaction in vivo seems to occur in parallel with the activation of muscle contraction. Therefore, how initiation signals are relayed onward and amplified in cascades and which types of phosphatases dephosphorylate twitchin to initiate the catch state are still not clear. Castellani and Cohen (12) reported that skinned catch muscles, from which endogenous phosphatase was extensively washed out, restored their ability to maintain catch tension following activation of exogenous bovine calcineurin (12). Calcineurin is a Ca²⁺-calmodulin-dependent phosphatase (13) classified as a Ser/Thr protein phosphatase 2B (PP2B) (14). Thus, calcineurin-like phosphatases might be involved in the regulation of the catch, and they could dephosphorylate the target protein, i.e. twitchin. As Castellani and Cohen (12) describe in their report, however, “a more complicated scheme involving a cascade of kinases/phosphatases is, of course, also possible.” It has not yet been determined whether calcineurin-like phosphatase directly or indirectly dephosphorylates twitchin in muscles.

By using thick and thin filaments isolated from catch muscles of the mussel Mytilus galloprovincialis, our group devised a powerful assay based on direct visualization of the binding of thin filaments to thick filaments in vitro under conditions corresponding to the catch state (15). These in vitro experiments are much more useful than those using muscle fibers to identify components essential for the catch state and to reveal phosphorylation/dephosphorylation cascades relaying signals onward since the catch state can be reconstituted with purified proteins, and each interaction is separately analyzed without interference of other components. Indeed, our group showed that the myofilament components essential for the catch state are myosin, twitchin, and actin, whereas other components such as paramyosin, catchin (16), and tropomyosin are not (15).

By extending this in vitro reconstitution assay, we have now clarified the relationship between the extent of twitchin phosphorylation and the binding of actin filaments to thick filaments, and we have determined the type of phosphatase involved in the initiation of the catch state based on the degree of inhibition of its initiation by phosphatase inhibitors. In addition, purified twitchin is dephosphorylated by PP2B, and this dephosphorylation is coupled with the initiation of the catch state in vitro. With this study, together with the previous one (15), we have completely reconstituted the catch-relaxation

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¶ The abbreviations used are: PKA, cAMP-dependent protein kinase; PP2B, protein phosphatase 2B; DTT, dithiothreitol; PIPES, 1,4-piperazineethanesulfonic acid; TRITC, tetramethylrhodamine isothiocyanate.
cycle by using only the purified and characterized proteins myosin, twitchin, actin, PKA, PP2B, and calmodulin, and we completely clarified the regulation scheme of the catch contraction.

**EXPERIMENTAL PROCEDURES**

**Proteins**—The soluble protein fraction was prepared from the byssus retractor and posterior smooth adductor muscles of *M. galloprovincialis*. The muscles were homogenized in buffer A containing 80 mM NaCl, 2 mM MgCl₂, 0.5 mM EGTA, 2 mM dithiothreitol (DTT), and 20 mM PIPES-NaOH (pH 7.0). After centrifugation at 300,000 × g for 30 min, the supernatant was extensively dialyzed against buffer A, and the soluble protein fraction was obtained as the supernatant of the same centrifugation. Contaminated myosin and twitchin were removed as described elsewhere (15).

Synthetic thick filaments were prepared from the precipitate of the muscle homogenate after the extraction of the soluble protein fraction. The precipitate was extracted by using 0.4 M KCl, 4 mM MgCl₂, 4 mM ATP, 4 mM EGTA, 2 mM DTT, and 10 mM potassium phosphate buffer (pH 7.0). After centrifugation at 300,000 × g for 30 min, 5 volumes of cold water were added to the supernatant. The formed filaments were collected by the same centrifugation, and the precipitate was dissolved in the same solution. Synthetic thick filaments were obtained by dialysis of this solution against buffer A without stirring. They were treated to either the relaxed or catch state as follows.

To prepare synthetic thick filaments in the relaxed state, the filaments were first mixed with the soluble protein fraction (about 0.1 mg/ml) in the presence of 1 mM ATP and 10⁻⁵ M cAMP. After 1 h at room temperature (24 °C), the filaments were centrifuged at 9,000 × g for 20 min and suspended in buffer A. To prepare synthetic thick filaments in the catch state, the filaments and the soluble protein fraction were first mixed in the presence of about 10⁻⁵ M free Ca²⁺. After 1 h at room temperature, an excess amount of EGTA was added to reduce the free Ca²⁺ concentration to about 10⁻⁷ M, and the filaments were collected by centrifugation and suspended in buffer A.

Myosin and twitchin were purified from the same muscles as described elsewhere (15). Myosin filaments were again made by dialysis. To get twitchin in the catch state (the dephosphorylated state), it was first mixed with the soluble protein fraction (0.2 mg/ml protein) or 2 mM MgATP, 4 mM EGTA, 2 mM DTT, and 20 mM PIPES-NaOH (pH 7.0). After centrifugation at 300,000 × g for 30 min, 5 volumes of cold water were added to the supernatant. The formed filaments were collected by the same centrifugation, and the precipitate was dissolved in the same solution. Synthetic thick filaments were obtained by dialysis of this solution against buffer A without stirring. They were treated to either the relaxed or catch state as follows.

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The following "catch treatment" was used to convert thick filaments from the relaxed to catch state. They were mixed with either the soluble protein fraction of muscle (about 0.2 mg/ml protein) or 0.2 μM bovine calcineurin (Sigma, C-1907) and 2 μM bovine calmodulin (Sigma, P-2277) in the presence of 10⁻⁵ M free Ca²⁺. After 10 min at room temperature, an excess amount of EGTA was added to terminate the treatment. Conversely, the following "relaxation treatment" was used to convert thick filaments from the catch to relaxed state. They were mixed with either the soluble protein fraction (0.2 mg/ml protein) or 2 μM bovine PKA (Sigma, P-5511) in the presence of 10⁻⁵ M cAMP and 1–4 mM ATP. In autoradiography experiments, [γ⁻³²P]ATP was added in the relaxation treatment. The mixture was kept at room temperature for 10 min.

The inhibitors of kinase and phosphatase used in this study were purchased from the following: okadaic acid (Wako, 150-01653), microcystin LR (Wako, 136-12241), cyclosporin A (Alexis Biochemicals, 380-002-M100), FK506 (Calbiochem, 342500), calcineurin autoinhibitory peptide (Sigma, C-9397), and the peptide inhibitor of PKA (Sigma, P-0565).

**Gel Electrophoresis and Autoradiography—SDS-PAGE** was performed under the discontinuous buffer system (17). After the gel was dried, it was exposed to an x-ray film (Fuji, RX-U) with intensifying screens at ~80 °C for 2–4 days and developed.

**RESULTS**

**Reconstitution of Catch State Using Synthetic Thick Filaments**—In our previous study, the catch state was reconstituted by using native thick filaments, and nearly half of them did not bind to fluorescence-labeled actin filaments tightly in the catch state (15). This heterogeneity of the native thick filaments can lead to misinterpretation of the results of the in vitro catch assay, so we needed an assay system that would enable us to judge the state more clearly and easily.

We prepared synthetic thick filaments after high salt extraction of the precipitate of the muscle homogenate (see "Experimental Procedures" for details). They consisted of the main components of native thick filaments twitchin, myosin (a heavy chain and light chains), catchin (16), and paramyosin, with a small amount of actin (Fig. 4B, lane 1). They were observed as bundles of long filaments (see Fig. 1, for example) with a dark field light microscope. When these were pretreated with the soluble protein fraction of muscles and cAMP (i.e., relaxation treatment; see "Experimental Procedures" for details), they did not bind to fluorescence-labeled actin filaments in the presence of MgATP at a low free Ca²⁺ concentration (Fig. 1A, left panels, relaxed state). After treatment with the soluble protein fraction and Ca²⁺ (i.e., catch treatment), they bound to actin filaments (Fig. 1A, middle panels, catch state), and subsequent relaxation treatment caused detachment of actin filaments (Fig. 1A, right panels, relaxed state) under the same solution conditions.

These results are essentially the same as those for the native thick filaments (15), indicating that the synthetic ones reproduced the catch and relaxed states of muscle. Furthermore, they did not show the heterogeneity found in the native thick filaments; all bundles bound to many actin filaments in the

![Fig. 1. Initiation and termination of the catch state of the synthetic thick filaments using the soluble protein fraction.](http://www.jbc.org/Downloaded from/July 25, 2018)
catch state and bound to almost none in the relaxed state. The difference between the catch and relaxed states was thus more evident with the synthetic thick filaments (as indicated in a comparison of Fig. 1A with Fig. 2 of Ref. 15). Therefore, we used synthetic thick filaments for our in vitro studies of the catch state.

Effects of Phosphatase Inhibitors on Initiation of Catch State—At the termination of the catch state in vivo, the intracellular cAMP concentration increases and PKA is activated (4). Recent studies with skinned muscle fibers (9) and in vitro reconstitution systems (15) suggest that phosphorylation of twitchin controls the catch state. These studies also suggest that twitchin is dephosphorylated by protein phosphatases during the initiation of the catch state. In our in vitro assay system, the initiation of the catch state occurred during the catch treatment (15), so dephosphorylation of twitchin should have occurred during the catch treatment.

To identify which types of phosphatases work during the initiation of the catch state using the in vitro assay system, we studied the effects of several types of phosphatase inhibitors during catch treatment on the binding of actin filaments to synthetic thick filaments. Some phosphatase inhibitors, such as okadaic acid and microcystin LR, inhibit different types of phosphatases at particular concentrations. The concentrations (K$\text{v}$ values) of okadaic acid where phosphatase activity is inhibited are half and 150 nM for protein phosphatase 1, 0.032 nM for protein phosphatase 2A, and 5 μM for PP2B (18). The K$\text{v}$ values of microcystin LR are 0.05 nM for protein phosphatase 1, 0.008 nM for protein phosphatase 2A, and 200 nM for PP2B (19). These inhibitors do not inhibit protein phosphatase 2C. Thus, the concentrations of these inhibitors where the activity becomes about half would suggest the type of phosphatase involved.

When the catch treatment was performed with a smaller amount of soluble protein fraction, fewer actin filaments bound to the synthetic thick filaments (Fig. 1B). When the phosphatase activity was about 1⁄4, only a few actin filaments bound to synthetic thick filaments. The catch treatment was then performed in the presence of various concentrations of either okadaic acid (Fig. 2A) or microcystin LR (Fig. 2B). Only a few actin filaments bound when the catch treatment was performed in the presence of 50 μM okadaic acid or 1 μM microcystin LR. Thus, the K$\text{v}$ values for okadaic acid and microcystin LR were estimated to be about 20 and 0.5 μM, respectively. These results indicate that PP2B is most likely involved in the initiation of the catch state.

To confirm the involvement of PP2B, we studied the effects of specific inhibitors for PP2B. Cyclosporin A and FK506 bind to and specifically inhibit PP2B in the presence of immunophilins (20). Calcineurin autoinhibitory peptide is a specific inhibitor for PP2B (21). In our experiments, actin filaments did not bind to thick filaments after the catch treatment in the presence of 1 μM cyclosporin A, 1 μM FK506, or 0.2 mM calcineurin autoinhibitory peptide (Fig. 2C). These results indicate that immunophilins were present in the Mytilus catch muscles, and PP2B was involved in the initiation of the catch state. The fact that Ca$^{2+}$ is required for the initiation of the catch state (15) is consistent with this, since PP2B is a Ca$^{2+}$-dependent protein phosphatase (13). It should be noted that after the catch treatment without these phosphatase inhibitors, actin filaments bound to synthetic thick filaments even in the presence of these inhibitors (data not shown). The results indicate that these inhibitors did not inhibit the binding of actin filaments in the catch state but did inhibit the initiation of the catch state.

Although the above results indicate that PP2B is involved in the initiation of the catch state, it is not clear whether PP2B and its activator calmodulin (13) are the only components in the soluble protein fraction of muscles essential for the initiation of the catch state. We thus performed the catch treatment with bovine calcineurin and calmodulin instead of the soluble protein fraction. We found that actin filaments again bound to synthetic thick filaments. Further relaxation treatment with bovine PKA terminated the catch state (Fig. 3). Thus, PP2B and calmodulin are essential components in the soluble protein fraction for the initiation of the catch state, and PKA is essential for its termination. It should be noted that the initiation of the catch state by PP2B and calmodulin did not require ATP.

Protein Phosphorylation during Termination and Initiation of Catch State—To study protein phosphorylation during the termination and initiation of the catch state, we first prepared synthetic thick filaments in the catch state. The state was identified by observing the binding of actin filaments to the filaments in the presence of MgATP at a low free Ca$^{2+}$ concentration. Each observation consists of two images, as in Fig. 1, and the bar indicates 20 μm in all photographs.

![Fig. 2. Effects of phosphatase inhibitors on the initiation of the catch state.](image-url)
myosin light chains were not (Fig. 4B, lanes 2 and 3). Twitchin was the most phosphorylated in both experiments, which is consistent with the results for skinned catch muscle fibers (9). After the relaxation treatment in the presence of \( \gamma^{32}\text{P}\)ATP, catch treatment was performed. Note that \( \gamma^{32}\text{P}\)ATP still existed in the medium during this catch treatment. Following SDS-PAGE and autoradiography experiments revealed that twitchin was dephosphorylated, whereas the other proteins were not, and that the myosin light chains were not phosphorylated (Fig. 4B, lanes 4 and 5). These results indicate that dephosphorylation of twitchin is correlated to the initiation of the catch state and that the phosphorylation of other proteins such as myosin, catchin, and paramyosin are not involved in the regulation of the catch state.

**Effects of Kinase and Phosphatase Inhibitors on Phosphorylation**—We examined the effects of a specific inhibitor of PKA to confirm the type of kinase in the soluble protein fraction that is responsible for phosphorylation of twitchin. When a peptide inhibitor of PKA (22) was added during relaxation treatment, actin filaments did not bind to synthetic thick filaments (data not shown). Autoradiography experiments showed that in the presence of this inhibitor, twitchin was not phosphorylated (Fig. 5A), whereas myosin and catchin were to some extent. The phosphorylations of myosin and catchin were thus due to other types of kinases.

Dephosphorylation of twitchin during catch treatment was inhibited by 100 \( \mu \text{M} \) okadaic acid, 2 \( \mu \text{M} \) microcystin LR, 1 \( \mu \text{M} \) cyclosporin A, 1 \( \mu \text{M} \) FK506, or 0.2 \( \mu \text{M} \) calcinulin autoinhibitory peptide (Fig. 5B). Because the catch treatment with these inhibitors failed to initiate the catch state in the *in vitro* reconstitution assays (see above), the results are consistent with the conclusion that dephosphorylation of twitchin initiates the catch state. It should be noted that twitchin was doublet bands in the SDS gels (Fig. 4). The band of higher mobility seemed to be a proteolytic product (see below). Even in its presence, synthetic thick filaments showed both the catch and relaxed states, as mentioned above.

**Reconstitution of Catch State and Protein Phosphorylation with Purified Myosin and Twitchin**—As reported previously (15), actin filaments bind to thick filaments of purified myosin and twitchin in the catch state. We performed autoradiography experiments with these purified proteins to exclude possible effects of other components that the synthetic thick filament preparation contains. Twitchin was first treated with the soluble protein fraction and Ca\(^2+\) and was retrieved using Mono S cation-exchange chromatography (see "Experimental Procedures"). This treatment putatively dephosphorylated twitchin. When myosin filaments were mixed with this twitchin, they bound fluorescent actin filaments in the presence of MgATP at a low free Ca\(^2+\) concentration (Fig. 6A, panel 1). After relaxation treatment with the soluble protein fraction, only a few actin filaments bound to the thick filaments (Fig. 6A, panel 2). Further catch treatment with the soluble protein fraction restored the binding of actin filaments to thick filaments (Fig. 6A, panel 4). Bovine PKA was substituted for the soluble protein fraction in the relaxation treatment (Fig. 6A, panel 3). Further catch treatment with bovine calcineurin and calmodulin resulted in binding of actin filaments to the thick filaments (Fig. 6A, panel 5).

Autoradiography experiments under the same conditions, but \( \gamma^{32}\text{P}\)ATP was added during the relaxation treatment, showed that twitchin was phosphorylated during the relaxation treatment and dephosphorylated during the catch treatment (Fig. 6B, *Myosin + twitchin*). These results were the same as those obtained with synthetic thick filaments containing...
paramyosin and catchin (see above). Such phosphorylation and dephosphorylation of twitchin was also observed without myosin (Fig. 6B, Twitchin). Overexposure of the SDS gel to the film (Fig. 6B, bottom panel) showed that the myosin heavy chain either with or without twitchin was slightly phosphorylated by the soluble protein fraction. However, no phosphorylation of the myosin heavy chain was found with treatments with the bovine enzymes. This confirms that the phosphorylation of the myosin heavy chain was due to kinases other than PKA and that this phosphorylation was not involved in the regulation of the catch state. It should be noted that purified twitchin was doublet bands and that the band of higher mobility in the SDS gel became thicker after the catch treatment with the soluble protein fraction (Fig. 6B, lane 4). The proteolysis was apparently because of Ca^{2+}-dependent proteases in the soluble fraction. Such proteolysis was not found in the catch treatment with bovine calcineurin and calmodulin (Fig. 6B, lane 5). The same experiments were performed using myosin filaments alone (Fig. 6B, "Myosin"). The in vitro reconstitution assays revealed that they bound to no actin filaments even after the catch treatment (15). The myosin heavy chain was slightly phosphorylated by treatment with the soluble protein fraction (Fig. 6B, lanes 2 and 4) but was not phosphorylated with the bovine enzymes (lanes 3 and 5). Myosin light chains were not phosphorylated at all in any of the steps (data not shown).

**DISCUSSION**

**Reconstitution Assay of Catch State with Synthetic Thick Filaments**—In this study we used synthetic thick filaments for reconstituting the catch state instead of native thick filaments, nearly half of which did not bind to actin filaments even in the catch state (15). This previous observation might have been

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**Fig. 5. Effects of kinase and phosphatase inhibitors on the phosphorylation of twitchin.** A, relaxation treatment of the synthetic thick filaments (lane 1) with the soluble protein fraction and [γ-32P]ATP was performed (lane 2), and phosphorylated proteins were analyzed. When the same treatment was performed in the presence of the peptide inhibitor of PKA either with (lane 3) or without (lane 4) the soluble protein fraction, twitchin was not phosphorylated. B, the synthetic thick filaments in the catch state (lane 1) were first treated to be in the relaxed state with the soluble fraction and [γ-32P]ATP (lane 2). Then the catch treatment with the soluble fraction was performed either without (lane 3) or with various inhibitors of protein phosphatases (lanes 4–8). Dephosphorylation of twitchin was inhibited by 100 μM okadaic acid (lane 4), 2 μM microcystin LR (lane 5), 1 μM cyclosporin A (lane 6), 1 μM FR506 (lane 7), or 0.2 mM calcineurin autoinhibitory peptide (lane 8). Tw, twitchin; MHC, myosin heavy chain; Cat, catchin; PM, paramyosin; Ac, actin; CBB, Coomassie Brilliant Blue.

**Fig. 6. Protein phosphorylation during the termination and initiation of the catch state of purified protein filaments.** A, assays were performed as in Fig. 4A using thick filaments of purified myosin and twitchin instead of the synthetic thick filaments. Each panel consists of two images, as in Fig. 4A, and the bar indicates 20 μm in all photographs. B, [γ-32P]ATP was added in the relaxation treatment, and the phosphorylated proteins present in each step (corresponding to A, panels 1–5) were analyzed by SDS-PAGE and autoradiography (Twitchin + Myosin). Either purified twitchin (Twitchin) or purified myosin filaments (Myosin) was also treated and analyzed in the same manner. The gel (upper) was exposed to a film either for 2 (center) or 4 days (lower). Tw, twitchin; MHC, myosin heavy chain. Note that twitchin was phosphorylated by the relaxation treatment (lanes 2 and 3) and dephosphorylated by the following catch treatment (lanes 4 and 5). Although the myosin heavy chain was slightly phosphorylated by the treatment with the soluble fraction, it was not phosphorylated at all with the bovine enzymes. CBB, Coomassie Brilliant Blue.
because of the heterogeneity of thick filaments or cells in muscle. Because the synthetic thick filaments were made by dialysis of the homogeneous solution of muscle proteins, they were also considered to be homogeneous. However, there might be other reasons for the apparent heterogeneity of the native ones. First, in the preparation of the native thick filaments, it was difficult to remove thin filaments completely from the muscle homogenate, and any remaining thin filaments without fluorescence might have occupied some part of the surface of the native thick filaments in the catch state. The present synthetic thick filaments contained only a small amount of actin (Fig. 4B), so more actin filaments with fluorescence could bind to the synthetic thick filaments than did to the native filaments.

Second, it is well known that native thick filaments of catch muscles contain a much larger amount of paramyosin than myosin in their cores (23, 24). In contrast, the present synthetic thick filaments contained approximately equal amounts of paramyosin and myosin (Fig. 4B), and as we showed, paramyosin is not involved in the catch state. The density of myosin molecules in the synthetic thick filaments should be higher than in native ones, so the synthetic ones should have greater ability to bind to actin filaments in the catch state. In addition, they tended to form bundles, and these bundles were easier to observe with a dark field light microscope. These factors indicate that reconstitution assay of the catch state with synthetic thick filaments is more sensitive than that with native ones. Future examination of possible catch-like properties in other types of muscles should thus use synthetic thick filaments rather than native ones.

**PP2B Dephosphorylates Twitchin in Catch Muscles**—Castellani and Cohen (12) reported that although chemically skinned catch muscles lose their calcineurin-like phosphatase and their ability to maintain catch tension with extensive washing, the ability is restored by adding bovine brain calcineurin. They concluded that calcineurin-like phosphatase is required for catch contraction. Because the catch state is terminated by PKA (4), its reverse reaction, dephosphorylation, is required for the initiation of the catch state. Experiments with skinned muscles suggested that phosphorylation of twitchin is correlated with the relaxation of the catch state (9) and that purified twitchin is phosphorylated by PKA (10, 11). These results indicate that the target of the PKA is twitchin, the phosphorylation of which terminates the catch state of molluscan muscles.

As for the reverse reaction, calcineurin-like phosphatase PP2B can apparently dephosphorylate twitchin in a $Ca^{2+}$-dependent manner. However, no direct evidence has been presented that twitchin is the direct target for PP2B. A more complicated phosphatase cascade such as shown in vertebrate skeletal muscles was also possible.

In vertebrate skeletal muscles, the activity of protein phosphatase 1 is regulated by a protein termed “inhibitor 1.” This protein is phosphorylated by PKA and dephosphorylated by PP2B, and its phosphorylated form inhibits protein phosphatase 1. Thus, PP2B dephosphorylates phosphoinhibitor 1 activating protein phosphatase 1, which dephosphorylates other phosphoproteins such as phosphorylase $a$ (25). In the present study, we found that low concentrations of okadaic acid or microcystin LR, enough to inhibit protein phosphatase 1, did not affect the initiation of the catch state (Fig. 2, A and B). This indicates that the phosphatase cascade of PP2B and protein phosphatase 1, as found in vertebrate skeletal muscles (25), does not initiate the catch state.

We also found that inhibitors specific to PP2B such as cyclosporin A, FK506, and calcineurin autoinhibitory peptide inhibited dephosphorylation of twitchin and initiation of the catch state. This indicates that phosphatases other than PP2B do not dephosphorylate twitchin, initiating the catch state. In addition, we succeeded in initiating the catch state with myosin, twitchin, actin, bovine brain calcineurin (PP2B), and its activator calmodulin. In this step, twitchin was dephosphorylated, and this dephosphorylation occurred even without myosin. These results indicate that twitchin is the direct target of PP2B and that its dephosphorylation initiates the catch state.

**Phosphorylation-Dephosphorylation Cycle Caused by PKA and PP2B**—Our *in vitro* studies have clarified not only the protein components essential for the catch state and its regulation but also their relationships during the contraction-catch-relaxation cycle of the muscle. These components are actin, myosin, twitchin, PKA, PP2B, and calmodulin, and their relationships are summarized in Fig. 7. Twitchin is associated with myosin filaments (15). In the relaxed state, twitchin is phosphorylated, and actin filaments are dissociated from the myosin filaments. When the muscle is stimulated by acetylcholine, the intracellular free $Ca^{2+}$ concentration increases, and the $Ca^{2+}$ activates not only myosin but also the PP2B. Thus, the active PP2B directly dephosphorylates twitchin during active

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**Fig. 7. Schematic drawing showing the regulation of the catch state in molluscan catch muscle.** At a low free $Ca^{2+}$ concentration, muscle is either in the relaxed state (left) or the catch state (right). In the relaxed state, twitchin (light blue), which is associated with myosin (yellow) is phosphorylated, and the actin filament (purple) is detached from the myosin filament. Twitchin is dephosphorylated by the protein phosphatase 2B (PP2B) activated by $Ca^{2+}$ and calmodulin (CaM). When it is dephosphorylated, the actin filament tightly binds to the myosin filament at a low free $Ca^{2+}$ concentration (the catch state). Twitchin is phosphorylated by the protein kinase A (PKA) activated by cAMP, resulting in the dissociation of the actin filament from the myosin filament (the relaxed state). P indicates phosphate.
contraction of the muscle. It should be noted that the present study showed no other component is required for this dephosphorylation. Cessation of the stimulation results in a decrease in the intracellular free Ca\(^{2+}\) concentration, which terminates the motile activity of the myosin molecules. Because twitchin is in the dephosphorylated state at this time, actin filaments tightly bind to the myosin filaments (the catch state). Phosphorylation of twitchin by the active PKA causes dissociation of the actin filaments from the myosin filaments, resulting in the termination of the catch state (the relaxed state).

The two second messengers, cAMP and Ca\(^{2+}\), have the opposite effects in terms of the regulation of the catch state. If they simultaneously increase in catch muscle cells, both phosphorylation and dephosphorylation of twitchin are promoted, resulting in the waste of ATP consumption. In bovine heart, PKA phosphorylates and inhibits cyclic nucleotide phosphodiesterase, resulting in a decrease in the concentration of cAMP. When the intracellular Ca\(^{2+}\) concentration increases, PP2B dephosphorylates and activates the cyclic nucleotide phosphodiesterase, resulting in a decrease in the cAMP concentration (26). Although it is unclear whether such a system also works in catch muscles, it could be effective for precisely regulating the catch and preventing the waste of ATP consumption.

**Functions of Twitchin in Muscles—In vitro reconstitution experiments have revealed that twitchin is essential for the catch state (15). Twitchin, or mini-titin, is a member of the titin/connectin family present in nematodes (5, 6) and molluscs (7, 8, 27). The family also includes arthropod projectin and vertebrate titin/connectin. Their amino acid sequences show that they contain one protein kinase domain that has a primary sequence similar to that of vertebrate smooth muscle myosin light chain kinase near their C terminus (5, 6, 10, 11). Because of this similarity and because bacterially expressed *Aplysia* twitchin kinase domain phosphorylates myosin regulatory light chains (28), their physiological substrates are considered to be myosin regulatory light chains. In our *in vitro* reconstitution experiments, however, we found no incorporation of \(^{32}\)P into the myosin light chains during either the relaxation or the catch treatment (Fig. 4B). Thus, the kinase activity of twitchin, if it exists, is not related to the regulation of the catch state. The physiological role of its putative kinase activity remains to be clarified.

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Protein Phosphatase 2B Dephosphorylates Twitchin, Initiating the Catch State of Invertebrate Smooth Muscle

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