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Twitchin as a regulator of catch contraction in molluscan smooth muscle

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Abstract

Molluscan catch muscle can maintain tension for a long time with little energy consumption. This unique phenomenon is regulated by phosphorylation and dephosphorylation of twitchin, a member of the titin/connectin family. The catch state is induced by a decrease of intracellular Ca^{2+} after the active contraction and is terminated by the phosphorylation of twitchin by the cAMP-dependent protein kinase (PKA). Twitchin, from the well-known catch muscle, the anterior byssus retractor muscle (ABRM) of the mollusc *Mytilus*, incorporates three phosphates into two major sites D1 and D2, and some minor sites. Dephosphorylation is required for re-entering the catch state. Myosin, actin and twitchin are essential players in the mechanism responsible for catch during which force is maintained while myosin cross-bridge cycling is very slow. Dephosphorylation of twitchin allows it to bind to F-actin, whereas phosphorylation decreases the affinity of the two proteins. Twitchin has been also shown to be a thick filament-binding protein. These findings raise the possibility that twitchin regulates the myosin cross-bridge cycle and force output by interacting with both actin and myosin resulting in a structure that connects thick and thin filaments in a phosphorylation-dependent manner.

Introduction

Molluscan smooth muscles such as bivalve adductor and mussel anterior byssus retractor muscles exhibit a unique contraction referred to as 'catch', which uses little energy during maintenance of tension. Since the discovery of catch almost a century ago (reviewed in Bayliss, 1927), many studies have been performed to investigate the mechanism responsible for tension maintenance.

The schematic representation of the catch contraction is shown in Fig. 1. Molluscan catch muscle is controlled by cholinergic and serotonergic nerves. The stimulation of the former brings about an increase of the intracellular Ca^{2+} concentration, initiating phasic contraction, and the subsequent decrease of Ca^{2+} shifts it to the catch state. Catch tension is maintained until serotonin is released causing an accumulation of cAMP in the muscle cell. It has been thought that a cAMP-dependent protein kinase (PKA) activated by cAMP phosphorylates a myofibrillar protein which is the key component in regulation of catch (Cole and Twarog, 1972; Achazi *et al.*, 1974).

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Components of molluscan muscle seem similar to those of vertebrate muscle, but the structure of the thick filament is extremely different. Paramyosin is a dimer of a 100 kDa subunit, and it makes up the core of thick filament which is wrapped by a monolayer of myosin (Kendrick-Jones *et al.*, 1969; Szent-Györgyi *et al.*, 1971; Epstein *et al.*, 1975; Cohen, 1982). Paramyosin is widely distributed in all invertebrate muscles. Its content varies depending on species and tissues, and correlates with the tension which the muscle can develop (Lowy *et al.*, 1964). Paramyosin is most abundant in catch muscles (Winkelman, 1976). Therefore, it has been suggested that paramyosin plays a role in the catch contraction (Watabe and Hartshorne, 1990). Paramyosin is thought to be necessary for the thick filament to bear very high tension (up to 15 kg/cm²; Millman, 1964), but the function of paramyosin is not yet clearly identified. Another unique protein is myosin rod-like protein called myorod (Shelud'ko *et al.*, 1999), of which an homologous protein exists in the insect flight muscle (Standiford *et al.*, 1997). The inert protein, which shares the rod region with myosin heavy chain, is expressed by alternative splicing from the myosin heavy chain gene (Yamada *et al.*, 2000). Yamada *et al.* 2000 named the protein 'catchin' because they thought that it might be a regulator of the catch contraction, but as is discussed later, this does not seem to be the case. The name myorod, which emphasizes the relationship to myosin thus seems to be more appropriate. Myorod has unique N-terminal sequences, depending on species (Yamada *et al.*, 2000). At present, its function in the muscle cell is unknown. Molluscan myosin is quite similar to vertebrate myosin, except for the regulatory system. Molluscan myosin is activated by direct binding of Ca²⁺ to myosin (Kendrick-Jones *et al.*, 1970; Szent-Györgyi and Chantler, 1994). Unbinding of Ca²⁺ from myosin inactivates the molecule. Thus, molluscan catch muscle has a myosin-linked regulatory system somewhat similar to vertebrate smooth muscle, but differs from vertebrate skeletal muscle, which employs the actin-linked regulatory system.

It is difficult to explain the mechanism of catch contraction using only the contractile proteins myosin and actin, and it has been thought that the most likely third player was the protein phosphorylated by PKA when the muscle relaxes from catch. The target protein of PKA had been explored for a long time. Myosin heavy chain (Castellani and Cohen, 1987), myosin light chain (Sohma *et al.*, 1985, 1988a, Sohma *et al.*, b), paramyosin (Achazi, 1979a,b; Watabe *et al.*, 1989) and myorod (Yamada *et al.*, 2000) were all reported to be candidates for the regulator of catch because they are phosphorylated by PKA *in vitro*. However, these proteins were rejected because their phosphorylation state did not change when catch tension was released in skinned muscles.

Phosphorylation of twitchin associated with the release of catch

A study using ABRM skinned fibers, ³²P-ATP and caged ³²P-ATP with a photoflash technique, has identified the protein phosphorylated by PKA when catch is released (Siegman *et al.*, 1997). The protein of ~600 kDa specifically incorporated phosphate, whereas the phosphorylation level of other ABRM components, myosin heavy chain, myosin light chain, paramyosin and myorod remained unchanged. A PKA inhibitor completely inhibited this phosphorylation as well as the relaxation of catch tension. Partial protein and cDNA sequencing illustrated that the phosphorylated protein is *twitchin*, a member of the titin/connectin family, well-known as a myosin-binding protein (Siegman *et al.*, 1998). In ABRM skinned fibers, twitchin is irreversibly thiophosphorylated in the presence of cAMP and ATPγS. This causes the muscle to lose the ability to enter the catch state, but has no effect on force output in a high calcium-containing solution. These results showed that twitchin is the regulator of the catch state.

Twitchin was first reported as the *unc-22* gene product of *Caenorhabditis elegans* (Moreman *et al.*, 1988; Benian *et al.*, 1989), and mutation of it caused abnormal twitching movements (Waterston *et al.*, 1980). The collapse of the sarcomere structure of body wall muscle in the

unc-22 mutant raises the possibility that twitchin functions as does titin/connectin in vertebrate skeletal muscle, namely, a ruler of thick filaments. However, it is unlikely that twitchin in catch muscle plays a role equivalent to titin/connectin in skeletal muscle because molluscan smooth muscle has no distinct sarcomere structure. The discovery of the new function of twitchin as the regulator of catch has facilitated elucidation of the regulatory system of the unique energy-saving contraction.

Structure of *Mytilus* twitchin

The full sequence of *Mytilus* twitchin was determined by cDNA cloning with 3' and 5' RACE methods (Funabara *et al.*, 2003). *Mytilus* twitchin is composed of a single polypeptide of 530 kDa consisting of 4736 amino acids, which is a substantially smaller than that of *C. elegans* (~754 kDa) (Benian *et al.*, 1989, 1993). *Mytilus* twitchin has 24 immunoglobulin (Ig) and 15 fibronectin type 3 (Fn) motifs as well as one kinase domain, making it a member of the titin/connectin family. The alignments of the Ig and Fn motifs are very similar to those of other invertebrate twitchin related proteins, *C. elegans* twitchin and Crayfish projectin (Oshino *et al.*, 2003) (Fig. 2).

The proteins of the titin/connectin family usually have a PEVK domain whose length depends on species and/or tissue source (Labeit and Kolmerer, 1995). The PEVK domain is a unique region rich in the amino acid residues P, E, V and K, and it is thought to contribute to muscle elasticity. *Mytilus* twitchin has a short PEVK domain in the sequence between the sixth and seventh Ig domain, composed of only 79 amino acids, and the content of PEVK is lower than those of other vertebrate proteins (Funabara *et al.*, 2003). The function of the short PEVK in *Mytilus* twitchin is unknown.

Phosphorylation of twitchin by PKA

Twitchin purified from *Mytilus* ABRM is a good *in vitro* substrate for the catalytic subunit of PKA (Funabara *et al.*, 2001a). Phosphorylation occurs very rapidly, and 3 mol phosphate are incorporated into 1 mol twitchin within a few minutes. This indicates that there are at least three phosphorylation sites in the twitchin molecule. The rapid phosphorylation of twitchin corresponds to the rapid release of catch caused by the addition of cAMP *in vivo*. So far, two of the phosphorylation sites, referred to as D1 and D2, have been identified (Funabara *et al.*, 2003). D1 and D2 exist in the linker regions connecting the 7th and 8th Ig motifs from the N-terminus and the 3rd and 4th Ig motifs from the C-terminus (Fig. 2). D1 and D2 contain the typical recognition sequence for PKA, RRXS. Three additional RRXS sites are located in the region N-terminal to the D1 site raising the possibility that additional phosphorylation occurs in the same linker region as D1.

The expressed protein including the 7th and 8th Ig domains was phosphorylated *in vitro* even though the D1 serine was replaced with an alanine residue (D. Funabara, unpublished observation). Two-dimensional phosphopeptide mapping of tryptic digests of the phosphorylated twitchin purified from ABRM showed several small spots in addition to major ones representing D1 and D2. *In vivo* experiments using skinned ABRM muscles and phosphorylation dependent antibodies for the D1 and D2 sites showed that relaxation of catch occurs only when both sites are phosphorylated. The endogenous PKA that phosphorylates twitchin has not been identified, but it is known that a soluble fraction of the ABRM homogenate phosphorylates twitchin *in vitro*.

Kinase activity of twitchin

Twitchin and its related proteins have near the C-terminus a kinase domain that is highly homologous to the catalytic domain of myosin light chain kinase. This suggests that twitchin should act as a kinase *in vivo*. Indeed, it has been reported that native twitchin, its kinase domain

and projectin (the arthropod equivalent of twitchin) phosphorylate myosin light chains and are capable of autophosphorylation *in vitro* (Maroto *et al.*, 1992; Heierhorst *et al.*, 1994, 1995; Lei *et al.*, 1994; Weitkamp *et al.*, 1998). It has been reported that twitchin is activated by binding of Ca^{2+} /S100 to the kinase domain (Heierhorst *et al.*, 1996). On the other hand, in twitchin from molluscan catch muscle, phosphorylation assays showed no autophosphorylation (Funabara *et al.*, 2001a). Furthermore, SDS-PAGE and autoradiography of proteins from permeabilized ABRM treated with cAMP showed no incorporation of phosphate into myofibrillar proteins other than twitchin. Thus, twitchin does not seem to function as a kinase during relaxation of catch (Siegmán *et al.*, 1997). It is possible that the kinase activity of twitchin differs in catch and non-catch muscles, or that it phosphorylates a yet unknown substrate that plays a role other than regulation of catch.

Dephosphorylation of twitchin

Phosphorylated twitchin must be dephosphorylated before the muscle can enter the catch state. The phosphatase that acts on twitchin is active when the intracellular Ca^{2+} concentration is high during activation of the muscle (Funabara *et al.*, 2003). The most likely candidate for the phosphatase is calcineurin, also called protein phosphatase IIB, which is activated by Ca^{2+} /CaM (Castellani and Cohen, 1988; Yamada *et al.*, 2004). It is well-known that calcineurin dephosphorylates sites phosphorylated by PKA. In addition, in an *in vitro* system, it was shown that bovine calcineurin dephosphorylated twitchin and initiated the catch state (Yamada *et al.*, 2004).

Interaction of twitchin with myofibrillar proteins

There is some evidence concerning the interaction of twitchin with other myofibrillar proteins. Since the identification of twitchin in *C. elegans*, it has been thought that it associates with myosin because the loss of twitchin causes alteration of the thick filaments. Recently, the interaction between *Mytilus* twitchin and myosin was shown by a co-sedimentation assay *in vitro* (Yamada *et al.*, 2001). However, how twitchin binds to myosin is unknown. Our study on the nucleotide sequence of *Mytilus* twitchin raised the possibility that twitchin binds to myosin in the same ways as does myosin binding protein C (MyBP-C) in striated muscle, because the twitchin structure including the phosphorylation site D1 shows some resemblance to C-protein (Funabara *et al.*, 2003). Furthermore, there is a common sequence for actin-binding near D1, suggesting that twitchin has the ability to interact with actin. Shelud'ko *et al.* 2004 recently showed that twitchin binds to F-actin in a phosphorylation-dependent manner. However, Yamada *et al.* 2001 concluded from co-sedimentation assays that twitchin does not bind to actin. Our preliminary studies showed that *Mytilus* twitchin has the ability to bind to actin in a phosphorylation-independent manner (D. Funabara, unpublished). The data thus suggest that twitchin may bind to both myosin and actin, and may, in doing so, regulate the actin-myosin cross-bridge cycle in a phosphorylation dependent manner.

As discussed above, the most abundant protein in catch muscle is paramyosin. Projectin, a counterpart of twitchin in arthropods, affects paramyosin organization in synthetic filaments *in vitro* (Kolsch *et al.*, 1995) and has a dramatic effect on formation of thick filament *in vitro* (Fährmann *et al.*, 2002). In catch muscle, purification of twitchin involves treatment with a high-salt buffer, which is usually used to extract the salt-soluble proteins such as myosin and paramyosin (Funabara *et al.*, 2001). This is consistent with the idea that twitchin is on the thick filament and interacts with myosin and/or paramyosin.

How is twitchin involved in catch contraction?

The mechanism of maintenance of the catch state is unknown. In an *in vitro* assembly system it has been shown that the essential components to form the catch state are myosin, actin and twitchin (Yamada *et al.*, 2001). Myosin crossbridge cycling in catch must be very slow since

the energy consumption is very low (Lowy *et al.*, 1964; Lowy and Poulsen, 1982). Molluscan actin-activated myosin ATPase is activated by direct binding of Ca^{2+} , and it has a very low ATPase in the absence of Ca^{2+} binding (see, Szent-Györgyi and Chantler, 1994). The low intracellular Ca^{2+} concentration during catch is undoubtedly responsible for the low ATPase in catch, but the tension maintenance in the catch state means that the interaction between myosin and actin persists despite the low Ca^{2+} . Phosphorylation of twitchin removes the interaction between actin and myosin at low Ca^{2+} and allows relaxation of force. Since twitchin interacts with actin in a phosphorylation-dependent manner, it is possible that binding of dephosphorylated twitchin to actin would strengthen the cross-bridge interaction, leading to the catch state. The details of how phosphorylation of twitchin modifies the actin-myosin interaction to give rise to relaxation of force at low Ca^{2+} concentration remain to be elucidated.

'Latch' in vertebrate smooth muscle

Vertebrate smooth muscles are capable of exhibiting catch-like contraction named 'latch' (Dillon *et al.*, 1981), which is induced when the intracellular Ca^{2+} concentration decreases after an active contraction, as in the case of molluscan catch muscle (Remboldt and Murphy, 1986). It is assumed that during tension maintenance the cross-bridge interaction remains or cycles very slowly. Two proteins have been proposed as regulators of latch contraction so far. Caldesmon well-known as a thin-filament binding protein, first isolated as a Ca^{2+} /calmodulin binding protein (Sobue *et al.*, 1981), is thought to regulate vertebrate smooth muscle contractility by binding to thin filament. It has been shown that the protein has effects on the latch state of skinned vertebrate smooth muscles (Katsuyama *et al.*, 1992; Albrecht *et al.*, 1997). Calponin, also known as a thin-filament binding protein, may be another regulator of the latch contraction (Szymanski, 2004). Calponin inhibits actomyosin ATPase (Winder and Walsh, 1990) and the sliding of actin filament in *in vitro* motility assay (Shirinsky *et al.*, 1992; Haeberle, 1994). Thus in vertebrate smooth muscles latch is possibly regulated by the thin-filament associated proteins. Is there the same regulatory system in molluscan catch muscle? Interestingly, calponin also expresses in molluscan catch muscle and inhibits actomyosin ATPase *in vitro* (Funabara *et al.*, 2001b). However there is no available information whether molluscan calponin is involved in the catch contraction at present. Further studies are needed to find if there is a common mechanism between the catch and latch contractions.

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Fig. 1.

Relationship between tension, intracellular Ca^{2+} concentration and phosphorylation of twitchin in catch muscles. Solid and dotted lines represent muscle tension and intracellular Ca^{2+} concentration, respectively. In the relaxed muscle twitchin is phosphorylated. Contraction is initiated by release of acetylcholine which induces an increase in intracellular Ca^{2+} and muscle contraction. Dephosphorylation of twitchin by a Ca^{2+} -dependent phosphatase occurs while intracellular Ca^{2+} is high. When Ca^{2+} concentration falls, catch force is maintained and energy consumption is very low. The catch state is terminated by release of serotonin which causes an increase of intracellular cAMP and a cAMP-dependent protein kinase-mediated phosphorylation of twitchin.

Fig. 2.
Schematic representation of *Mytilus* twitchin and related proteins. The phosphorylation sites D1 and D2 in *Mytilus* twitchin are shown.