The highly efficient holding function of the mollusc ‘catch’ muscle is not based on decelerated myosin head cross-bridge cycles

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Certain smooth muscles are able to reduce energy consumption greatly when holding without shortening. For instance, this is the case with muscles surrounding blood vessels used for regulating blood flow and pressure. The phenomenon is most conspicuous in ‘catch’ muscles of molluscs, which have been used as models for investigating this important physiological property of smooth muscle. When the shells of mussels are held closed, the responsible muscles enter the highly energy-efficient state of catch. According to the traditional view, the state of catch is caused by the slowing down of the force-generating cycles of the molecular motors, the myosin heads. Here, we show that catch can still be induced and maintained when the myosin heads are prevented from generating force. This new evidence proves that the long-held explanation of the state of catch being due to the slowing down of force producing myosin head cycles is not valid and that the highly economic holding state is caused by the formation of a rigid network of inter-myofilament connections based on passive molecular structures.

Keywords: mollusc catch muscles; catch state; smooth muscle; energy economy during holding; muscle relaxation; titin-like proteins

1. INTRODUCTION

The phenomenon of catch has been mostly investigated using the anterior byssus retractor muscle (ABRM) of edible mussels of the genus *Mytilus* (for a review, see Rüegg 1971). When the ABRM is stimulated with the neurotransmitter acetylcholine, it contracts actively and after cessation of stimulation it relaxes only very slowly or even completely fails to relax (figure 1). This state of slowly decaying force in the absence of stimulation represents the catch state. During catch, the energy consumption is low (Güth *et al.* 1984; Butler *et al.* 1998) and the intracellular free Ca^{2+} concentration is near basal levels (Ishii *et al.* 1989). In this state, the muscle is particularly stiff. The muscle is resistant to stretches—that is, when large loads are applied, the muscle lengthens only a little and slowly. Furthermore, when the muscle is in catch, a quick release in muscle length is followed by only little redevelopment of force (for a review, see Rüegg 1971). This type of response is similar to that occurring when the muscle is in rigor and is dissimilar to that occurring when the muscle is actively contracting.

The state of catch depends on the phosphorylation state of twitchin (Siegmán *et al.* 1997; Yamada *et al.* 2001), a thick filament protein of 530 kD, which resembles the giant protein titin (approx. 3 MD) of vertebrate striated muscle (Funabara *et al.* 2005). Dephosphorylation of twitchin by a Ca^{2+} -dependent calcineurin-like phosphatase (Castellani & Cohen 1992)

induces the state of catch. Thus, catch appears to be initiated during active contraction when, following stimulation, the cytosolic free Ca^{2+} concentration increases, causing activation of the Ca^{2+} -dependent phosphatase and dephosphorylation of twitchin. The state of catch is not formed if twitchin is kept phosphorylated by high activity of protein kinase A (PKA) (phasic contraction). PKA is activated by cyclic adenosine monophosphate (cAMP) which rises following the release of serotonin from catch-inhibiting neurons (Twarog 1954; Achazi *et al.* 1974). Catch depends not only on the phosphorylation state of twitchin but also on the intracellular pH. Thus, moderate alkalization both terminates and prevents catch (Rüegg 1964; Höpfinger *et al.* 2006).

For more than 40 years, it was thought that catch is due to a slowing of the molecular motors, the myosin heads (‘traditional catch model’; Lowy *et al.* 1964). The myosin heads project laterally from the thick filaments as pairs and pull on neighbouring actin filaments in a cyclical manner. The energy for these force-generating pulling cycles is provided by the hydrolysis of Mg-ATP to Mg-ADP and inorganic phosphate occurring within the myosin heads. The pulling cycles are turned on when the cytosolic free Ca^{2+} concentration increases during muscle stimulation and turned off when the free Ca^{2+} concentration returns back to basal levels after cessation of stimulation. In mollusc smooth and striated muscles, this Ca^{2+} regulation occurs via direct binding of Ca^{2+} to the necks of myosin heads (Kendrick-Jones *et al.* 1970). When Ca^{2+} dissociates from its binding site, the myosin heads detach from the actin filaments or remain weakly attached to the actin filaments without

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Figure 1. Current model for the regulation of the catch state in intact and permeabilized preparations. (a) shows the time course of the force response in intact preparations when exposed to acetylcholine and serotonin, while (b) indicates the time course of the force response of permeabilized preparations exposed to high Ca^{2+} concentration (pCa 4.5 or 5) and cAMP. Twitchin dephosphorylation and formation of catch linkages are illustrated below the panels. Corresponding references and further details are given in the text.

producing force. For simplicity, these processes will be called 'myosin head detachment'. According to the traditional model, the rate of myosin head detachment is considerably decreased during the catch state (i.e. when twitchin is not phosphorylated) because it is assumed that the attached myosin heads with Ca^{2+} -free necks are unable to release ADP rapidly (Takahashi *et al.* 1988; Butler *et al.* 1998, 2001). The view that catch is based on a slowing of myosin heads was challenged soon after it was proposed. Thus, Regg (1964) observed that the catch state persisted in intact ABRM preparations after application of membrane-permeable inhibitors of myosin function, such as thiourea. Based on this result, it was concluded that the state of catch is not based on a slowing of myosin heads (Regg 1964). However, Lowy *et al.* (1964) argued that thiourea may not have completely inhibited the myosin heads in these experiments, and therefore the view of decreased myosin head detachment rates for explaining the state of catch became widely accepted, although not all scientists agreed with this assumption (cf. Elliott 1982; Sugi *et al.* 1999). Recent studies, in which specific myosin head inhibitors such as vanadate were used on permeabilized ABRM preparations, again showed that the catch state persisted when myosin was inhibited (Galler *et al.* 2005; Butler *et al.* 2006). However, in these experiments, the myosin head inhibitors were applied during the catch state and one could argue that, when a muscle is in catch, not all myosin heads can be blocked if, for example, they become less sensitive to inhibitors.

In the current study, a new approach was used to overcome this problem. With this new approach, the specific inhibitors were applied before muscle stimulation. The inability to develop force in the presence of inhibitors was used as evidence that myosin heads were completely blocked. In intact ABRM preparations, complete force

inhibition was obtained with the newly reported specific myosin head inhibitor blebbistatin (Kovacs *et al.* 2004). In permeabilized ABRM preparations, complete force inhibition was obtained with blebbistatin (0.1–0.4 mM) and/or vanadate (5–10 mM). To test if a state of catch was induced, we determined the relative stiffness as ratio of the measured forces. High stiffness that was sensitive to either serotonin in the intact ABRM preparation or cAMP in the permeabilized preparation was taken as an indicator for the catch state. When no force was generated in the presence of myosin head inhibitors, stimulation by acetylcholine or Ca^{2+} was followed by a marked increase in stiffness, which was sensitive to serotonin/cAMP in intact and permeabilized ABRM preparations, respectively. Therefore, it can be concluded that catch does not require force-generating myosin head cycles, and consequently the traditional explanation for the state of catch is finally disproved: catch is not caused by decelerated myosin head cycles but must rather be due to the formation of a rigid network of structures interconnected by passive elements.

2. MATERIAL AND METHODS

The methods and apparatus have been described previously (Galler *et al.* 2005; Andrucho *et al.* 2006). Briefly, ABRMs of fresh blue mussels (*M. galloprovincialis*), obtained from a local sea-food supplier, were isolated. Experiments with intact muscles were carried out in artificial sea water (ASW) containing 450 mM NaCl, 10 mM KCl, 10 mM CaCl_2 , 50 mM MgCl_2 and 10 mM Tris-HCl, pH 7.2 (Chick & Stephenson 1995). ABRM preparations with permeabilized cell membranes were obtained by incubating the preparations for 30 min in a relaxing solution containing 0.05 per cent (w/v) saponin or in a rigor solution containing 1 per cent (v/v) Triton-X100. Experiments with permeabilized ABRMs were carried out in solutions containing 3 mM ATP (added as $\text{Na}_2\text{H}_2\text{ATP}$), 3 mM free Mg^{2+} , 50 mM potassium-EGTA, 1 mM DTT, 30 mM MOPS and 40 μM leupeptin. The pH value of the solutions was 6.7 or 6.8 adjusted with KOH; solutions for providing 'moderate alkalization' had a pH of 7.4. In rigor solutions, ATP was substituted by EGTA. The relaxation and rigor solutions had a pCa value greater than 8 (with $\text{pCa} = -\log_{10} [\text{Ca}^{2+}]$), and the activating solutions had a pCa value of 4.5 or 5 by adding CaCO_3 . The chemicals were obtained from SIGMA-ALDRICH; blebbistatin was dissolved in DMSO (20 mM stock solution); vanadate and cAMP were dissolved in double distilled water for stock solutions with concentrations of 214 and 10 mM, respectively. Serotonin was dissolved in ASW (1 mM stock solution). Unless otherwise indicated, the experiments were carried out at room temperature (22–28°C).

Stiffness was determined on the same preparation by applying identical rectangular length changes (10 min intervals) and recording the change in force. When the force level in the same preparation differed between two conditions, the length of the preparation was either decreased (2–5%) or increased (1–2%) to bring the force to the same level for the two conditions, before stiffness was determined. This was necessary for creating similar conditions with respect to pre-stretch force when comparing stiffness values. The stretches were 1 min in duration and 4–8% in magnitude in experiments where the force in the preparation

was near zero, and 0.2 s in duration and about 0.3% in magnitude in experiments where there was a high level of force in the preparation, to avoid damage. The change in force was always measured at the end of the length step.

Results of stiffness are expressed relative to each other and given as mean $\pm$ SEM (n). For analysis, paired Student's t -tests were applied.

3. RESULTS

In the present study, the part of stiffness that was sensitive to serotonin (intact preparations) or cAMP and moderate alkalization (pH 7.4; permeabilized preparations) was taken as an indicator for catch. As mentioned in §1, a defining feature of catch is the high stiffness, which remains high even when the catch force completely disappears during the course of an experiment carried out under isometric conditions (for a review, see Rüegg 1971). The first notable feature of the experimental approach used in this study is that the function of the myosin heads was effectively blocked by the inhibitors. Thus, experiments using inhibitors were considered for evaluation only when stimulation by acetylcholine (intact preparations) or Ca^{2+} (permeabilized preparations) did not result in any measurable force development. The second notable feature of the strategy is that analyses were performed only on muscle preparations that were not in a state of partial catch at the beginning of an experiment. A state of partial catch was identified by a lower stiffness in the preparation following application of catch releasing factors (serotonin, cAMP or pH 7.4) at the end of an experiment compared with that at the beginning of the experiment.

(a) Experiments on intact muscle preparations

Figure 2a shows the length and force trace of a typical experiment with an untreated intact ABRM preparation. Stiffness was determined by applying rectangular length changes of constant amplitude when the force was near zero (to allow quantitative comparisons with measurements made when the preparations were relaxed or when myosin head inhibitors were used) and by measuring the amplitude of the force response owing to stretch at the end of the length step (before returning to the initial length). Changes in the relative amplitude of the stretch-induced force responses were used as a simple measure of changes in stiffness in a particular preparation. As shown in figure 2a, the stiffness was first determined before stimulation with acetylcholine, when the preparation was fully relaxed. Then, as expected, 0.2 mM acetylcholine induced force, and its subsequent removal was followed by only slow and incomplete relaxation (catch state). At the moment indicated by the arrow in figure 2a, when the preparation was in catch, the length of the preparation was reduced to bring the force close to zero and the stiffness was determined afterwards. The magnitude of the stretch-induced force response when the muscle was in catch was considerably greater than before stimulation with acetylcholine. After the application of 10 μM serotonin, the stiffness decreased to a value close to that determined before stimulation with acetylcholine, when the preparation was relaxed. The average relative stiffness during catch compared to that before stimulation with acetylcholine was 2.7 ± 0.4 -fold ($p < 0.01$) in eight

ABRM preparations. Subsequent addition of 10 μM serotonin reduced stiffness 2.7 ± 0.7 -fold ($n = 8$; $p < 0.01$). Figure 2b shows an example of serotonin reducing the stretch-dependent force response when serotonin was added at the time indicated by the arrow, indicating the serotonin-induced reduction of stiffness.

In intact ABRM preparations, blebbistatin (a compound that permeates membranes) was added before mounting the preparations to the apparatus to inhibit the myosin heads. Figure 2c shows the length and force trace of a typical experiment with an intact ABRM preparation pre-treated with 0.1 mM blebbistatin before stimulation with 0.2 mM acetylcholine. As expected, acetylcholine was not able to induce force, but stiffness increased considerably in its presence. The average increase was 2.5 ± 0.4 times ($n = 4$; $p < 0.05$). After acetylcholine was removed, stiffness remained high, indicating that the muscle was in a state of catch. Addition of 10 μM serotonin reduced stiffness by a factor of 2.5 ± 0.5 ($n = 3$; $p < 0.05$). Moreover, when serotonin was present during the application of acetylcholine (figure 2d), no increase in stiffness was observed (0.99 ± 0.03 ; $n = 7$; $p = 0.5$). Thus, serotonin both abolishes and prevents the acetylcholine-induced increase of stiffness as it both terminates and inhibits catch force in muscles with functioning myosin heads.

(b) Experiments on permeabilized muscle preparations

In permeabilized preparations, catch was induced by Ca^{2+} activation (pCa 5 or 4.5) and subsequent removal of Ca^{2+} (pCa > 8 ; figure 3). In the absence of myosin head inhibitors (figure 3a), the stiffness increased 2.2 ± 0.2 -fold ($n = 9$; $p < 0.01$) in the catch state compared with the control before Ca^{2+} activation. Moderate alkalization (pH 7.4) produced a 1.7 ± 0.1 -fold reduction ($n = 5$; $p < 0.01$). Addition of 0.1 mM cAMP produced a 1.3 ± 0.1 -fold reduction ($n = 4$; $p < 0.05$). The relatively smaller effect of cAMP can be explained by a partial loss of PKA from the permeabilized preparation during the experiment, which could prevent full phosphorylation of twitchin.

In permeabilized preparations, inhibitors were added at least 20 min before application of Ca^{2+} . Immediately after the inhibitor application, stiffness decreased by less than 5 per cent of the initial value becoming stable before the beginning of the experiment. Figure 3b shows typical stretch-induced force responses from permeabilized ABRM preparations treated with blebbistatin. The same type of experiment was also performed using the inhibitor vanadate in concentrations of 5–10 mM (Galler et al. 2005) or vanadate and blebbistatin together. Ca^{2+} application (pCa 5 or 4.5) was not able to induce force in the presence of the myosin head inhibitors, but stiffness increased 1.8 ± 0.3 -fold ($n = 6$; $p < 0.05$). After removal of Ca^{2+} , stiffness remained high. Addition of cAMP reduced stiffness 1.6 ± 0.2 -fold ($n = 4$; $p > 0.05$). Stiffness was also reduced (1.6 ± 0.1 -fold; $n = 2$; $p < 0.05$) when moderate alkalization (pH 7.4) was applied instead of cAMP administration. Exposure of the inhibitor-treated preparations to pCa 5 or 4.5, when cAMP was continuously present, did not significantly increase stiffness (0.96 ± 0.04 ; $p = 0.4$; $n = 9$).

Figure 2. Experiments for the determination of relative stiffness in intact ABRM preparations kept in artificial sea water (ASW). Constant, repetitive stretches were applied on each preparation and the resulting force response at the end of the stretch step was taken as a measure for relative stiffness. The upper traces in each panel indicate the change in the muscle length and the lower traces in each panel indicate the time course of the force response. Length and force traces shown in (a) and (b) are from control, untreated preparations, while those shown in (c) and (d) are from preparations treated with 400 μM of the myosin head inhibitor blebbistatin, which prevented generation of active force. The temperature was around 25°C in (a), (b) and (d), and 10°C in (c). For stimulation, the preparations were exposed to the excitatory neurotransmitter acetylcholine (ACh, 0.2 mM). As shown in (a), (c) and (d), ACh induced active force only in the untreated preparations. Wash-out of ACh induced a slow and incomplete relaxation in (a), indicating that a state of catch was induced. During this catch phase, the muscle was released in length to reach near-zero force (arrow in (a)) and stiffness was determined before and after the application of serotonin. A sustained increase in stiffness was observed in all intact preparations, independent of whether active force was generated (a) or not (c). The increase in stiffness was reversed (a,c) or prevented (d) by 10 μM serotonin. (b) shows responses from an untreated preparation in which 10 μM serotonin reduced the stretched-induced force response when applied during the time course of a prolonged stretch. Scale bars for length, tension and time given in (d) are also valid for (a)–(c).

In permeabilized ABRM preparations, it is possible to directly compare stiffness on the same preparation before and after inhibition of myosin heads with inhibitors. To achieve this comparison, the following experiment was carried out: the catch state was induced at pH 6.8 by

Figure 3. Experiments for the determination of relative stiffness in permeabilized ABRM preparations kept in artificial solutions mimicking the intracellular ionic milieu (pH 6.8 in (a) and pH 6.7 in (b)). The upper traces in (a) and (b) show the changes in muscle length and the lower traces show the force responses from a control, untreated preparation (a) and from a preparation treated with 100 μM blebbistatin that completely prevented the generation of active force (b). For stimulation, the preparations were exposed to elevated free Ca^{2+} concentration (pCa 4.5 or 5). This stimulation induced active force only in the untreated preparation (a). Removal of Ca^{2+} (pCa > 8) induced a slow and incomplete relaxation in (a), indicating that a state of catch was induced. During this catch phase, the muscle was released to bring the force to a near-zero value (arrow in (a)) and stiffness was measured before and after moderate alkalization (pH 7.4), which is a catch releasing factor. Ca^{2+} stimulation induced a sustained increase in stiffness in all permeabilized preparations, independent of whether active force was generated (a) or not (b). This increase in stiffness was reduced by moderate alkalization (a) or 0.1 mM cAMP (b). Scale bars for length, tension and time given in (b) are also valid for (a).

applying a pCa 5/pCa > 8 cycle and stiffness was determined (at high force). Thereafter, the preparation was relaxed at pH 7.4 in a relaxation solution. A further pCa 5/pCa > 8 cycle was repeated at pH 6.8 in the presence of 5 mM vanadate and 100 μM blebbistatin to induce a catch state again, this time with inhibited myosin heads. Stiffness was then determined at a force level similar to that observed during the initial catch state. This was achieved by slightly stretching the preparation. The stiffness determined in the catch state with blocked myosin heads was 0.88 ± 0.03 ($n = 4$, $p < 0.05$) of that determined in the catch state without myosin head blocking. The small difference is most likely due to slight deterioration of the preparation during the time course of the experiment (approx. 50 min). Thus, the results indicate that the catch state under conditions when the myosin heads are blocked and do not cycle is comparable with that of untreated preparations.

4. DISCUSSION

The present study provides an answer to the question whether the state of catch is due to a slowing down of the myosin head cycle. The evidence that a state of catch could still be induced in both intact and permeabilized preparations when the myosin heads were prevented from generating force (figures 2*c* and 3*b*) demonstrates that catch cannot be due to a deceleration of force-generating myosin head cycles.

The state of catch was monitored by an increase in stiffness that was sensitive to the catch releasing factors serotonin, cAMP and moderate alkalization. As shown in this study, stiffness remained high during catch even in the absence of force. This makes relative stiffness a better indicator for monitoring the state of catch than force, which has been used in most studies over the last 2–3 decades for this purpose. Furthermore, intact and ABRM preparations permeabilized with Triton-X100 (or saponin) for 30 min exhibited a similar increase in stiffness after stimulation by acetylcholine or Ca^{2+} , respectively. This shows unambiguously that permeabilized preparations enter into a state of catch after the removal of the activator Ca^{2+} . This state is not distinguishable from that following stimulation with acetylcholine of intact preparations, putting to rest previously expressed doubts regarding the occurrence of catch in permeabilized preparations. If so, then the enzymes involved in the regulation of the catch state—namely the Ca^{2+} -stimulated calcineurin-like phosphatase and the cAMP-dependent protein kinase—must remain (at least partially) within the permeabilized muscle cells for up to 1 h after exposure to the skinning agents.

This study also offers an answer to a long-standing question, highlighted in the review by Galler (2008): whether the linkage structures carrying catch force (catch linkages) are formed during the stimulation phase or only after the end of stimulation. As shown in figures 2*c* and 3*b*, a serotonin/cAMP-sensitive stiffness already appears during the stimulation phase (at least in a standby mode; Galler 2008) (acetylcholine/ Ca^{2+} in intact/permeabilized preparations, respectively) and remains high after the end of stimulation in the subsequent catch phase. This shows that catch linkages must evidently appear during the stimulation phase and remain formed during the subsequent catch phase where they are responsible for the maintenance of force and for the high stiffness. This finding directly validates the current view about the regulation of catch described in §1. Hitherto, this view was to a certain degree speculative, as it was based only on a number of (indirect) observations and conclusions (for a review, see Galler 2008). Thus, catch linkages (at least in a standby mode) are the immediate product of a dephosphorylation reaction executed by a calcineurin-like phosphatase that is activated by the Ca^{2+} increase during muscle stimulation. When Ca^{2+} returns to basal levels after the end of stimulation, catch linkages apparently remain stable as long as no cAMP-dependent phosphorylation occurs. Based on published data, catch appeared to occur only after an active contraction (see review by Galler 2008). However, this study has shown that a direct transition from the relaxed state to the catch state is possible without force activation, as long as the level of Ca^{2+} rises during stimulation to activate the calcineurin-like phosphatase

responsible for the dephosphorylation of twitchin. As catch linkages appear to be formed during the phase of active contraction, when the muscle is still able to shorten, catch linkages probably exhibit ratchet-like properties (Andruchov *et al.* 2006; Galler 2008), resisting stretch but not opposing shortening. Interestingly, ratchet-like protein interconnections were recently found in skeletal muscle, where connections between the proteins titin and telethonin in the Z-disc have been shown to resist very high forces in the ‘physiological working direction’, but only very low forces in other directions (Bertz *et al.* 2009).

The identity of structures responsible for the state of catch is a matter for speculation (Galler 2008). Catch could be due to passive linkages interconnecting thick filaments with actin filaments. Because of its regulatory role and large size, twitchin is a candidate for this interconnection (Shelud’ko *et al.* 2004; Funabara *et al.* 2007; Avrova *et al.* 2009; in a sense, also Yamada *et al.* 2001). Twitchin could either span from the thick filament backbone to the actin filaments (Butler *et al.* 2006; Shelud’ko *et al.* 2007) or it could strongly tie a fraction of myosin heads to the actin filaments (Funabara *et al.* 2007). Alternatively, twitchin binding to the myosin head could mechanically lock the myosin head in one direction (for opposing stretches) while keeping it freely mobile in the opposite direction (for force generation; Galler 2008). In the latter two cases, myosin heads would in fact be involved in the catch phenomenon, but only as severely transformed passive structures. As twitchin seems to be 15 times less abundant than myosin (Funabara *et al.* 2005), the number of twitchin-based catch linkages can only be small. Interconnections between longitudinally displaced thick filaments could further contribute to form a rigid myofilament network during catch (for reviews, see Rüegg 1971; Galler 2008). This hypothesis is supported by electron microscopic studies showing interconnections among thick filaments during catch (Heumann & Zebe 1968; Gilloteaux & Baguet 1977; Takahashi *et al.* 2003). As these results were sometimes challenged based on potential preparation artefacts (Bennett & Elliott 1989), further studies using new electron microscopic techniques would be helpful.

Taken together, observations presented in this paper and results obtained in previous studies indicate that the state of catch develops and persists despite blockage of myosin head cycling activity, implying that the catch state does not require force-generating myosin head cycles. Therefore, catch must be due to passive elements linking the myofilaments.

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