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The catch state of mollusc catch muscle is established during activation: experiments on skinned fibre preparations of the anterior byssus retractor muscle of *Mytilus edulis* L. using the myosin inhibitors orthovanadate and blebbistatin

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Summary

Catch is a holding state of muscle where tension is maintained passively for long time periods in the absence of stimulation. The catch state becomes obvious after termination of activation; however, it is possible that catch linkages are already established during activation. To investigate this, skinned fibre bundles of the anterior byssus retractor muscle of *Mytilus edulis* were maximally activated with Ca^{2+} and subsequently exposed to 10 mmol l^{-1} orthovanadate (V_i) or 5 μmol l^{-1} blebbistatin to inhibit the force-generating myosin head cross-bridges. Repetitive stretches of about 0.1% fibre bundle length were applied to measure stiffness. Inhibitor application depressed force substantially but never resulted in a full relaxation. The remaining force was further decreased by moderate alkalization (change of pH from 6.7 to 7.4) or by cAMP. Furthermore, the stiffness/force ratio was higher during exposure to V_i or blebbistatin than during partial Ca^{2+} activation producing the same submaximal force. The increased stiffness/force ratio was abolished by moderate alkalization or cAMP. Finally, the stretch-induced delayed force increase (stretch activation) disappeared, and the force recovery following a quick release of the fibre length, was substantially reduced when the force was depressed by V_i or blebbistatin. All these findings suggest that catch linkages are already established during maximal Ca^{2+} activation. They seem to exhibit ratchet properties because they allow shortening and resist stretches. In isometric experiments a force decrease is needed to stress the catch linkages in the high resistance direction so that they contribute to force.

Key words: catch muscle, mollusc smooth muscle, *Mytilus edulis*, skinned muscle preparations, myosin inhibitors, calcium activation.

Introduction

Certain bivalve mollusc smooth muscles have the ability to maintain force passively and resist stretch for long periods of time after cessation of stimulation. This phenomenon is called ‘catch state’ or, briefly, ‘catch’ (Jewell, 1959). Many of the studies on catch were carried out on the anterior byssus retractor muscle (ABRM) of *Mytilus edulis*. Catch is characterized by basal levels of both ATP consumption (Güth et al., 1984) and cytosolic free Ca^{2+} concentrations (Ishii et al., 1989). In experiments under isometric conditions, catch is indicated by a very slow force decrease after cessation of stimulation (Rüegg, 1965). Furthermore, a substantial force recovery after a quick release of the muscle length (active state) (Jewell, 1959) is absent. In intact ABRM preparations, catch force is relaxed by serotonin (5-hydroxytryptamin), which is released from synapses of specific neurons (Twarog, 1954). Serotonin induces an increase of intracellular cAMP (adenosine 3′5′-cyclic monophosphate) (Achazi et al., 1974). cAMP activates protein kinase A, which in turn phosphorylates twitchin (Siegman et al., 1998; Butler et al., 2001) resulting in termination of catch (Siegman et al., 1997; Siegman et al., 1998). Twitchin is a mini-titin (for a review, see Funabara et al., 2005) which is located on the thick filaments (Siegman et al., 1998).

In skinned ABRM preparations a catch-like state can be established by removal of Ca^{2+} after Ca^{2+}-induced activation at a moderately acidic pH (pH 6.5–6.8) (e.g. Rüegg, 1971; Siegman et al., 1998; Galler et al., 2005). At this stage, quick stretches induce a rigor-like force response whereas during Ca^{2+} activation the same length change is followed by a delayed force increase (stretch activation) (Gagelman et al., 1984). Similarly, in release experiments a substantial force recovery is present during Ca^{2+} activation but not after Ca^{2+} removal, indicating the absence of an active state. cAMP (Cornelius, 1982) or the catalytic subunit of protein kinase A (Pfitzer and Rüegg, 1982) leads to the termination of this catch-

like state due to phosphorylation of twitchin (Siegman et al., 1997). Catch is also terminated by a moderate alkalisation, e.g. when the pH is increased from 6.2 to 7.5 (Rüegg, 1964), from 6.5 to 7.7 (Rüegg, 1971) or from 6.7 to 7.4 (Galler et al., 2005). The termination of the catch state by moderate alkalisation seems to be independent of cAMP and protein kinase A (Höpflinger et al., 2006).

The molecular basis of catch is still unclear. Two different mechanisms have been proposed. The first explains catch in terms of myosin heads (cross-bridges) remaining sustainable attached to actin filaments (myosin head model) (Lowy et al., 1964). The very slow force decrease during catch is thought to be due to a very slow detachment of myosin heads. The second mechanism envisions the formation of link structures (interconnections), which are different from myosin head cross-bridges, between myofilaments [alternative linkage model (Rüegg, 1963; Rüegg, 1965)]. Recently, the alternative linkage model was supported by some physiological data (Galler et al., 1999; Sugi et al., 1999; Mukou et al., 2004; Galler et al., 2005; Andruchova et al., 2005; Höpflinger et al., 2006; Butler et al., 2006). However, the kind of linkages has not yet been identified.

The time point where catch is developed during an activation-relaxation cycle is not clear. The catch state becomes obvious after termination of activation; however, it is possible that catch linkages are established already during activation. Studies of intact ABRM preparations showed that a serotonin-sensitive stiffness is present not only in the catch state but also during active contraction (for a review, see Rüegg, 1971). Furthermore, in skinned ABRM preparations it was found that force is reduced by cAMP at submaximal Ca^{2+} activations (Butler et al., 1998; Siegman et al., 1998). The cAMP-sensitive force component decreases with increasing activation (Butler et al., 2006) and it is absent at maximal Ca^{2+} activation. The fraction of force that is sensitive to cAMP was thought to be catch force, since the application of cAMP was not associated with any significant change of ATPase activity (Butler et al., 1998). From these results it was concluded that catch linkages are already established during Ca^{2+} activation. Furthermore, the authors assumed that the number of catch linkages decreases with increasing activation and it reaches zero at maximal activation (Butler et al., 2006).

The reliability of these conclusions should be reflected in the context of the following considerations. (1) The force depression by cAMP at submaximal Ca^{2+} activation may not necessarily indicate the presence of catch, because, in principle, it could also be due to a change in the cross-bridge kinetics. In particular, a slowing of the myosin head attachment (rate constant, f) or an acceleration of the myosin head detachment (rate constant, g) would result in a force depression. This is a consequence of the two-state model of force generation (Huxley, 1957), where force is proportional to $f/(f+g)$. There are in fact some indications that cAMP slows myosin head attachment, which could be responsible for the observed force depression: cAMP significantly decelerates both the delayed force increase following a quick stretch (stretch activation) and the force rise following a stepwise increase of ATP concentration (caged-ATP) starting at low-force rigor (Andruchova et al., 2005). The assumption that cAMP in fact detaches catch linkages at submaximal Ca^{2+} activations was substantially corroborated by the finding of Butler et al. (Butler et al., 2006) that cAMP even depresses force after inhibition of the cycling myosin head cross-bridges. (2) The absence of a change in ATPase activity during cAMP-induced force depression at submaximal Ca^{2+} activations does not unequivocally indicate that the force change must be due to other factors than a change in cross-bridge kinetics: a decrease in force can be due to a drop of f and a simultaneous rise of g, which may not necessarily change the ATPase activity which is proportional to $gf/(f+g)$. (3) Furthermore, small differences in ATPase activity are difficult to detect, and thus, the significance of the available measurements should be carefully considered. A precise determination of ATPase activity is difficult in experiments where it is measured during the time course of Ca^{2+} activation before and after cAMP application because, as noted by Butler et al. (Butler et al., 1998) (their fig. 2), the ATPase activity is not constant during prolonged activation. Moreover, in such types of experiments the ATP cleavage due to the myosin head power strokes cannot be separated from the ATP cleavage due to the phosphorylation reaction (transfer of the γ-phosphate of ATP to the phosphorylation site) stimulated by cAMP. Precise measurements of ATPase activity are also difficult if preparations, which were thiophosphorylated before the measurement, are compared with control preparations [figs 4 and 5 of Butler et al. (Butler et al., 1998)], because the volume of the active muscle preparations is hard to investigate precisely. (4) The conclusion of Siegman et al. (Siegman et al., 1998) and Butler et al. (Butler et al., 2006) that catch linkages are established only at submaximal but not at maximal Ca^{2+} activation is questionable because Ca^{2+} removal leads to a catch state after both submaximal and maximal Ca^{2+} activation. Moreover, the time course of the force decay after Ca^{2+} removal is not apparently different after submaximal and maximal Ca^{2+} activation (e.g. Butler et al., 2001; Galler et al., 2005; Butler et al., 2006), and thus, it seems unlikely that at maximal Ca^{2+} activation the catch linkages are established *after* Ca^{2+} removal, whereas at submaximal activations the catch linkages are established *in* the presence of Ca^{2+}.

For all these reasons it seems important to further investigate whether, (1) catch linkages are already present during Ca^{2+} activation, and (2) if this applies only for submaximal activations but not for maximal activations. To answer these questions further methodological approaches are required. Therefore, in the present study we substantially extended the approach of Butler et al. (Butler et al., 2006) by using maximally Ca^{2+}-activated skinned ABRM preparations and by including rapid changes of the fibre length: as in the work of Butler et al. (Butler et al., 2006), we inhibited the active cycling cross-bridges by specific myosin inhibitors during Ca^{2+} activation. Orthovanadate (Herzig et al., 1981; Dantzig and Goldman, 1985; Chase et al., 1993; Jaworowski

et al., 1999; Galler et al., 2005) and blebbistatin (Kovacs et al., 2004; Butler et al., 2006) were chosen as myosin inhibitors. The force remaining after myosin inhibition was investigated by addressing the following questions. (1) Is the remaining force sensitive not only to cAMP but also to moderate alkalisation? (2) Does the remaining force show an increased stiffness/force ratio as it is known for the rigor or catch state (Pfitzer and Rüegg, 1982; Sugi et al., 1999; Höpflinger et al., 2006)? (3) Are rectangular length changes followed by rigor-like force responses, i.e. no delayed force increase following a stretch (stretch activation) or no substantial force recovery following a quick release (active state)? Our experimental results provide increasing evidence for the assumption that catch linkages are established already during Ca^{2+} activation. This seems to apply not only for submaximal but also for maximal Ca^{2+} activations. The implications of these findings for the understanding of the molecular mechanism of catch are discussed.

Materials and methods

Muscle preparations and skinning procedure

The preparation and skinning procedure of ABRM have been described previously (Galler et al., 2005). Briefly, ABRMs of fresh blue mussels (*Mytilus edulis* L.; shell length, 4–7 cm), obtained from a local sea-food supplier, were isolated. The dissected muscle was teased into several bundles about 0.4 mm wide in artificial seawater containing (in mmol l^{-1}): 490 NaCl, 8 KCl, 10 $CaCl_2$, 15 $MgCl_2$, 1 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes), pH 7.2. These bundles were exposed first to a sodium skinning solution and then to a potassium skinning solution both containing saponin (Cornelius, 1980; Castellani and Cohen, 1992; Chick and Stephenson, 1995; Galler et al., 2005). In addition to 0.05% (w/v) saponin, the sodium skinning solution contained (in mmol l^{-1}): 132 sodium propionate, 5 ethylene glycolbis(2-aminoethylether)-*N*,*N*,*N*,*N*-tetraacetic acid (EGTA), 5 Na_2H_2ATP, 7 $MgCl_2$, 10 3-morpholinopropanesulfonic acid (Mops), 2 dithioerythritol (DTE), 646 sucrose, pH adjusted to 6.9 with KOH. In the potassium skinning solution the sodium propionate was replaced by potassium propionate. The skinning procedure took about 30 min in total and was carried out at 20–24°C.

Solutions used for the mechanical experiments contained (in mmol l^{-1}): 150 sucrose, 5 EGTA, 5 Na_2H_2ATP, 5 disodium creatinphosphate, 3 free Mg^{2+}, 1 DTE, 1 NaN_3 and 30 i.u. ml^{-1} creatine phosphokinase. Solutions of pH 6.7 contained 20 mmol l^{-1} Mops and solutions of pH 7.4 contained 20 mmol l^{-1} Hepes. The ionic strength was adjusted to 0.20 mol l^{-1} with KCl, and the pH was adjusted to 6.7 or 7.4 using KOH. The pCa (=–log$[Ca^{2+}]_{free}$) was adjusted with $CaCl_2$ and measured using a calcium-sensitive electrode (Fluka 21188). The relaxation solution had a pCa of >8 and the activation solution had a pCa of 4.4. Submaximal activation solutions were prepared by mixing relaxation and activation solution in different proportions.

The solutions containing 10 mmol l^{-1} orthovanadate (Na_3VO_4; V_i) were prepared as described in our previous study (Galler et al., 2005). Briefly, a stock solution of 300 mmol l^{-1} V_i (pH 10) was prepared in double-distilled water and boiled until colourless. After adding the V_i, the pH of skinned fibre solutions was adjusted to either pH 6.7 or pH 7.4 with HCl. Blebbistatin was dissolved in DMSO at a concentration of 5 mmol l^{-1}. This stock solution was added to the activation solution to obtain a final blebbistatin concentration of 5 μmol l^{-1}. The final DMSO concentration was about 0.1% (v/v) and did not affect the contractile properties of the skinned ABRM muscle bundles (Andruchova et al., 2005).

Experimental setup and procedure

For mechanical experiments, skinned fibre bundles of 100–300 μm diameter and 2.5–4.9 mm length were used. The bundles were mounted horizontally between two vertically oriented pins of an isometric apparatus (compliance, 4 μm mN^{-1}). One pin was attached to a force sensor; the other pin was attached to a stepping motor. The apparatus and the methods for mechanical measurements have been described previously (Galler and Hilber, 1994). A force sensor, AE 801 (SensoNor, Horten, Norway) with a resonance frequency of ~7.5 kHz was used. Rapid changes (about 1–2 ms) in fibre length were achieved by a feedback-controlled stepping motor based on a Ling vibrator. A cuvette transporting system provided quick changes of solutions. Small repetitive rectangular stretches were continuously applied to measure stiffness. These stretches were about 0.1% of the skinned fibre bundle length (L_0) in amplitude and 20 ms in duration and the frequency was <0.5 s^{-1}. The stiffness is given by the ratio $\Delta F/\Delta L$, where ΔF represents the amplitude of the force spikes and ΔL the length change.

All mechanical experiments were carried out at 22.0–22.5°C. Results are expressed as means ± s.d.

Results

Ca^{2+} activation of skinned ABRM fibre bundles

Fig. 1A shows an original force recording of a saponin-skinned ABRM fibre bundle. Small repetitive rectangular stretches of constant amplitude (0.1% of L_0) and duration (20 ms) were applied to measure stiffness. The stiffness is proportional to the amplitude of a force spike. The preparation was subsequently exposed to solutions with increasing concentrations of free Ca^{2+}. An increase of Ca^{2+} was followed by an increase in force and stiffness. In order to find out if the force is maximal at pCa 4.4, four ABRM fibre bundles were exposed to pCa 5.51 and subsequently to pCa 4.4. The steady state force measured at pCa 5.51 was 96.7±1.8% of that at pCa 4.4, indicating saturating conditions. The stiffness/force diagram in Fig. 1B shows the increase of these two parameters with increasing Ca^{2+} activation in the experiment shown in Fig. 1A. The data points were obtained at the end of each individual Ca^{2+} activation. The insets in Fig. 1A shows force transients induced by stretches of about 0.2% L_0 and 5 s

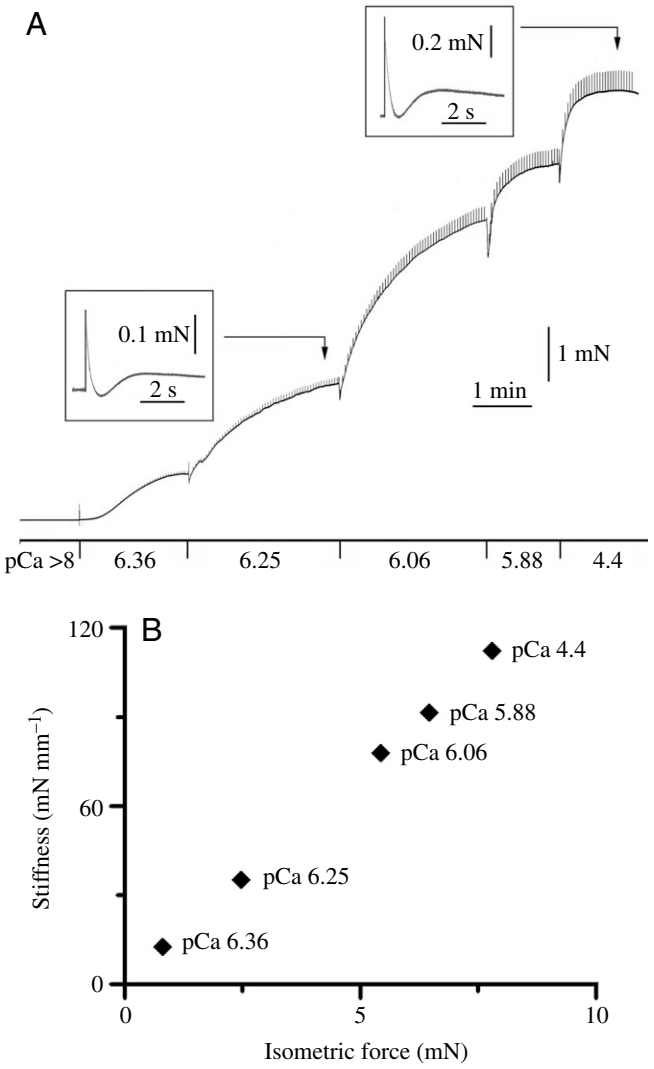

Fig. 1. Activation of skinned fibre bundles of anterior byssus retractor muscle (ABRM) with stepwise increase in Ca^{2+} concentrations. (A) Original recording of force and stiffness. The force spikes are induced by repetitive rectangular stretches of 20 ms duration and 0.1% L_0 in amplitude. The free Ca^{2+} concentration is expressed as pCa (= –log[Ca^{2+}]). The force recordings (insets) were obtained on another preparation; they show the force transients induced by rectangular stretches of about 5 s in duration and 0.2% L_0 in amplitude applied at different stages of the experiment. (B) Relationship between stiffness and force at different Ca^{2+} activations in the experiment shown in A. The data points were obtained at the end of each individual Ca^{2+} activation.

duration recorded at different stages of the experiment. The force transients were similar at both pCa 6.25 and pCa 4.4. In both cases the stretch resulted in an immediate rise of force followed by a decrease and a delayed increase (stretch activation).

Effect of orthovanadate on maximal Ca^{2+} activation

Fig. 2A shows the effect of the inhibitor orthovanadate on force and stiffness of a maximally Ca^{2+}-activated (pCa 4.4) skinned ABRM fibre bundle. The first and main part of the experiment was carried out under conditions which are favourable for the formation of the catch state: pH 6.7 and absence of cAMP. The exposure to pCa 4.4 induced an increase in force and stiffness to a plateau. The stiffness/force diagram in Fig. 2B (grey squares) shows the increase of these two parameters during the development of the activation. The relationship resembles that shown in Fig. 1B where the Ca^{2+} concentrations was increased in a stepwise manner, and data points were obtained at steady-state conditions. When the plateau of Ca^{2+} activation was reached, 10 mmol l^{-1} V_i were added. This resulted in a pronounced depression of force (values in Table 1). Stiffness was less decreased by V_i (open circles of Fig. 2B) than force, causing an increased stiffness/force ratio.

The force responses following rectangular length changes (insets in Fig. 2A) were substantially changed when force was depressed by V_i. The stretch-induced delayed force increase disappeared, and the overall force decay became smaller. Furthermore, the force recovery following a quick release of fibre length was substantially smaller after V_i inhibition. Theoretically, it is possible that these rigor-like force responses are simply due to the lower force during V_i exposition. However, Fig. 1 clearly shows that this is not the case. If a low force level is reached not by cross-bridge inhibition using V_i (or blebbistatin, see later) but by partial Ca^{2+} activation, a clear stretch-induced delayed force increase is present. Thus, a rigor-like response is present only if force is depressed by cross-bridge inhibitors.

In Fig. 2A the broken line represents a force trace of a V_i experiment carried out at pH 7.4. As shown by Höpflinger et al. (Höpflinger et al., 2006) the maximal force was similar at pH 7.4 and pH 6.7. However, in comparison with experiments at pH 6.7 (solid line in Fig. 2A), 10 mmol l^{-1} V_i depressed force significantly more at pH 7.4 (for values see Table 1). The plot in Fig. 2C shows the relationship between stiffness

Table 1. *Force depression by the cross-bridge inhibitors orthovanadate or blebbistatin depending on pH and cAMP*

Experimental background	Force depression (as a % of maximum) (*N*) Orthovanadate (10 mmol l^{-1})	Blebbistatin (5 μmol l^{-1})
pH 6.7	71.5±5.7 (15)	51.3±5.5 (7)
pH 7.4	90.2±2.7 (7)	79.1±2.8 (4)
pH 6.7 + cAMP*	91.2±2.6 (4)	81.3±2.3 (3)

The skinned anterior byssus retractor muscle (ABRM) fibre bundles were maximally Ca^{2+} activated at indicated pH, and orthovanadate (V_i) or blebbistatin were applied, resulting in a force depression (Fig. 2A, Fig. 3A). The depression was measured when the force decay reached a plateau after V_i application or 7–10 min after blebbistatin application. The values (means ± s.d.) represent the amount of force depression given as a percentage of the maximal value before cross-bridge inhibition.

*100 μmol l^{-1} cAMP and 2 μmol l^{-1} cyclosporine A.

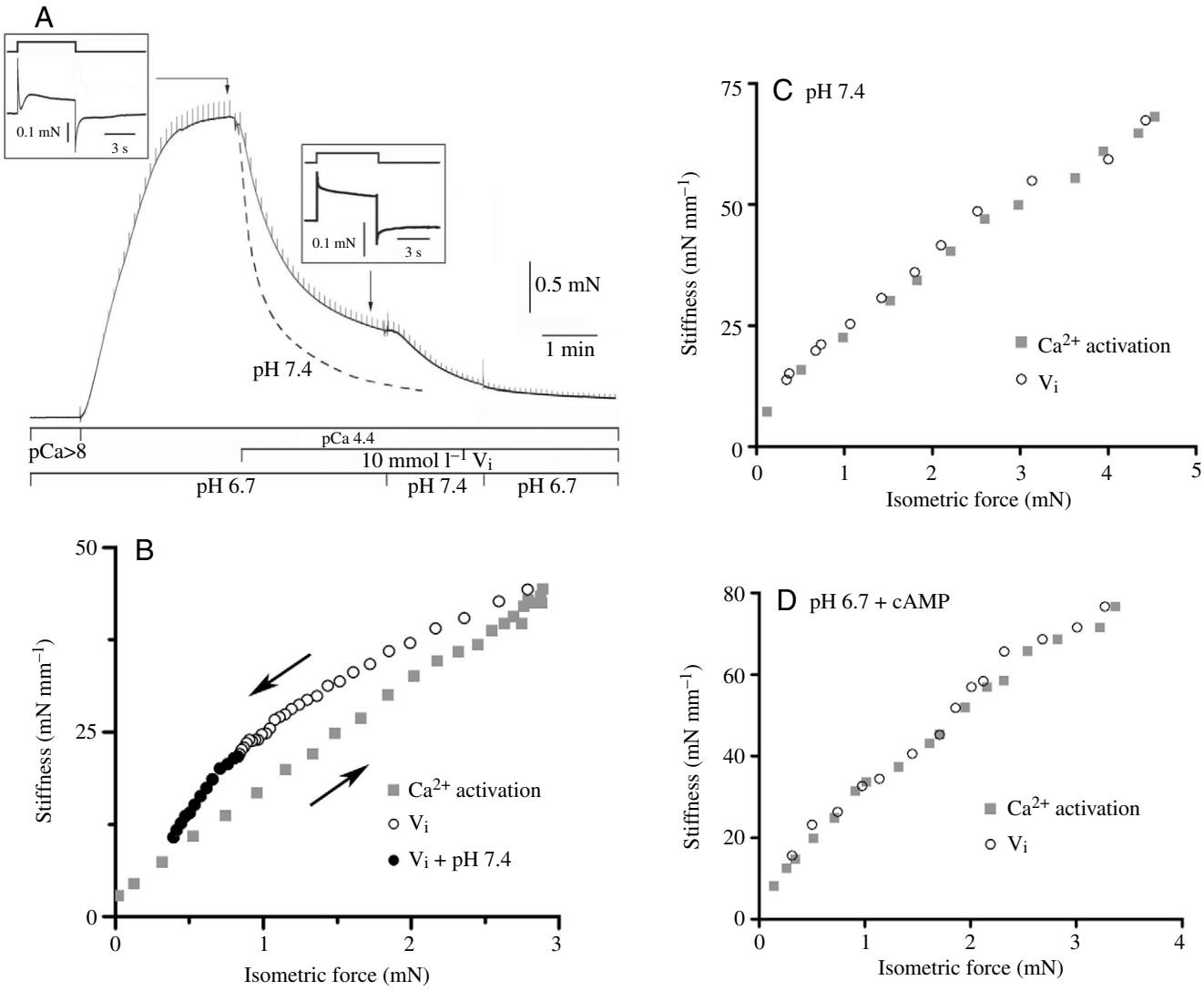

Fig. 2. Depression of force and stiffness of a maximally Ca^{2+}-activated skinned anterior byssus retractor muscle (ABRM) fibre bundle by 10 mmol l^{-1} orthovanadate (V_i) and subsequent further depression by moderate alkalization. (A) Original recording of force and stiffness during changes of solutions as indicated. The force spikes are induced by repetitive rectangular stretches of 20 ms in duration and 0.1% L_0 in amplitude. The broken line shows an experiment at pH 7.4. The insets show experiments on another ABRM fibre bundle; here force transients were induced by rectangular length changes of 0.2% L_0 at different stages of the experiment. (B) Diagram showing the stiffness/force relationship of the experiment of Fig. 2A. Grey squares, Ca^{2+} activation; open circles, V_i; solid circles, moderate alkalization (change from pH 6.7 to pH 7.4) in the presence of V_i. The arrows indicate the time course of the experiment. (C,D) Data from similar experiments carried out at pH 7.4 (C) or in the presence of 100 μmol l^{-1} cAMP and 2 μmol l^{-1} cyclosporine A (CsA; D). Grey squares, Ca^{2+} activation; open circles, V_i application.

and force during the time course of the experiment. In contrast to pH 6.7 (Fig. 2B), the stiffness/force relationship during force depression by V_i (open circles) does not deviate from that during the development of the Ca^{2+} activation (grey squares). Thus, the increase in the stiffness/force ratio present at pH 6.7 was not observed at pH 7.4. A larger force depression and no deviation of the stiffness/force relationship were also observed when 100 μmol l^{-1} cAMP and 2 μmol l^{-1} of the phosphatase 2B inhibitor cyclosporine A (CsA) were present during the whole experiment (Table 1 and stiffness/force plot of Fig. 2D).

Application of blebbistatin during the catch state

Orthovanadate, at concentrations of 1–10 mmol l^{-1}, has been shown to not affect the catch force (Galler et al., 2005). However, there was no information on the effect of the myosin inhibitor blebbistatin, and therefore, we examined the effect of blebbistatin on the catch state. The catch state was induced by Ca^{2+} removal after maximal Ca^{2+} activation. As shown in many other studies (e.g. Butler et al., 1998; Höpflinger et al., 2006), the Ca^{2+} removal results in an initial rapid relaxation which is followed by a much slower relaxation a few minutes later. This slow relaxation phase is thought to represent the

catch state. Application of blebbistatin (1–10 μmol l^{-1}, 2–10 min) during the slow phase of relaxation had no effect on force (N=4).

Effect of blebbistatin on maximal Ca^{2+} activation

Fig. 3A shows the effect of 5 μmol l^{-1} blebbistatin on force and stiffness of a maximally Ca^{2+}-activated (pCa 4.4) skinned ABRM fibre bundle. The experiment was carried out under conditions that are favourable for the formation of the catch state: pH 6.7 and absence of cAMP. Blebbistatin at the concentration of 5 μmol l^{-1} depressed force at a substantially slower rate than V_i at a concentration of 10 mmol l^{-1}. Likewise, removal of blebbistatin led to a much slower force recovery than removal of V_i (data not shown). The force levels (mean ± s.d.) measured at a time point when the force decay tended to reach a plateau (about 7–10 min after blebbistatin application) are given in Table 1. Like orthovanadate, blebbistatin also depressed force more than stiffness. The resulting increase in the stiffness/force ratio can be seen in the stiffness/force plot in Fig. 3B.

The stretch-induced delayed force increase (stretch activation) and the substantial force recovery following a quick release of fibre length which were present at maximal Ca^{2+} activation disappeared after force depression by blebbistatin (insets of Fig. 3A).

The broken line in Fig. 3A represents a force trace of a blebbistatin experiment carried out at pH 7.4. In comparison with experiments at pH 6.7 (solid line of Fig. 3A), 5 μmol l^{-1} blebbistatin depressed force substantially more at pH 7.4. The amount of force depression (mean ± s.d.) about 7–10 min after blebbistatin application is given in Table 1. It can be seen, that force decreased by a greater extent than at pH 6.7. The plot in

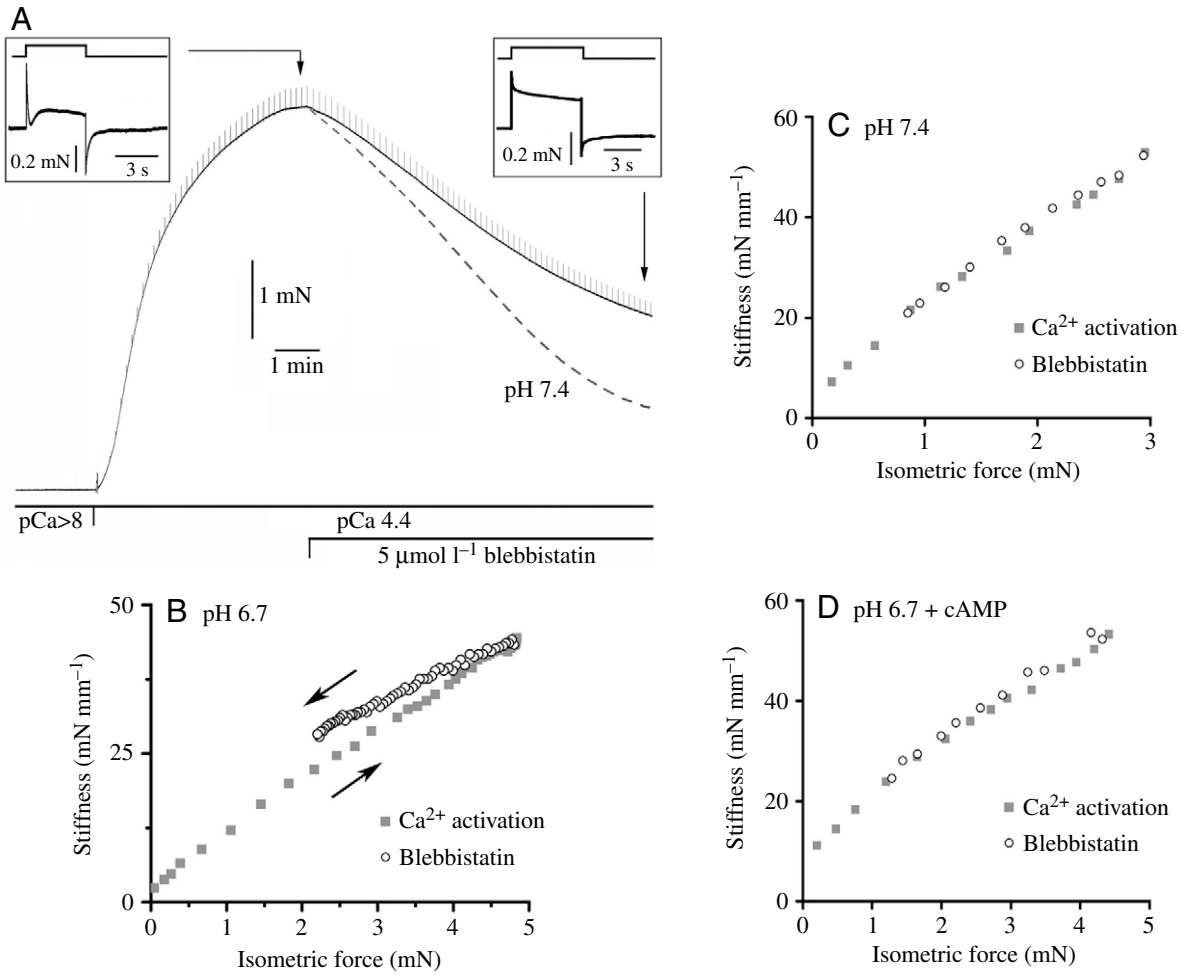

Fig. 3. Depression of force and stiffness of a maximally Ca^{2+}-activated skinned anterior byssus retractor muscle (ABRM) fibre bundle by 5 μmol l^{-1} blebbistatin. (A) Original recording of force and stiffness during changes of solutions as indicated. The force spikes are induced by repetitive rectangular stretches of 20 ms in duration and 0.1% L_0 in amplitude. The broken line shows an experiment at pH 7.4. The insets show experiments on another ABRM fibre bundle; here force transients were induced by rectangular length changes of 0.2% L_0 at different stages of the experiment. (B) Diagram showing the stiffness/force relationship of the experiment shown in A. Grey squares, Ca^{2+} activation; open circles, blebbistatin. (C,D) Data from similar experiments carried out at pH 7.4 (C) or in the presence of 100 μmol l^{-1} cAMP and 2 μmol l^{-1} cyclosporine A (CsA). Grey squares, Ca^{2+} activation; open circles, blebbistatin application.

Table 2. *Force depression by moderate alkalization (pH 7.4) or cAMP after cross-bridge inhibition by orthovanadate or blebbistatin during maximal Ca^{2+} activation*

	Force depression (as a % of maximum) (*N*)	
Influencing factor	Orthovanadate (10 mmol l^{-1})	Blebbistatin (5 μmol l^{-1})
pH 7.4	17.5±2.2 (3)	24.0±2.9 (2)
cAMP*	22.2±1.6 (3)	27.3 (1)

The skinned anterior byssus retractor muscle (ABRM) fibre bundles were maximally Ca^{2+} activated at pH 6.7 and force was subsequently depressed by 10 mmol l^{-1} orthovanadate or 5 μmol l^{-1} blebbistatin. A change to pH 7.4 or addition of cAMP resulted in a further depression of force (Fig. 2A). The amount of this depression is given as a percentage (mean ± s.d.) of the values before application of the inhibitors.

*100 μmol l^{-1} cAMP and 2 μmol l^{-1} cyclosporine A.

Fig. 3C shows the relationship between stiffness and force during the time course of a blebbistatin experiment carried out at pH 7.4. As in V_i experiments at pH 7.4, the stiffness/force relationship during force depression by blebbistatin (open circles) also does not deviate from that during the development of the Ca^{2+} activation (grey squares). Thus, the increase in the stiffness/force ratio present at pH 6.7 was not observed at pH 7.4. A larger force decrease and no deviation of the stiffness/force relationship was also observed in experiments where 100 μmol l^{-1} cAMP and 2 μmol l^{-1} CsA were continuously present (Table 1; Fig. 3D).

Effects of moderate alkalization or cAMP after cross-bridge inhibition by V_i or blebbistatin

In addition to the cross-bridge inhibition by V_i at maximal Ca^{2+} activation, Fig. 2A (second part) shows the effect of moderate alkalisation on depressed force. When the pH is changed from 6.7 to 7.4 in the presence of pCa 4.4 and 10 mmol l^{-1} V_i, the depressed force further decreased. As seen in Table 2, the total amount of force depression in these experiments was similar to that observed in experiments where pH 7.4 was continuously present during the whole experiment (see also broken line on the Fig. 2A). The pH change affected stiffness more than force, resulting in a reversal of the deviation of the stiffness/force relationship back to that during the development of the Ca^{2+} activation (solid circles of Fig. 2B). Thus, the increase of the stiffness/force ratio, which was observed during V_i inhibition was abolished by moderate alkalisation. The depression of force and stiffness by pH 7.4 was irreversible: a change of pH from 7.4 back to 6.7 did not result in a recovery of either force or stiffness (Fig. 2A). However, when the preparations were activated again with Ca^{2+} at pH 6.7, the catch state was re-established after removal of Ca^{2+} (data not shown).

Similar to the V_i experiments (Fig. 2A, second part), a change of pH from 6.7 to 7.4 after force depression by blebbistatin resulted in a further depression of force (Table 2) and in the abolition of the increased stiffness/force ratio. The total amount of force depression was similar to that observed in experiments conducted continuously at pH 7.4 (Table 1 and broken line in Fig. 3A).

Similar to moderate alkalisation, cAMP also depressed force and abolished the increased stiffness/force ratio when applied after cross-bridge inhibition by blebbistatin or V_i at pCa 4.4. The total amount of force depression was similar to that observed in experiments where cAMP was continuously present (Table 1). Furthermore, as shown in Table 2 the force depression by cAMP is similar to that reached by pH 7.4.

Discussion

In the present study, we investigated the question of the presence of catch linkages during maximal Ca^{2+} activation of skinned ABRM fibre bundles. To test this hypothesis, we inhibited force-generating myosin head cross-bridges at maximal Ca^{2+} activation (pCa 4.4) and examined the nature of the remaining force. The cycling of the cross-bridges was inhibited by 10 mmol l^{-1} orthovanadate or 5 μmol l^{-1} blebbistatin. V_i is thought to bind to the force-generating myosin heads with bound ADP (AM.ADP state) and induce an isomerisation to a stable non- or low-force-generating complex (Dantzig and Goldman, 1985; Chase et al., 1993). At a concentration of 10 mmol l^{-1} the force depression by V_i is saturated in skinned ABRM preparations (Galler et al., 2005). Blebbistatin is a recently discovered specific inhibitor of myosin ATPase activity (Kovacs et al., 2004). It is thought to bind to the detached myosin heads with bound ADP and inorganic phosphate (M.ADP.P_i state) and inhibit the transition into a force generating state (Allingham et al., 2005). At a concentration of 5 μmol l^{-1} the force depression by blebbistatin is close to saturation in skinned ABRM preparations (Butler et al., 2006). Whereas V_i and blebbistatin inhibit the cross-bridges they seem not to have any effects on catch linkages. This is suggested by the observation that application of 1–10 mmol l^{-1} V_i does not change force when the catch state is established (Galler et al., 2005). The same conclusion can be drawn for blebbistatin, since the present study showed that 1–10 μmol l^{-1} blebbistatin does not influence the force when catch is established after Ca^{2+} removal following Ca^{2+} activation.

Butler et al. (Butler et al., 2006) had already shown that saturating concentrations of blebbistatin depress the force of Ca^{2+}-activated ABRM preparations to intermediate levels. Furthermore, it was found that subsequent addition of 25 mmol l^{-1} phosphate did not influence force; however addition of cAMP caused a further substantial force depression. These results suggest that actively cycling myosin head cross-bridges are totally inhibited by blebbistatin and, thus, the remaining force should be based on passive catch linkages which are detached by cAMP. To further corroborate this assumption, in the present study we investigated the effects not only of cAMP but also of moderate alkalisation on force remaining after cross-bridge inhibition by V_i and blebbistatin.

In contrast to most of the experiments carried out by Butler et al. (Butler et al., 2006), our investigation concerned maximally Ca^{2+}-activated preparations. We further investigated the stiffness/force relationship and the force transients following rapid length changes before and during inhibitor application.

We found that the force remaining after cross-bridge inhibition is substantially reduced not only by cAMP but also by moderate alkalisation. This is an important hint for the presence of catch linkages because it is known that catch (induced by Ca^{2+} removal after Ca^{2+} activation) is abolished by these two factors [cAMP (Cornelius, 1982) and pH (Höpflinger et al., 2006)].

We further found that force inhibition by V_i or blebbistatin was accompanied by an increase of the stiffness/force ratio when compared with submaximal force levels reached either by partial Ca^{2+} activation under steady-state conditions (Fig. 1B) or during the development of the maximal Ca^{2+} activation (e.g. Fig. 2B). This is also an important hint for the presence of catch linkages, because a similar increase is usually observed when catch is induced by either Ca^{2+} removal after Ca^{2+} activation in skinned preparations (Pfitzer and Rüegg, 1982; Höpflinger et al., 2006) or removal of acetylcholine in intact preparations (Pfitzer and Rüegg, 1982; Sugi et al., 1999). In skinned skeletal muscle preparations [frog (Brozovich et al., 1988), rabbit psoas (Martyn and Gordon, 1992; Chase et al., 1993)] it was found that application of phosphate or orthovanadate increases the population of attached but non-force generating cross-bridges. This cross-bridge population is detectable only with very rapid length changes (<1 ms) at low temperature (5–13°C) indicating rapid detachments and reattachments (Chase et al., 1993; Dantzig and Goldman, 1985). Thus, this cross-bridge population markedly differs from long-lasting catch linkages. Furthermore, if this cross-bridge population is really present in ABRM, it may not contribute to our stiffness measurements which were performed with stepwise stretches (1–2 ms ramps) at 22.0–22.5°C. This assumption is further supported by our observation that the increased stiffness/force ratio during force depression by V_i or blebbistatin is abolished by moderate alkalization or cAMP. Thus, the increased stiffness/force ratio is rather due to the catch linkages and not to a population of short-living attached but non-force generating cross-bridges.

Finally, we found that the stretch-induced delayed force increase disappeared, and the force recovery following a release of the fibre length was substantially decreased, when the force was depressed by V_i or blebbistatin. These rigor-like responses are also a strong hint of the presence of catch linkages, because similar force transients are observed in ABRM during catch induction either by Ca^{2+} removal after Ca^{2+} activation in skinned preparations (Gagelmann et al., 1984; Cornelius, 1982; Galler et al., 2005; Butler et al., 1998) or by removal of acetylcholine in intact preparations (Pfitzer and Rüegg, 1982; Cornelius, 1980; Cornelius, 1982).

All these results strongly suggest that the force component that remains after cross-bridge inhibition by V_i or blebbistatin is largely due to catch linkages. The observation that the force did not fall to zero, but only to low levels (<10% of maximal force) when orthovanadate or blebbistatin and pH 7.4 or cAMP were applied concomitantly may be mainly because exposure times to the various factors was too short.

Are catch linkages also present at the maximum of Ca^{2+} activation?

A change in pH from 6.7 to 7.4 (Höpflinger et al., 2006) or addition of cAMP (Butler et al., 1998; Andruchova et al., 2005) does not affect the force of maximally activated skinned ABRM preparations. At first glance, this finding suggests that during maximal Ca^{2+} activation no catch linkages are established. However, it could also be that catch linkages are established but they are not under tension, so that their detachment (by pH 7.4 or cAMP) cannot be detected by measuring stiffness with short length changes (about 0.1% L_0). A detachment of such catch linkages would also not result in a change of isometric force. Our experiments clearly showed that catch force appears when myosin inhibitors are applied to maximally Ca^{2+}-activated skinned ABRM preparations. Therefore, unlike Siegman et al. (Siegman et al., 1998) or Butler et al. (Butler et al., 2006), we conclude that catch linkages are present also during maximal Ca^{2+} activation. However, the catch linkages are not under tension under isometric conditions as long as the force does not decline due to myosin inhibitors (or by the removal of Ca^{2+}) (see below).

Mechanism of catch

The fact that catch linkages are present already during Ca^{2+} activation has an important implication on the molecular mechanism underlying the catch phenomenon. Skinned ABRM fibre bundles are able to shorten both at maximal and submaximal Ca^{2+} activation with remarkable velocity (Butler et al., 1998). Firmly attached catch linkages would substantially prohibit shortening independently of their nature and location. The contradiction, however, can be resolved, if catch linkages behave like a ratchet. That is, the catch linkages exhibit a high resistance when the fibre is stretched and a low resistance when the fibre shortens. Based on these considerations we interpret important experimental findings around the catch phenomenon as follows.

When considering catch experiments under isometric conditions it appears that the state of catch is developed only after a decrease, but never during an increase, of force. This applies for intact muscles where catch is established after a force decrease resulting from cessation of stimulation (Rüegg, 1971). This also applies for experiments with skinned fibres where catch is established by a force decrease due to one of the following experimental procedures: (1) Ca^{2+} removal following Ca^{2+}-activated contraction (Rüegg, 1971; Siegman et al., 1998; Galler et al., 2005); (2) application of myosin inhibitors to Ca^{2+}-activated preparations (Butler et al., 2006) (this study); or (3) stepwise ATP increase in the high-force rigor at basal Ca^{2+} concentration (Galler et al., 1999; Butler et al., 2001).

That the appearance of catch properties requires a decrease of force can be seen in the present study. At about 30% of

maximal force a clear stretch activation response was present if this force was reached by partial Ca^{2+} activation starting at relaxing conditions. However, if the same force was reached by depressing the force during maximal Ca^{2+} activation, using myosin inhibitors, a rigor-like response was present, indicating catch. Likewise, an extra-stiffness indicating catch-linkages (increased stiffness/force ratio) could also be observed only during a force decrease, i.e. after Ca^{2+} removal following Ca^{2+} activation (Pfitzer and Rüegg, 1982; Höpflinger et al., 2006) or after myosin inhibition during Ca^{2+} activation (this study). By contrast, during the force increase at ongoing Ca^{2+} activation this extra stiffness was absent.

These observations can be explained using the ratchet model. During Ca^{2+} activation, cycling cross-bridges generate a pulling force. This force produces an intrinsic shortening (including a short filament sliding) and a stretch of series elastic components. The catch linkages are progressively (and slowly) established during Ca^{2+} activation, but they do not appreciably resist to the intrinsic shortening because, (1) the movement is in the low resistance direction and (2) the shortening is small. The catch linkages will not be stressed in the high resistance direction as long as the force rises or remains at a plateau. Under this condition the catch linkages have no contribution to stiffness (investigated with length changes of only 0.1% L_o) and isometric force. The situation changes when force decreases as a result of Ca^{2+} removal or myosin inhibition. The force decrease is associated with a reversal of the intrinsic shortening, which is driven by the stretched series elastic components. The reversal of the intrinsic shortening represents an internal stress which puts the catch linkages under tension because they are pulled in the high resistance direction. With decreasing force the number of catch linkages under tension increases. However, obviously not all catch linkages are put under tension; some of them remain unstrained. They only become visible if they are stretched during stiffness measurements. These unstrained catch linkages are responsible for the extra stiffness.

Since a force decrease seems to be necessary for putting the catch linkages under tension, it seems at first glance surprising that cAMP is able to depress force at stable plateau values during submaximal Ca^{2+} activations. This finding might be explained by the dual effects of cAMP on both catch linkages and cross-bridge kinetics (Andruchova et al., 2005). Thus, the force decrease is probably initialized by the cAMP-dependent change of cross-bridge kinetics (e.g. drop of f); it is then augmented, because force is not sustained by the catch linkages which are detached by cAMP. The initial force decrease due to a change in cross-bridge kinetics may occur only at submaximal but not at maximal Ca^{2+} activations. It is possible that f is in the order of g only at submaximal activations, but f is much larger than g at maximal Ca^{2+} activation. Consequently a change in f would affect the ratio $f/(f+g)$ (force) only at submaximal, but not at maximal, activation levels.

In contrast to our interpretation, Butler et al. (Butler et al., 2006) assumed that the whole cAMP-sensitive force component is catch force. This assumption and the dependency of the cAMP-sensitive force component on the grade of activation resulted in the hypothesis that actively cycling myosin head cross-bridges and catch linkages compete for a common binding site (Butler et al., 2006). As long as changes in the cross-bridge kinetics due to cAMP cannot be ruled out (see Introduction) these conclusions are debatable.

The molecular interpretation of catch reported here is compatible with the myosin head model of catch where the catch linkages are represented by the myosin head cross-bridges remaining attached to the actin filaments. If this model is true, it must be specified by assuming that the attached myosin heads exhibit ratchet properties. However, recent findings make the myosin head model of catch unlikely: cAMP (Siegman et al., 1998) or moderate alkalisation induce relaxation of catch force but do not accelerate myosin head detachment (Andruchova et al., 2005; Höpflinger et al., 2006). However, orthovanadate, phosphate and 2,3-butanedione monoxime (BDM) accelerate myosin head detachment but do not influence the catch state (Galler et al., 2005; Butler et al., 2006). Therefore, it is more likely that catch is based on link structures other than the myosin head cross-bridges.

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