The Journal of Experimental Biology 209, 668-676
Published by The Company of Biologists 2006
doi:10.1242/jeb.02033

# Effect of pH on the rate of myosin head detachment in molluscan catch muscle: are myosin heads involved in the catch state?

Marion Christine Höpflinger, Olena Andruchova, Oleg Andruchov, Herbert Grassberger and Stefan Galler*

*Department of Cell Biology, University of Salzburg, Hellbrunnerstr. 34, A-5020 Salzburg, Austria*

*Author for correspondence (e-mail: Stefan.Galler@sbg.ac.at)

*Accepted 13 December 2005*

## Summary

**Moderate alkalisation is known to terminate the catch state of bivalve mollusc smooth muscles such as the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* L. In the present study, we investigated the effect of moderate alkalisation (pH 7.2–7.7 *vs* control pH 6.7) on the myosin head detachment rate in saponin-skinned fibre bundles of ABRM in order to investigate the possible role of myosin heads in the force maintenance during catch. The detachment rate of myosin heads was deduced from two types of experiments. (1) In stretch experiments on maximally $Ca^{2+}$-activated fibre bundles (pCa 4.5), the rate of force decay after stepwise stretch was assessed. (2) In ATP step experiments, the rate of force decay from high force rigor (pCa>8) was evaluated. The ATP step was induced by photolysis of caged ATP. We found that moderate alkalisation induces relaxation of skinned fibres in catch, thereby reducing both force and stiffness, whereas it does not accelerate the rate of myosin head detachment. This acceleration, however, would be expected if catch would be simply due to myosin heads remaining sustainably attached to actin filaments. Thus, the myosin heads may be less involved in catch than generally assumed. Catch may possibly depend on a different kind of myofilament interconnections, which are abolished by moderate alkalisation.**

Key words: catch muscle, mollusc smooth muscle, *Mytilus edulis*, pH effect, caged ATP.

## Introduction

Certain bivalve mollusc smooth muscles have the ability to maintain force for long periods of time even after cessation of stimulation. This phenomenon is called ‘catch state’ or, briefly, ‘catch’ (Jewell, 1959). In the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* L., catch is used to attach the animal to the substrate at low energy expenditure. Catch is characterized by high levels of force maintained both at basal levels of ATP consumption (Güth et al., 1984) and at cytosolic free $Ca^{2+}$ concentrations similar to those in the resting state (Ishii et al., 1989). In experiments under isometric conditions, catch is characterized by a very slow rate of force decrease after cessation of stimulation (Rüegg, 1965).

In intact ABRM preparations, catch is induced by removal of acetylcholine after acetylcholine-induced activation. 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). Twitchin is a mini-titin (Funabara et al., 2003) located on thick filaments (Siegman et al., 1998). Phosphorylation of twitchin results in the termination of catch (Siegman et al., 1997, 1998).

In skinned ABRM preparations, removal of $Ca^{2+}$ after $Ca^{2+}$-induced activation leads to a catch-like 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). The maintenance of catch force is favoured by moderate acidic pH. Therefore, most studies investigating catch have been carried out at pH 6.5–6.8 (e.g. Rüegg, 1971; Siegman et al., 1998; Galler et al., 2005). A change in pH from 6.2 to 7.5 (Rüegg, 1964), from 6.5 to 7.7 (Rüegg, 1971) or from 6.7 to 7.7 (Galler et al., 2005) induces relaxation of catch force. The pH sensitivity of catch force is also present in intact ABRM preparations. Zange et al. (1990) observed a relaxation of catch force when the intracellular pH was artificially increased from about pH 7.4 to about pH 7.6. Furthermore, they found that relaxation of catch induced both by serotonin application and by an artificial increase of intracellular cAMP concentration, was accompanied by an intracellular alkalisation from about pH 7.4 to about pH 7.6.

The molecular basis of catch is still unclear. Two principally different mechanisms have been proposed. The first explains

catch in terms of myosin heads (cross-bridges) remaining sustainably attached to actin filaments. The very slow force decrease during catch is thought to be due to a very slow detachment of myosin heads (myosin head model; Lowy et al., 1964; Butler et al., 2001). The second envisions the formation of link structures (interconnections), which are different from myosin head cross-bridges, between myofilaments (alternative linkage model; Rüegg, 1963, 1965).

The mechanism of how moderate intracellular alkalisation induces relaxation of catch force is explained differently in the two models. Based on the myosin head model of catch (Lowy et al., 1964; Butler et al., 2001), moderate alkalisation may initiate detachment of myosin heads firmly attached to actin or may extensively accelerate the rate of slowly detaching myosin heads. The initiation or acceleration of myosin head detachment would induce rapid relaxation of the catch state. Based on the alternative linkage model of catch (Rüegg, 1963, 1965), moderate alkalisation may induce abolishment of interconnections linking myofilaments without affecting myosin heads. If the myosin head model is valid, an acceleration of myosin head detachment due to moderate alkalisation would be expected. If this acceleration is not observed, this model is questionable. Therefore, measurements of myosin head detachment at different pH should distinguish between the two proposed models for the mechanism of catch.

There are two approaches for investigation of myosin head detachment rates in skinned muscle fibres: (1) application of stepwise stretches to $Ca^{2+}$-activated fibres (stretch experiments) and (2) stepwise increase in ATP concentration under high-force rigor conditions provided by photolysis of caged ATP (ATP step experiments). Stepwise stretches applied to $Ca^{2+}$-activated fibres are accompanied by an instantaneous force increase, followed by force decay and, subsequently, a delayed force increase. This delayed force increase is called stretch activation. These force transients were demonstrated in insect flight muscle (Pringle, 1978), cardiac muscle (Steiger, 1971), vertebrate skeletal muscle (Huxley and Simmons, 1971; Heinl et al., 1974) and ABRM (Gagelmann et al., 1984). The force decay after stretch seems to represent the detachment of myosin heads, whereas the delayed force increase seems to represent the reattachment of myosin heads (Saeki et al., 1991; Kawai and Zhao, 1993). In ATP step experiments starting at high-force rigor (pCa>8), the initial rapid force decay seems to be determined by myosin head detachment following a sudden binding of ATP (Goldman et al., 1984). In our report, for reasons of simplicity, we do not distinguish between weakly attached (Schoenberg et al., 1984) and detached cross-bridges states. Therefore, the term ‘attachment’ implies transitions from non-force generating states (either detached or weakly attached) to force generating states, and the term ‘detachment’ implies transitions from force-generating states to non-force-generating states.

In the present study, we investigated the effect of pH on myosin head detachment in both stretch and ATP step experiments. No acceleration of myosin head detachment was found due to moderate alkalisation. Based on these results, the myosin head model of catch could only remain valid if one assumes that moderate alkalisation accelerates myosin head detachment only in the presence, but not in the absence, of catch. This seems unlikely and, therefore, our results rather support the alternative linkage model, where catch-maintaining interconnections other than the myosin heads are abolished by moderate alkalisation.

## 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.), obtained from a local sea-food supplier, were isolated (shell length, 4–7 cm). The dissected muscle was teased into several bundles about 0.4 mm wide in artificial seawater [490 mmol $l^{-1}$ NaCl, 8 mmol $l^{-1}$ KCl, 10 mmol $l^{-1}$ $CaCl_2$, 15 mmol $l^{-1}$ $MgCl_2$, 1 mmol $l^{-1}$ 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes), pH 7.2]. These bundles were skinned in two different solutions containing 0.05% (w/v) saponin (Cornelius, 1980; Chick and Stephenson, 1995; Galler et al., 2005). The first skinning solution (sodium skinning solution) comprised: 132 mmol $l^{-1}$ sodium propionate, 5 mmol $l^{-1}$ ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 7 mmol $l^{-1}$ $Na_2H_2ATP$, 2 mmol $l^{-1}$ $MgCl_2$, 10 mmol $l^{-1}$ 3-morpholinopropanesulfonic acid (Mops), 2 mmol $l^{-1}$ dithioerythritol (DTE), 646 mmol $l^{-1}$ sucrose, 0.05% (w/v) saponin, pH adjusted to 6.9 with KOH. The second skinning solution (potassium skinning solution) comprised: 5 mmol $l^{-1}$ EGTA, 5 mmol $l^{-1}$ $MgCl_2$, 1 mmol $l^{-1}$ $NaN_3$, 20 mmol $l^{-1}$ imidazole, 5 mmol $l^{-1}$ $Na_2H_2ATP$, 150 mmol $l^{-1}$ KCl, 150 mmol $l^{-1}$ sucrose, 1 mmol $l^{-1}$ DTE, 0.05% (w/v) saponin, pH adjusted to 6.7 with KOH. The skinning procedure took about 30 min in total and was carried out at 20–24°C.

### *Experimental setup and procedure*

For mechanical experiments, skinned fibre bundles of 50–200 μm diameter and 1.9–4.3 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 (within ~1 ms) of fibre length were achieved by a feedback-controlled stepping motor based on a Ling vibrator. A cuvette transporting system provided quick changes of solutions. For ATP step experiments, fibre bundles were immersed in a 10 μl droplet of either $Ca^{2+}$- or EGTA-rigor solution containing caged ATP (disodium adenosine 5′-triphosphate, $P^3$-1-(2-nitrophenyl)ethyl ester; Calbiochem, La Jolla, CA, USA). A xenon flash lamp (Optoelektronik,

Hamburg, Germany) was used to generate UV flashes for photolysis of caged ATP.

All mechanical experiments were carried out at 22.0–22.5°C. Results are expressed as means ± s.d. The significance of differences between means was calculated using Student's *t*-test for paired samples in stretch experiments and for unpaired samples in ATP step experiments.

### *Solutions*

Solutions used for the mechanical experiments contained 150 mmol $l^{-1}$ sucrose, 5 mmol $l^{-1}$ EGTA, 0.9 mmol $l^{-1}$ free $Mg^{2+}$ and 1 mmol $l^{-1}$ DTE. The ionic strength was adjusted to 0.20 mol $l^{-1}$ with KCl, and the pH was adjusted to 6.7, 7.2, 7.4 or 7.7 using KOH. The free [$Ca^{2+}$] was measured using a calcium-sensitive electrode (Fluka 21188) and was expressed as pCa=–log[$Ca^{2+}$]$_{free}$. Rigor solutions (EGTA-rigor, pCa>8; $Ca^{2+}$-rigor, pCa 4.5) additionally contained 50 mmol $l^{-1}$ Mops, whereas activation (pCa 4.5) and relaxation solutions (pCa>8) contained 20 mmol $l^{-1}$ imidazole, 5 mmol $l^{-1}$ $Na_2H_2ATP$, 5 mmol $l^{-1}$ disodium creatine phosphate, 1 mmol $l^{-1}$ $NaN_3$, 1 mmol $l^{-1}$ DTE and 30 U $ml^{-1}$ creatine phosphokinase.

To prevent force development during induction of low-force rigor, 50 mmol $l^{-1}$ 2,3-butanedione monoxime (BDM) was added to these rigor solutions by replacing 50 mmol $l^{-1}$ sucrose. In rigor solutions used for photolysis experiments, 10 mmol $l^{-1}$ caged ATP was added by replacing 55 mmol $l^{-1}$ KCl. These solutions contained 10 mmol $l^{-1}$ DTE in order to quench free radicals, which are produced by caged ATP photolysis. The ATP concentration after flash photolysis was assumed to be ~1.5 mmol $l^{-1}$. This estimation is based on a study which showed that, under comparable conditions, the same type of xenon flash lamp photolysed ~15% of present caged ATP (Arner et al., 1987).

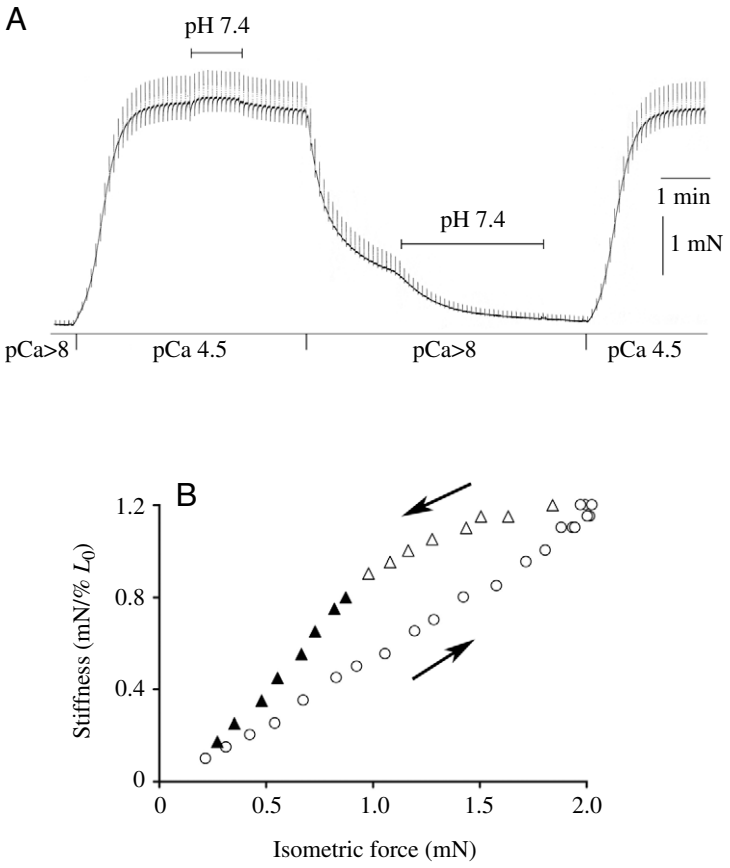


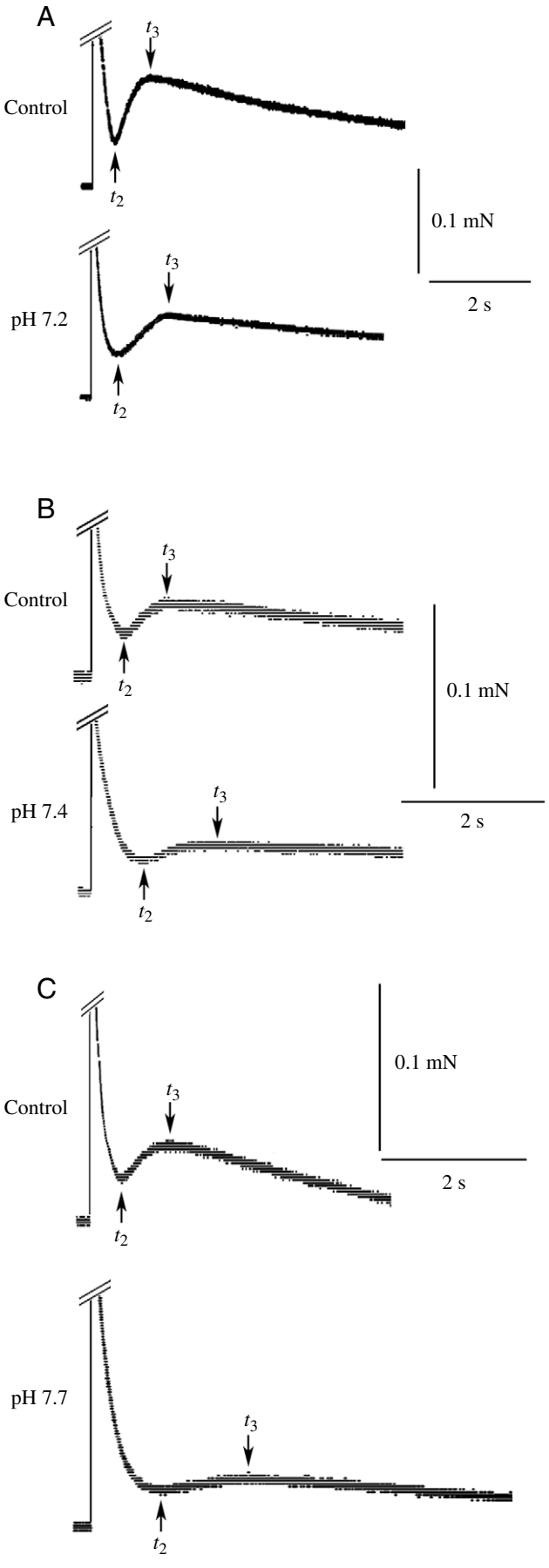

Fig. 1. (A) Force recording of a skinned fibre bundle of anterior byssus retractor muscle (ABRM) of common muscle *Mytilus edulis*. The spikes represent the force responses following repetitive stretches (0.2 Hz, 0.2% of the fibre length in amplitude and 0.2 s in duration), which serve as a measure of stiffness. The solution sequence is indicated. (B) Force–stiffness diagram of another ABRM fibre bundle undergoing the protocol shown in A. The stiffness (mN/%$L_0$) was calculated by relating the maximal positive amplitude of the stretch-induced force peaks (mN) to the (constant) relative length change (%$L_0$). Open circles, activation solution at pH 6.7; open triangles, relaxation solution at pH 6.7; filled triangles, relaxation solution at pH 7.4.

## Results

### *Effect of moderate alkalisation on force and stiffness*

Fig. 1A shows a recording of force and stiffness in a skinned ABRM fibre bundle. The stiffness is characterized by the amplitude of the spikes, which represent force responses following repetitive rectangular stretches. These stretches were applied with 0.2 Hz, 0.2% of fibre length in amplitude and 0.2 s in duration. The length changes were obtained within ~1 ms. After activation at pCa 4.5, a change in pH from control (pH 6.7) to pH 7.4 and back to control was applied. The pH change was associated with only small changes in force and stiffness. Removal of $Ca^{2+}$ (pCa>8) induced a relaxation, which progressively slowed down with time, resulting in a tension remnant (catch). Subsequently, a change in pH from pH 6.7 to pH 7.4 accelerated relaxation and reduced force and stiffness to basal values, thereby abolishing catch. A return from pH 7.4 to pH 6.7 caused no changes in stiffness and force. Subsequent new $Ca^{2+}$ activation led to an increase of both force and stiffness to values similar to those observed at the previous maximal activation, while removal of $Ca^{2+}$ induced again a progressively slow relaxation, i.e. it caused catch (not shown). Fig. 1B shows the relationship between stiffness and force in another fibre bundle undergoing the protocol presented in Fig. 1A. Note that during $Ca^{2+}$ activation, stiffness increased in proportion to force, whereas after removal of $Ca^{2+}$ (at pH 6.7) a comparatively large decay of force was associated with only a small decrease in stiffness, indicating a significant increase in the ratio of stiffness to force. Subsequently, the change from pH 6.7 to pH 7.4 caused a decay of both stiffness and force back to basal levels.

The experiment shown in Fig. 1A was repeated on seven ABRM fibre bundles. In each case, qualitatively the same result was obtained. Thus, it appears that moderate alkalisation




enforces a decline of both stiffness and force during catch, but not during $Ca^{2+}$ activation. In further experiments, the force relaxation was investigated at various pH values. The enhancement of relaxation increased in a graded manner with increasing pH (pH 7.2, 7.4 and 7.7; data not shown). All seven experiments clearly showed that the pH-induced abolition of catch, represented by the decline of stiffness and force in relaxation solution, was not reversible simply by changing the pH back to the control value. For the re-establishment of catch, a new $Ca^{2+}$ activation is required.

### *Stretch-induced force transients*

For investigating stretch-induced force transients, rapid stretches (within ~1 ms), 0.2–0.3% of fibre length in amplitude and 10–15 s in duration, were applied on maximally $Ca^{2+}$-activated fibre bundles. During the $Ca^{2+}$ activation, the pH was changed in the sequence 'control–test–control', where 'control' means pH 6.7, and 'test' means moderate alkalisation (pH 7.2, 7.4 or 7.7). In some experiments with pH 7.4, the sequence was test–control–test. The stretches caused a simultaneous rise in force, followed by a decrease and delayed increase, called stretch activation (Fig. 2A–C). The following time parameters of force transients were evaluated: $t_2$, the time from the beginning of stretch to the onset of delayed rise in force, and $t_3$, the time from beginning of stretch to the peak of delayed rise in force. The reproducibility of force transients in solutions of the same pH was ±1% for $t_2$ and ±4% for $t_3$ on average.

As shown in Fig. 2 and summarized in Table 1, stretch-induced force transients became slower with increasing pH. Compared with control conditions (pH 6.7), $t_2$ increased ~1.4 times at pH 7.2, ~1.8 times at pH 7.4 and ~2.3 times at pH 7.7. $t_3$ increased ~1.4 times at pH 7.2, ~1.6 times at pH 7.4 and ~1.8 times at pH 7.7. In control–test–control experiments with pH 7.4 ($N$=3), the following results were obtained: 435±58 ms at pH 6.7 and 771±63 ms at pH 7.4 for $t_2$; 1069±130 ms at pH 6.7 and 1779±143 ms at pH 7.4 for $t_3$. In test–control–test experiments with pH 7.4 ($N$=3), the following results were obtained: 473±66 ms at pH 6.7 and 835±105 ms at pH 7.4 for $t_2$; 1185±80 ms at pH 6.7 and 1733±91 ms at pH 7.4 for $t_3$. Since the values of both solution sequences were similar, they were combined in Table 1.

### *ATP step experiments*

The effect of pH on detachment rates of myosin heads was also investigated in experiments in which ATP was liberated from caged ATP. Fig. 3 shows original force recordings of an

Fig. 2. Original force recordings of stretch experiments on maximally $Ca^{2+}$-activated (pCa 4.5) skinned anterior byssus retractor muscle (ABRM) fibre bundles. (A–C) High time resolution force recordings during stretch experiments under indicated conditions. Control (pH 6.7) and test recordings of each set were obtained on the same fibre bundle and at the same stretch amplitude. Note that the delayed force increase (time parameter $t_3$) and the immediate force decay following stretch (time parameter $t_2$) are slowed by moderate alkalisation.

Table 1. *Parameters obtained from stretch and ATP step experiments at different pH*

| | pH 6.7, control | pH 7.2 | pH 7.4 | pH 7.7 |
|---|---|---|---|---|
| Stretch experiments | | | | |
| $t_2$ (ms) | 454±60 (17) | 639±89 (4) | 803±84 (6) | 1059±104 (7) |
| $t_3$ (ms) | 1127±105 (17) | 1596±126 (4) | 1756±117 (6) | 2040±131 (7) |
| ATP step experiments | | | | |
| $\Delta T^{\text{fast decay}}$ (%) | 44.8±7.2 (5) | – | 65.5±8.3 (7) | – |
| $r^{\text{slow decay}}$ (% min$^{-1}$) | 2.0±0.1 (5) | – | 2.2±0.1 (5) | – |
| $\tau^{\text{fast decay}}$ (ms) | 495±90 (5) | – | 478±70 (7) | – |
| $t_{½}^{\text{fast decay}}$ (ms) | 331±42 (5) | – | 319±37 (7) | – |
| $\tau^{\text{force rise}}$ (ms) | 590±43 (7) | – | 1339±502 (7) | – |
| $t_{½}^{\text{force rise}}$ (ms) | 441±48 (7) | – | 1024±125 (7) | – |

Values are means ± s.d. (*N*).

Parameters obtained from stretch experiments: $t_2$, time from the beginning of stretch to the onset of delayed force increase; $t_3$, time from the beginning of stretch to the peak of delayed force increase.

Parameters obtained from ATP step experiments: $\Delta T^{\text{fast decay}}$, extent of force decay within the initial rapid phase; $r^{\text{slow decay}}$, the rate of force decay per min within the slow (almost linear) phase; $\tau^{\text{fast decay}}$, $t_{½}^{\text{fast decay}}$, time constant and half-time, respectively, of force decay in the initial rapid phase; $\tau^{\text{force rise}}$, $t_{½}^{\text{force rise}}$, time constant and halftime, respectively, of force rise.

Significances for statistic differences: $t_2$ and $t_3$, $P<0.01$ for each test condition against control condition (paired Student-*t*-tests); $\tau^{\text{force rise}}$, $t_{½}^{\text{force rise}}$ and $\Delta T^{\text{fast decay}}$, $P<0.01$ for test and control condition (unpaired Student's *t*-tests); $r^{\text{slow decay}}$, $P<0.05$ for test and control condition (unpaired Student's *t*-tests). The time parameters $\tau^{\text{fast decay}}$ and $t_{½}^{\text{fast decay}}$ are not significantly different between test and control condition ($P>0.23$, unpaired Student's *t*-test).

exemplary ATP step experiment at low (A) and high (B) time resolution. For induction of high-force rigor, $Ca^{2+}$-activated fibre bundles were transferred from activation solution to $Ca^{2+}$-rigor solution followed by EGTA-rigor solution. Subsequently, fibre bundles were transferred to EGTA-rigor solutions containing 10 mmol l$^{-1}$ caged ATP either of pH 6.7 (control condition) or of pH 7.4 (test condition). Flash photolysis of caged ATP led to an initial rapid force decay (rapid phase) followed by a much slower force decay (slow phase). As shown by Butler et al. (2001), the force decay of this slow phase is strongly accelerated by cAMP, and thus it may represent the catch state of ABRM. For analyzing the force responses, the following parameters were evaluated: (1) the amplitude of force decay within the initial rapid phase from 0 to 5 s after flash, $\Delta T^{\text{fast decay}}$; (2) the rate of force decay within the slow (almost linear) phase from 4 to 5 min after flash, $r^{\text{slow decay}}$; (3) the time constant of force decay within the initial rapid phase from 0.1 s to 1.5 s after flash (single exponential function with $r^2>0.998$, $\tau^{\text{fast decay}}$); (4) the half-time of force decay within the initial rapid phase from 0 to 5 s after flash ($t_{½}^{\text{fast decay}}$). The means ± s.d. of these parameters for fibre bundles measured either at control pH or pH 7.4 are given in Table 1. $\Delta T^{\text{fast decay}}$ was 1.5 times larger at pH 7.4 ($P<0.01$). In

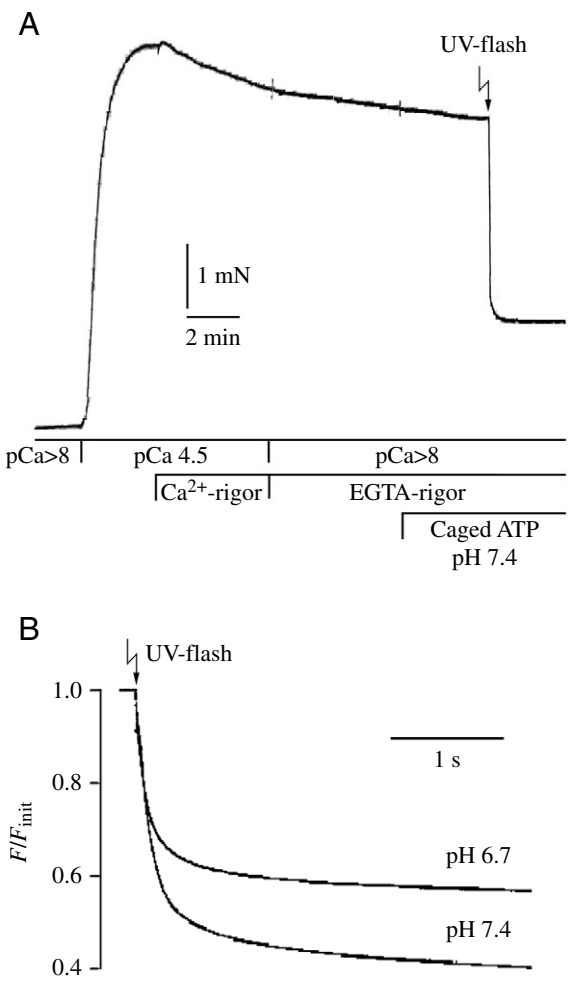



Fig. 3. ATP step experiments starting at high force rigor. A stepwise increase of ATP concentration was induced by flash photolysis of caged ATP. (A) Force recording at low time resolution, indicating the conditions for induction of high-force rigor and subsequent force decay induced by an ATP step. (B) Superimposed force recordings at high time resolution of ATP step under control (pH 6.7) and test conditions (pH 7.4).

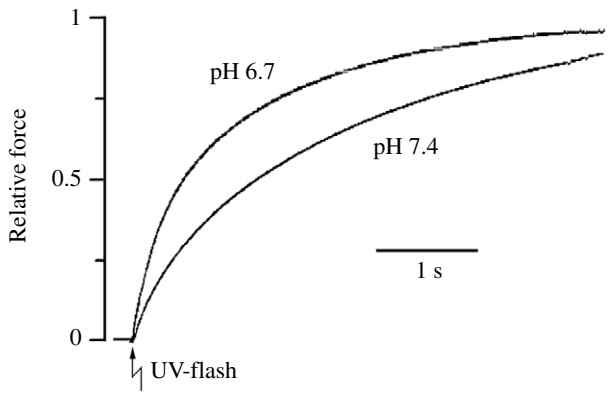



Fig. 4. Superimposed force recordings of ATP step experiments starting at low force rigor at pH 6.7 and pH 7.4. The force was normalised to that measured 10 s after flash. The force increase was induced by a stepwise increase of ATP concentration after induction of low-force rigor in the presence of high $Ca^{2+}$ concentration (pCa 4.5).

addition, $r^{\text{slow decay}}$ was 1.1 times faster at pH 7.4 ($P<0.05$). However, $\tau^{\text{fast decay}}$ and $t_{½}^{\text{fast decay}}$ did not differ significantly between pH 6.7 and pH 7.4 ($P>0.23$).

In other experiments, we investigated the force response induced by an ATP step in low-force rigor (Fig. 4). For induction of low-force rigor, preparations were transferred initially from relaxation solution to EGTA-rigor and then to $Ca^{2+}$-rigor solution. To avoid force development during rigor induction, 50 mmol $l^{-1}$ BDM was present in these solutions. After induction of rigor, the fibre bundle was transferred to $Ca^{2+}$-rigor without BDM. Subsequently, fibre bundles were transferred to $Ca^{2+}$-rigor solutions containing 10 mmol $l^{-1}$ caged ATP either of pH 6.7 (control condition) or of pH 7.4 (test condition). The ATP step produced a rapid force increase. For analysis, the time constant of the force rise within 0.05 s and 1.5 s after flash ($\tau^{\text{force rise}}$) was determined (single exponential function with $r^2>0.992$). In addition, the half-time of force rise ($t_{½}^{\text{force rise}}$) was evaluated within 0 s and 5 s after flash. Both $\tau^{\text{force rise}}$ and $t_{½}^{\text{force rise}}$ were ~2.3 times larger at pH 7.4 than at pH 6.7 (Table 1).

## Discussion

Catch is a physiologically important phenomenon for maintaining force at low energy expenditure. The phenomenon is present, for example, in the adductor muscles and in ABRM of *Mytilus edulis*; the former hold the shell closed, whereas the latter helps to attach the animal to the substrate. The molecular events underlying catch are still unclear. A moderate alkalisation of the intracellular environment is known to enforce relaxation of catch force in both intact (Zange et al., 1990) and glycerol-extracted ABRM preparations (Rüegg, 1964, 1971). This was confirmed in saponin-treated fibre bundles in the present study and in one of our previous studies (cf. fig. 1A in Galler et al., 2005). When catch is abolished by serotonin in intact ABRM, the pH changes from approximately pH 7.4 and pH 7.6 (Zange et al., 1990). The pH changes applied in the present study were larger, ranging stepwise from pH 6.7 (control) to 7.2, 7.4 and 7.7. The reason for the different pH sensitivity of intact and skinned ABRM preparations is not known; it was observed also by other investigators (Rüegg, 1971; Siegman et al., 1998; Galler et al., 2005).

The effect of moderate alkalisation on catch seems not to depend on cAMP-induced twitchin phosphorylation. In one of our previous studies (cf. fig. 1A of Galler et al., 2005), we showed that moderate alkalisation enforced relaxation of catch force after a low force level was reached by addition of cAMP. In the present study, we showed that moderate alkalisation accelerated the relaxation of catch force even in the absence of cAMP. Furthermore, the present study also showed for the first time that the stiffness is irreversibly reduced when catch force is abolished by moderate alkalisation. This means that the linkages maintaining catch force are abolished by a pH change in the physiological range (from 6.7 to 7.4) and cannot be re-established simply by reversing the pH change. The re-establishment of catch linkages requires a process that is associated with new $Ca^{2+}$ activation followed by $Ca^{2+}$ removal under favourable conditions, e.g. from pH 6.5 to 6.8.

Pfitzer and Rüegg (1982) showed for intact ABRM that force and stiffness are apparently coupled during activation, but they are uncoupled during the prolonged time course of relaxation (catch state). The same is the case in skinned fibre bundles of ABRM (Fig. 1B). This observation suggests that during the long time course of relaxation (i.e. in the catch state), linkages exist that contribute to stiffness but not to force. These non-force-generating linkages may be represented by rigor-like myosin head cross-bridges (as suggested by Butler et al., 2001; myosin head model) or by a different kind of linkage (alternative linkage model).

Our results indicate that moderate alkalisation does not accelerate the detachment of myosin heads, either at low (pCa>8; catch condition) or at high $Ca^{2+}$ concentration (pCa 4.5). This acceleration would be expected if catch were due to myosin heads remaining sustainably attached to actin filaments. As discussed below, this was deduced from ATP step experiments performed at pCa>8 and from stepwise stretch experiments performed at maximal $Ca^{2+}$ activation (pCa 4.5). Therefore, it is suggested (see also Andruchova et al., 2005; Galler et al., 2005) that the myosin head model may not be appropriate for explanation of the catch state. Catch may rather be attributed to structural linkages between myofilaments other than the myosin heads (Rüegg, 1963).

### *Stretch experiments*

The time course of stretch-induced force transients is a measure of specific steps of cross-bridge cycle. This assumption is mainly based on the following findings obtained on maximally $Ca^{2+}$-activated skinned fibres. First, there is a correlation between the time parameters ($t_2$ and $t_3$) of stretch-induced force transients of mammalian striated muscle fibres and the content of myosin heavy chain isoforms (Galler et al., 1997, 2002; Andruchov et al., 2004a,b). Thus, the stretch-

induced force transients seem to be caused by a transient stretch-synchronisation of a group of myosin heads and, consequently, reflect kinetic properties of myosin heads during force-generating cycles. Second, the kinetics of the force transients depends on concentrations of phosphate, MgATP and MgADP (e.g. Kawai and Brandt, 1980; Kawai and Zhao, 1993). Based on the effects of MgATP and phosphate, it was concluded that the force decrease after stretch (time parameter $t_2$) represents detachment of myosin heads following ATP binding (Kawai and Zhao, 1993). Moreover, it was concluded that the delayed force increase (time parameter $t_3$) represents myosin reattachment and force generation prior to the release of phosphate (Saeki et al., 1991; Kawai and Zhao, 1993).

Our experiments show that moderate alkalisation of intracellular environment (pH 7.2–7.7) did not increase, but decreased, the rate of force decay following stepwise stretch of maximally $Ca^{2+}$-activated fibres, although catch force and stiffness were relaxed at these pH values. It can thus be concluded that a moderate alkalisation is not increasing but decreasing the rate of myosin head detachment. Moderate alkalisation caused an increase of both $t_2$ and $t_3$, suggesting slower kinetics of both detachment and attachment of myosin heads.

### *ATP step experiments*

The force decay following an ATP step in high-force rigor is thought to represent the detachment of myosin heads (Goldman et al., 1984). On the other hand, the force rise following an ATP step in low-force rigor may not be determined only by myosin head attachment. Rather it may depend on the rates of the whole cross-bridge cycle. This is because an appreciable elasticity of the contractile apparatus is expanded during the force rise (Huxley et al., 1994; Wakabayashi et al., 1994), and this is most likely associated with more than one cross-bridge cycle. This is suggested by a recent study (Sleep et al., 2005) showing that the force increase in an ATP step experiment has the same kinetics as the force redevelopment following a period of isotonic shortening. The latter was shown to be correlated with ATPase activity (Brenner and Eisenberg, 1986). Therefore, it is likely that both the force redevelopment following a period of isotonic shortening and the force increase in ATP step experiments are determined by the rate of the whole cross-bridge cycle.

Moderate alkalisation did not affect the rate of initial rapid force decay (rapid phase) following an ATP step at high-force rigor ($\tau^{\text{fast decay}}$, $t_{½}^{\text{fast decay}}$; Table 1). However, it increased the extent of the initial rapid relaxation ($\Delta T^{\text{fast decay}}$) and it slightly accelerated the force decay during the subsequent slow relaxation phase ($r^{\text{slow decay}}$; Table 1), both suggesting a decline of catch. Thus, it can be concluded that a moderate alkalisation, although it is releasing catch, does not accelerate myosin head detachment. If stretch experiments are compared with ATP step experiments, a discrepancy is observed. The force decay after stretch ($t_2$) was slowed by moderate alkalisation, whereas the force decay following an ATP step ($\tau^{\text{fast decay}}$, $t_{½}^{\text{fast decay}}$; Table 1) was not affected. There is no straightforward explanation for this discrepancy, but differences in the experimental conditions should be considered: the $Ca^{2+}$ concentration is high in stretch experiments (pCa 4.5) but low in ATP step experiments (pCa>8). Furthermore, the concentration of MgATP was calculated to be ~4 mmol $l^{-1}$ in stretch experiments and ~1 mmol $l^{-1}$ in ATP step experiments (Dantzig et al., 1998).

Moderate alkalisation (pH 7.4) decreased the rate of force rise ($\tau^{\text{force rise}}$, $t_{½}^{\text{force rise}}$) from low-force rigor following an ATP step. This corresponds to the decelerating effect of pH 7.4 on the stretch activation time parameter $t_3$ (Table 1). The parameter $t_3$ was slowed by a factor of ~1.6 (Table 1) whereas both $\tau^{\text{force rise}}$ and $t_{½}^{\text{force rise}}$ were slowed by a factor of ~2.3. Thus, it appears that moderate alkalisation decelerates the attachment of myosin heads.

The mechanism by which pH is able to affect myosin head attachment and detachment rates is not clear. Effects of pH on contractile properties were also found on other muscles. In skinned cardiac and skeletal muscle preparations, a moderate alkalisation produced an increase of maximum tension and $Ca^{2+}$ sensitivity (Ashley and Moisescu, 1977; Fabiato and Fabiato, 1978; Robertson and Kerrick, 1979; Chase and Kushmerick, 1988). By contrast, in some smooth muscles, opposite pH effects on maximum tension were observed (Spurway and Wray, 1987; Smith et al., 1998). For cardiac muscle and smooth muscle, troponin C (Ding et al., 1996) and tropomyosin (Yamaguchi et al., 1984), respectively, were considered as pH sensors, which influence actin–myosin interaction.

### *Catch mechanism*

The myosin head model of catch implies that factors which induce catch relaxation are expected to accelerate myosin head detachment; on the other hand, factors that accelerate myosin head detachment are expected to induce relaxation of catch force. At this point, our studies revealed a mismatch of expectation and experimental observation. Twitchin phosphorylation (Siegman et al., 1998; Butler et al., 2001) induces relaxation of catch force but does not accelerate the rate of myosin head detachment (Andruchova et al., 2005). Likewise, the present study showed that moderate alkalisation of intracellular environment also induces relaxation of catch force but does not accelerate the rate of myosin head detachment as well. On the other hand, orthovanadate, phosphate and BDM accelerate myosin head detachment but do not influence the catch state (Galler et al., 2005). Summarizing, it appears that catch is not based on myosin heads remaining attached to actin filaments. Catch may rather depend on other myofilament interconnections that are abolished due to moderate alkalisation.

Our current ideas about the molecular mechanism underlying catch can be described as follows. Catch linkages are established while myosin heads are sustainably producing force. The contribution of these linkages to the maintenance of isometric force starts (or it becomes apparent) after detachment of force-generating myosin heads. Depending on the number

of catch linkages, the force level after this detachment is high or low. The subsequent slow force relaxation characterizes abolishment (by detaching or yielding) of catch linkages. This view would lead to the following interpretation of the ATP step experiment shown in Fig. 3; the number of catch linkages is determined by the pH present before the flash-induced ATP step. This number is lower at pH 7.4 than at pH 6.7. The following ATP step leads to the detachment of force-generating myosin heads (rapid phase). The force level reached after this detachment is higher at pH 6.7, because more catch linkages were established at this pH. The subsequent slow phase of force decay is a little faster at pH 7.4 than at pH 6.7, because a moderate alkalisation favours the abolishment of catch linkages.

A number of observations confirm the assumption that the initial rapid and the subsequent slow phase of force decay (Fig. 3) are based on different structures: (1) the two phases exhibit totally different kinetics and, (2) as already mentioned above, the two phases are affected by the same factors (vanadate, BDM and phosphate; twitchin phosphorylation; moderate alkalisation) in different ways.

The results of our present study challenge, but do not exclude, the myosin head model of catch. For this model to be valid, unusual assumptions are necessary. It would have to be assumed that a moderate alkalisation accelerates myosin head detachment only in the presence, but not in the absence, of catch. This would imply that a moderate alkalisation would accelerate myosin head detachment at low (catch condition) but not at high $Ca^{2+}$ concentration. Our findings contradict this idea, because no acceleration of myosin head detachment was found either at low $Ca^{2+}$ (ATP step experiment) or at high $Ca^{2+}$ concentrations (stretch experiments). Moreover, our stretch experiments at high $Ca^{2+}$ concentration suggest that moderate alkalisation induces a deceleration rather than an acceleration of myosin head detachment. If the myosin head model of catch is still valid, factors other than $Ca^{2+}$ have to be responsible for switching between a pH-sensitive and a pH-insensitive state of myosin head detachment.

As more extensively discussed by Galler et al. (2005), there are some structural hints in favour of the alternative linkage model of catch. Electron micrographs showed visible interconnections of adjacent thick filaments that seem to be formed by distinct projections (Sobieszek, 1973; Takahashi et al., 2003). These interconnections are much more frequent during catch (Takahashi et al., 2003). Therefore, they could be involved in force maintenance during catch. Twitchin could be the structure interconnecting the thick filaments (Mukou et al., 2004). This is supported by a study on isolated proteins (Shelud'ko et al., 2004) that showed that twitchin of mollusc muscles can aggregate with F-actin of rabbit skeletal muscle. This aggregation is diminished when twitchin is phosphorylated. It is plausible to speculate that twitchin spans from one myosin filament to a neighbouring one while contacting adjacent actin filaments. A moderate alkalisation (and twitchin phosphorylation) could be able to abolish the twitchin interconnections, resulting in the termination of catch.

In summary, the results of our work provide further evidence for the view that catch may be due to interconnections between myofilaments other than the myosin heads. The most likely candidate for these interconnections is the large, titin-like protein twitchin. Moderate alkalisation abolishes these interconnections, which results in rapid relaxation of catch force.

The work was supported by a grant of the Austrian Science Foundation (FWF-P16709-B09) and the South Tyrolean Sparkasse.

## References

**Achazi, R. K., Dolling, B. and Haakshorst, R.** (1974). 5-HT-induced relaxation and cyclic AMP in a molluscan smooth muscle. *Pflügers Arch. Eur. J. Physiol.* **349**, 19-27.

**Andruchov, O., Andruchova, O., Wang, Y. and Galler, S.** (2004a). Kinetic properties of myosin heavy chain isoforms in mouse skeletal muscle: Comparison with rat, rabbit and human and correlation with amino acid sequence. *Am. J. Physiol.* **287**, C1725-C1732.

**Andruchov, O., Wang, Y., Andruchova, O. and Galler, S.** (2004b). Functional properties of skinned rabbit skeletal and cardiac muscle preparations containing α-cardiac myosin heavy chain. *Pflügers Arch. Eur. J. Physiol.* **448**, 44-53.

**Andruchova, O., Höpflinger, M. C., Andruchov, O. and Galler, S.** (2005). No effect of twitchin phosphorylation on the rate of myosin head detachment in molluscan catch muscle: are myosin heads involved in the catch state? *Pflügers Arch. Eur. J. Physiol*. **450**, 326-334.

**Arner, A., Goody, R. S., Rapp, G. and Rüegg, J. C.** (1987). Relaxation of chemically skinned guinea pig taenia coli smooth muscle from rigor by photolytic release of adenosine-5′-triphosphate. *J. Muscle Res. Cell Motil*. **8**, 377-385.

**Ashley, C. C. and Moisescu, D. G.** (1977). Effect of changing the composition of the bathing solutions upon the isometric tension-pCa relationship in bundles of crustacean myofibrils. *J. Physiol. Lond.* **270**, 627-652.

**Brenner, B. and Eisenberg, E.** (1986). Rate of force generation in muscle: correlation with actomyosin ATPase activity in solution. *Proc. Natl. Acad. Sci. USA* **83**, 3542-3546.

**Butler, T. M., Narayan, S. R., Mooers, S. U., Hartshorne, D. J. and Siegman, M. J.** (2001). The myosin cross-bridge cycle and its control by twitchin phosphorylation in catch muscle. *Biophys. J.* **80**, 415-426.

**Chase, P. B. and Kushmerick, M. J.** (1988). Effects of pH on contraction of rabbit fast and slow skeletal muscle fibers. *Biophys. J.* **53**, 935-946.

**Chick, J. J. and Stephenson, D. G.** (1995). The effect of temperature on contractile activation of intact and chemically skinned 'catch' muscle fibre bundles of Mytilus edulis. *J. Muscle Res. Cell Motil.* **16**, 285-294.

**Cornelius, F.** (1980). The regulation of tension in a chemically skinned molluscan smooth muscle: effect of $Mg^{2+}$ on the $Ca^{2+}$-activated tension generation. *J. Gen. Physiol.* **75**, 709-725.

**Cornelius, F.** (1982). Tonic contraction and the control of relaxation in a chemically skinned molluscan smooth muscle. *J. Gen. Physiol*. **79**, 821-834.

**Dantzig, J. A., Higuchi, H. and Goldman, Y. E.** (1998). Studies of molecular motors using caged compounds. *Meth. Enzymol*. **291**, 307-348.

**Ding, X. L., Akella, A. B., Sonnenblick, E. H., Rao, V. G. and Gulati, J.** (1996). Molecular basis of depression of $Ca^{2+}$ sensitivity of tension by acid pH in cardiac muscles of the mouse and the rat. *J. Card. Fail*. **2**, 319-326.

**Fabiato, A. and Fabiato, F.** (1978). Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. *J. Physiol. Lond.* **276**, 233-255.

**Funabara, D., Watabe, S., Mooers, S. U., Narayan, S., Dudas, C., Hartshorne, D. J., Siegman, M. J. and Butler, T. M.** (2003). Twitchin from molluscan catch muscle: primary structure and relationship between site-specific phosphorylation and mechanical function. *J. Biol. Chem.* **278**, 29308-29316.

**Gagelmann, M., Güth, K. and Rüegg, J. C.** (1984). Stretch induced tension rise in a molluscan smooth muscle skinned by freeze drying. *J. Comp. Physiol.* **154**, 187-189.

**Galler, S. and Hilber, K.** (1994). Unloaded shortening of skinned mammalian

skeletal muscle fibres: effects of the experimental approach and passive force. *J. Muscle Res. Cell Motil.* **15**, 400-412.

**Galler, S., Hilber, K. and Pette, D.** (1997). Stretch activation and myosin heavy chain isoforms of rat, rabbit and human skeletal muscle fibres. *J. Muscle Res. Cell Motil.* **18**, 441-448.

**Galler, S., Puchert, E., Gohlsch, B., Schmid, D. and Pette, D.** (2002). Kinetic properties of cardiac myosin heavy chain isoforms in rat. *Pflügers Arch. Eur. J. Physiol.* **445**, 218-223.

**Galler, S., Höpflinger, M. C., Andruchov, O., Andruchova, O. and Grassberger, H.** (2005). Effects of vanadate, phosphate and 2,3-butanedione monoxime (BDM) on skinned molluscan catch muscle. *Pflügers Arch. Eur. J. Physiol.* **449**, 372-383.

**Goldman, Y. E., Hibberd, M. G. and Trentham, D. R.** (1984). Relaxation of rabbit psoas muscle fibers from rigor by photochemical generation of adenosine-5′-triphosphate. *J. Physiol. Lond.* **354**, 577-604.

**Güth, K., Gagelmann, M. and Rüegg, J. C.** (1984). Skinned smooth muscle: time course of force and ATPase activity during contraction cycle. *Experientia* **40**, 174-176.

**Heinl, P., Kuhn, H. J. and Rüegg, J. C.** (1974). Tension responses to quick length changes of glycerinated skeletal muscle fibres from the frog and tortoise. *J. Physiol. Lond.* **237**, 243-258.

**Huxley, A. F. and Simmons, R. M.** (1971). Proposed mechanism of force generation in striated muscle. *Nature* **233**, 533-538.

**Huxley, H. E., Stewart, A., Sosa, H. and Irving, T.** (1994). X-ray diffraction measurements of the extensibility of actin and myosin filaments in contracting muscle. *Biophys. J.* **67**, 2411-2421.

**Ishii, N., Simpson, A. W. and Ashley, C. C.** (1989). Free calcium at rest during "catch" in single smooth muscle cells. *Science* **243**, 1367-1368.

**Jewell, B. R.** (1959). The nature of the phasic and the tonic responses of the anterior byssal retractor muscle of Mytilus. *J. Physiol. Lond.* **149**, 154-177.

**Kawai, M. and Brandt, P. W.** (1980). Sinusoidal analysis: a high resolution method for correlating biochemical reactions with physiological processes in activated skeletal muscles of rabbit, frog and crayfish. *J. Muscle Res. Cell Motil.* **1**, 279-303.

**Kawai, M. and Zhao, Y.** (1993). Cross-bridge scheme and force per cross-bridge state in skinned rabbit psoas muscle fibres. *Biophys. J.* **65**, 638-651.

**Lowy, J., Millman, B. M. and Hanson, J.** (1964). Structure and function in smooth tonic muscle of lamellibranch molluscs. *Proc. R. Soc. Lond. B Biol. Sci.* **160**, 525-536.

**Mukou, M., Kishi, H., Shirakawa, I., Kobayashi, T., Tominaga, K., Imanishi, H. and Sugi, H.** (2004). Marked load-bearing ability of Mytilus smooth muscle in both active and catch states as revealed by quick increases in load. *J. Exp. Biol.* **207**, 1675-1681.

**Pfitzer, G. and Rüegg, J. C.** (1982). Molluscan catch muscle: regulation and mechanics in living and skinned anterior byssus retractor muscle of *Mytilus edulis*. *J. Comp. Physiol. B* **147**, 137-142.

**Pringle, J. W.** (1978). The Croonian Lecture, 1977. Stretch activation of muscle: function and mechanism. *Proc. R. Soc. Lond. B* **201**, 107-130.

**Robertson, S. P. and Kerrick, W. G.** (1979). The effects of pH on $Ca^{2+}$-activated force in frog skeletal muscle fibers. *Pflügers Arch. Eur. J. Physiol.* **380**, 41-45.

**Rüegg, J. C.** (1963). Actomyosin inactivation by thiourea and the nature of viscous tone in a molluscan smooth muscle. *Proc. R. Soc. Lond. B* **158**, 177-195.

**Rüegg, J. C.** (1964). Tropomyosin–paramyosin system and 'prolonged contraction' in a molluscan smooth muscle. *Proc. R. Soc. Lond. B* **160**, 536-542.

**Rüegg, J. C.** (1965). Physiologie und Biochemie des Sperrtonus. *Helv. Physiol. Pharmacol. Acta* **16**, S1-S76.

**Rüegg, J. C.** (1971). Smooth muscle tone. *Physiol. Rev.* **51**, 201-248.

**Saeki, Y., Kawai, M. and Zhao, Y.** (1991). Comparison of cross-bridge dynamics between intact and skinned myocardium from ferret right ventricles. *Circ. Res.* **68**, 772-781.

**Schoenberg, M., Brenner, B., Chalovich, J. M., Greene, L. E. and Eisenberg, E.** (1984). Cross-bridge attachment in relaxed muscle. *Adv. Exp. Med. Biol.* **170**, 269-284.

**Shelud'ko, N. S., Matusovskaya, G. G., Permyakova, T. V., Matusovsky, O. S.** (2004). Twitchin, a thick-filament protein from molluscan catch muscle, interacts with F-actin in a phosphorylation-dependent way. *Arch. Biochem. Biophys.* **432**, 269-277.

**Siegman, M. J., Mooers, S. U., Li, C., Narayan, S., Trinkle-Mulcahy, L., Watabe, S., Hartshorne, D. J. and Butler, T. M.** (1997). Phosphorylation of a high molecular weight (approximately 600 kDa) protein regulates catch in invertebrate smooth muscle. *J. Muscle Res. Cell Motil.* **18**, 655-670.

**Siegman, M. J., Funabara, D., Kinoshita, S., Watabe, S., Hartshorne, D. J. and Butler, T. M.** (1998). Phosphorylation of a twitchin-related protein controls catch and calcium sensitivity of force production in invertebrate smooth muscle. *Proc. Natl. Acad. Sci. USA* **95**, 5383-5388.

**Sleep, J., Irving, M. and Burton, K.** (2005). The ATP hydrolysis and phosphate release steps control the time course of force development in rabbit skeletal muscle. *J. Physiol.* **563**, 671-687.

**Smith, R. D., Eisner, D. A. and Wray, S.** (1998). The effects of changing intracellular pH on calcium and potassium currents in smooth muscle cells from the guinea-pig ureter. *Pflügers Arch. Eur. J. Physiol.* **435**, 518-522.

**Sobieszek, A.** (1973). The fine structure of the contractile apparatus of the anterior byssus retractor muscle of *Mytilus edulis*. *J. Ultrastruct. Res.* **43**, 313-343.

**Spurway, N. C. and Wray, S.** (1987). A phosphorus nuclear magnetic resonance study of metabolites and intracellular pH in rabbit vascular smooth muscle. *J. Physiol. Lond.* **393**, 57-71.

**Steiger, G. J.** (1971). Stretch activation and myogenic oscillation of isolated contractile structures of heart muscle. *Pflügers Arch. Eur. J. Physiol.* **330**, 347-361.

**Takahashi, I., Shimada, M., Akimoto, T., Kishi, T. and Sugi, H.** (2003). Electron microscopic evidence for the thick filament interconnections associated with the catch state in the anterior byssal retractor muscle of *Mytilus edulis*. *Comp. Biochem. Physiol.* **134A**, 115-120.

**Twarog, B. W.** (1954). Responses of a molluscan smooth muscle to acetylcholine and 5-hydroxytryptamine. *J. Cell Physiol.* **44**, 141-163.

**Wakabayashi, K., Sugimoto, Y., Tanaka, H., Ueno, Y., Takezawa, Y. and Amemiya, Y.** (1994). X-ray diffraction evidence for the extensibility of actin and myosin filaments during muscle contraction. *Biophys. J.* **67**, 2422-2435.

**Yamaguchi, M., Ver, A., Carlos, A. and Seidel, J. C.** (1984). Modulation of the actin-activated adenosinetriphosphatase activity of myosin by tropomyosin from vascular and gizzard smooth muscles. *Biochemistry* **23**, 774-779.

**Zange, J., Pörtner, H. O., Jans, A. W. H. and Grieshaber, M. K.** (1990). The intracellular pH of a molluscan smooth muscle during a contraction-catch-relaxation cycle estimated by the distribution of [14C]DMO and by 31P-NMR spectroscopy. *J. Exp. Biol.* **150**, 81-93.