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Effects of vanadate, phosphate and 2,3-butanedione monoxime (BDM) on skinned molluscan catch muscle

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Abstract The effects of orthovanadate (V_i), inorganic phosphate (P_i) and 2,3-butanedione monoxime (BDM) on tension, force transients and the catch state (passive tension maintenance) were investigated in saponin-skinned fibre bundles of the anterior byssus retractor muscle (ABRM) of the bivalve mollusc *Mytilus edulis* at pH 6.7. During maximal Ca^{2+} activation isometric force was depressed by V_i (0.03–10 mM), P_i (10 mM) and BDM (50 mM). Force transients following quick stretches (0.1–0.3% of fibre length) were accelerated substantially by 1 mM V_i , 10 mM P_i or 50 mM BDM. These compounds also accelerated force responses in experiments in which ATP was released rapidly from caged ATP by flash photolysis at both pCa 4.7 (force rise) and at pCa > 8 (force decline). The effects on the catch state were investigated in two types of experiments: (1) Ca^{2+} removal after maximal Ca^{2+} activation and (2) rapid ATP release during high-force rigor at pCa > 8. In both cases rapid relaxation was followed by slow relaxation (slower than 2% of initial force per min). This later slow relaxation (catch) was insensitive to V_i (1–10 mM), P_i (10 mM) and BDM (50 mM) but was accelerated by 0.12 mM cAMP. Complete relaxation to almost zero force was attained by changing pH from 6.7 to 7.7 (pCa > 8). We conclude that catch depends on cAMP- and pH-sensitive structures linking the myofilaments and not on the force-generating actomyosin cross-bridges that are sensitive to V_i , P_i and BDM.

Keywords Catch muscle · Vanadate effects on muscle · Phosphate effects on muscle · 2,3-Butanedione

monoxime (BDM) · Caged-ATP · Muscle relaxation · Skinned invertebrate smooth muscle · Stretch activation

Introduction

The anterior byssus retractor muscle (ABRM) of the mussel *Mytilus edulis* is a molluscan smooth muscle that has been investigated extensively because of its ability to maintain tension with very low energy consumption. To a lesser extent this phenomenon also occurs in vertebrate smooth muscle where it is called the “latch state” (e.g. [11]). When activated with acetylcholine (ACh) or by direct current the ABRM contracts actively, but relaxes very slowly, and may even fail to relax completely, after cessation of stimulation. This slowly decaying tension remnant is known as the catch state, or “catch”, and is characterised by passive tension maintenance. Serotonin (5-hydroxytryptamin, 5-HT) abolishes the catch state and induces fast relaxation [51]. During this relaxation the intracellular concentration of cAMP increases [1] and twitchin, a protein located together with myosin on the paramyosin-containing thick filaments, is phosphorylated by a cAMP-dependent protein kinase (PKA) [45]. Twitchin is related to skeletal muscle titin but is much smaller [13].

During catch the cytosolic free Ca^{2+} level is as low as in the resting state [25] and tension is maintained with little or even no energy expenditure [27]. The basis of such force maintenance is still not understood. It may be associated with a very slow rate of myosin head (cross-bridge) detachment from the actin filament (see [32] for review). Alternatively, other structural proteins may link the myofilaments (see [40, 41] for review) and produce catch. A catch-like state is also observed in skinned ABRM preparations in which cell membranes are removed by freeze-drying or detergent treatment. In this case removal of Ca^{2+} after Ca^{2+} -induced activation

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leads to very slow relaxation with tension being maintained passively at basal levels of the actomyosin ATPase activity [20]. cAMP [8] or the catalytic subunit of PKA cause rapid relaxation and abolishment of this catch-like state in skinned ABRM fibres [38, 44].

In molluscan muscle, force development and actomyosin ATPase are initiated by binding of Ca^{2+} to the myosin heads [30]. Ca^{2+} accelerates the release of inorganic phosphate (P_i) and promotes the subsequent release of ADP after ATP hydrolysis. P_i release leads to the formation of the strongly bound and force-generating cross-bridge state AM.ADP (A = actin, M = myosin); ADP release leads to the formation of the strongly bound and force-generating AM state. Binding of ATP leads to myosin head detachment that allows further cycling to be initiated. Attempts have been made to explain the catch phenomenon on the basis of the effect of Ca^{2+} on the release of ADP. The decrease in free $[\text{Ca}^{2+}]$ after a tonic contraction [25] could slow ADP release markedly so that cross-bridges remain in a force-generating state. According to this hypothesis, catch would be related to the persistence of a Ca^{2+} -depleted, strongly bound cross-bridge (AM.ADP) state [3, 4, 17, 41, 45, 50], which detaches very slowly from actin because of the slow dissociation of ADP (locked AM.ADP state model).

Orthovanadate (V_i), a well-known phosphate analogue, inhibits force and ATPase activity of skinned muscle preparations of skeletal, cardiac and smooth muscle [5, 9, 19, 23, 26]. The inhibition is thought to result from V_i binding to the AM.ADP state, which induces isomerisation to a stable non- or low-force-generating complex resulting in depression of force and ATPase activity of skinned muscle fibres. Increased concentrations of inorganic phosphate (P_i) elicit the same effects, but to a lesser extent [5, 23, 29, 35]. 2,3-Butanedione monoxime (BDM), a non-competitive inhibitor of myosin ATPase, also depresses force of skinned striated and smooth muscle preparations [22, 36, 43, 54, 55]. Therefore, it was interesting to determine the effects of V_i , P_i and BDM on skinned ABRM preparations during maximal Ca^{2+} activation and the catch state. All three compounds had significant effects on skinned ABRM fibre bundles in the maximally Ca^{2+} -activated state, but no effects were observed during the catch state. cAMP markedly increased the rate of relaxation during catch, but this relaxation was never complete. Further relaxation was induced when pH was changed from 6.7 to 7.7. Our results do not support the hypothesis that catch is based on a locked AM.ADP state. We suggest that other structures that link the myofilaments may be responsible for maintaining force during catch.

Materials and methods

Muscle preparations

Common mussels (*Mytilus edulis*) with a shell length of 4–7 cm were obtained from a local sea-food supplier.

After opening the shell by cutting off the adductor muscles, the ABRMs were removed in the presence of artificial seawater (ASW) of the following composition (mM): 490 NaCl, 8 KCl, 10 CaCl_2 , 15 MgCl_2 , 1 HEPES; pH 7.2. During the dissection the ABRM was kept at 0–5 °C in ASW containing additionally 10 μM 5-HT. Using a scalpel ABRMs were cut off from the shell at one end and from posterior byssus retractor muscle at the other end [6]. The muscle was then dissected in ice-cooled ASW to obtain thin bundles containing 13–36 fibres of 50–200 μm diameter and 7–19 mm length. The bundles were mounted horizontally in an auxotonic apparatus. For experiments that included quick changes of the fibre length or for experiments in which ATP was released rapidly by flash photolysis of caged ATP a more sophisticated isometric apparatus was used. In this case the bundles were 50–150 μm in diameter and 2.7–3.5 mm in length and were skinned before attachment.

Experimental set-up

The isometric apparatus of ca. 4 $\mu\text{m mN}^{-1}$ compliance and the methods for mechanical measurements have been described previously [15]. The attachment points for the muscle fibres on the mechanical apparatus consisted of two approximately 2-mm-long, vertically oriented epoxy carbon fibre needles with tips of about 100 μm diameter. They were connected to the apparatus by silicon plates from force transducer elements (AE 801, SensoNor, Norway). One element, the force sensor (resonance frequency about 7.5 kHz), was connected mechanically to a micrometer screw and electrically to a force bridge amplifier. The other element was glued to the lever arm of a stepping motor. Rapid changes of fibre length (≤ 1 ms) were achieved by a feedback-controlled stepping motor based on a Ling vibrator. The ability to make rapid changes of solutions were provided by a cuvette transporting system.

An auxotonic apparatus was used for measurements of intact preparations and after their subsequent skinning. The ends of muscle bundles were clamped by vertically oriented, approximately 100- μm -wide, stainless-steel, forceps-like clips. One of the clips was connected to a force transducer (KG7a, Scientific Instruments, Heidelberg, Germany) while the other was attached to a fixed support point. The resonance frequency of the force measuring system was ca. 200 Hz with compliance of about 400 $\mu\text{m mN}^{-1}$. Due to this compliance the fibre bundles shortened during maximal activation by about 20–40% of their initial length.

For the experiments with rapid ATP release the fibre bundles were immersed in a 10- μl drop of rigor solution containing caged ATP (see below). A xenon flash-lamp (Optoelektronik, Hamburg, Germany) was used to generate UV flashes for photolysis of caged ATP. The experimental set-up is described more extensively elsewhere [2, 17].

Solutions and skinning procedure

Before skinning, control experiments with 20 μM ACh and 20 μM 5-HT were carried out in ASW at room temperature (20–24 °C). The fibres were skinned in two steps in solutions containing 0.05% (w/v) saponin [7]. The first skinning solution (sodium skinning solution) had the following composition (mM): 132 sodium propionate, 5 EGTA, 7 $\text{Na}_2\text{H}_2\text{ATP}$, 2 MgCl_2 , 10 3-(*N*-morpholino)propanesulphonic acid (MOPS), 2 dithioerythritol (DTE), 30 BDM, 646 sucrose, saponin 0.05% (w/v), adjusted to pH 6.9 with KOH. The second skinning solution (potassium skinning solution) had the following composition (mM): 5 EGTA, 5 MgCl_2 , 1 NaN_3 , 20 imidazole, 5 $\text{Na}_2\text{H}_2\text{ATP}$, 150 KCl, 150 sucrose, 1 DTE, 0.05% saponin, adjusted to pH 6.7 with KOH. The skinning procedure took about 20 min in total and was carried out at 20–24 °C.

After skinning, mechanical experiments were carried out in the following solutions (pH 6.7, 20–24 °C): relaxation solution (pCa > 8) (mM): 5 EGTA, 5 MgCl_2 , 1 NaN_3 , 20 imidazole, 5 $\text{Na}_2\text{H}_2\text{ATP}$, 5 sodium phosphocreatine, creatine kinase 20 U ml^{-1} , 150 KCl, 150 sucrose, 1 DTE, 0.9 free Mg^{2+} ; activation solution (pCa 4.7) (mM): 4.5 CaCl_2 , 5 EGTA, 5 MgCl_2 , 1 NaN_3 , 20 imidazole, 5 $\text{Na}_2\text{H}_2\text{ATP}$, 5 sodium phosphocreatine, creatine kinase 20 U ml^{-1} , 150 KCl, 150 sucrose, 1 DTE, 0.9 free Mg^{2+} . When required, orthovanadate (0.01–10 mM, Na_3VO_4) or phosphate (10 mM, mixture of Na_2HPO_4 and NaH_2PO_4) was added to these solutions and removing KCl to keep the ionic strength constant. The exact $[\text{V}_i]$ was determined by absorption measurement at 265 nm, assuming an extinction coefficient of $2925 \text{ M}^{-1} \text{ cm}^{-1}$ [26]. A stock solution of 300 mM V_i (pH 10) was prepared in double-distilled water and boiled until colourless [26]. After adding, the pH of skinned fibre bath solutions was readjusted to 6.7 with HCl. A Ca^{2+} -sensitive electrode (Fluka 21188) was used for measuring the free $[\text{Ca}^{2+}]$ of the solutions. For solutions containing 10 mM V_i no noticeable decrease of free $[\text{Ca}^{2+}]$ was detectable. This was checked, because in principle Ca^{2+} could bind to V_i . In some experiments, 50 mM BDM was added to the activation or relaxation solution by substituting it for sucrose. In some experiments the pH of the relaxation solution was adjusted to pH 7.7 by addition of an appropriate amount of KOH. In specific experiments 0.5 or 1 mM MgADP was added to the relaxation solution from a 100 mM MgADP stock solution prepared by dissolving 100 mM NaH_2ADP and 100 mM MgCl_2 in 20 mM imidazole and adjusting pH to 6.7.

In experiments with caged ATP two rigor solutions (pH 6.7, 20–24 °C) were used: EGTA-rigor solution (pCa > 8) containing (in mM): 5 EGTA, 0.93 MgCl_2 , 1 NaN_3 , 1 DTE, 20 imidazole, 155 KCl, 150 sucrose, 0.9 free Mg^{2+}) and Ca^{2+} -rigor solution (pCa 4.7) containing (mM): 4.43 CaCl_2 , 5 EGTA, 0.90 MgCl_2 , 1 NaN_3 , 1 DTE, 20 imidazole, 155 KCl, 150 sucrose, 0.9 free Mg^{2+} . To prevent force development during induction of low-force rigor, 50 mM sucrose was replaced by

50 mM BDM in the EGTA- and Ca^{2+} -rigor solutions. Rapid ATP release was induced in EGTA- or Ca^{2+} -rigor solutions in which 50 mM KCl had been replaced by 10 mM caged ATP [adenosine 5'-triphosphate, P^3 -1-(2-nitrophenyl)ethyl ester]. The $[\text{ATP}]$ after photolysis was assumed to be 1.5 mM. This estimation is based on a study showing that, under comparable conditions, the same type of xenon flash-lamp photolyses 15% of the caged ATP present [2]. The DTE concentration was 10 mM so as to quench the free radicals that are produced by ATP photolysis. In photolysis experiments, $[\text{MgCl}_2]$ was 4.0 mM in the EGTA- and 3.93 mM in the Ca^{2+} -rigor solutions. The calculated free $[\text{Mg}^{2+}]$ was always 0.9 mM after photolysis. The apparent binding constants of caged ATP were taken from [10]. To investigate the effects of V_i , P_i and BDM on the force transients following rapid ATP release, 1 mM V_i , 10 mM P_i or 20 mM BDM were added to the respective caged ATP-containing rigor solutions by substituting for KCl (V_i , P_i) or sucrose (BDM).

For calculations of tension, the force was related to the cross-sectional area of the muscle fibre bundle determined in relaxing solution. This area was calculated assuming a circular shape. Results are expressed as means \pm SD and the significance of differences between means determined using Students-*t*-test for paired samples.

Results

Contractile properties of intact and skinned ABRM fibre bundles

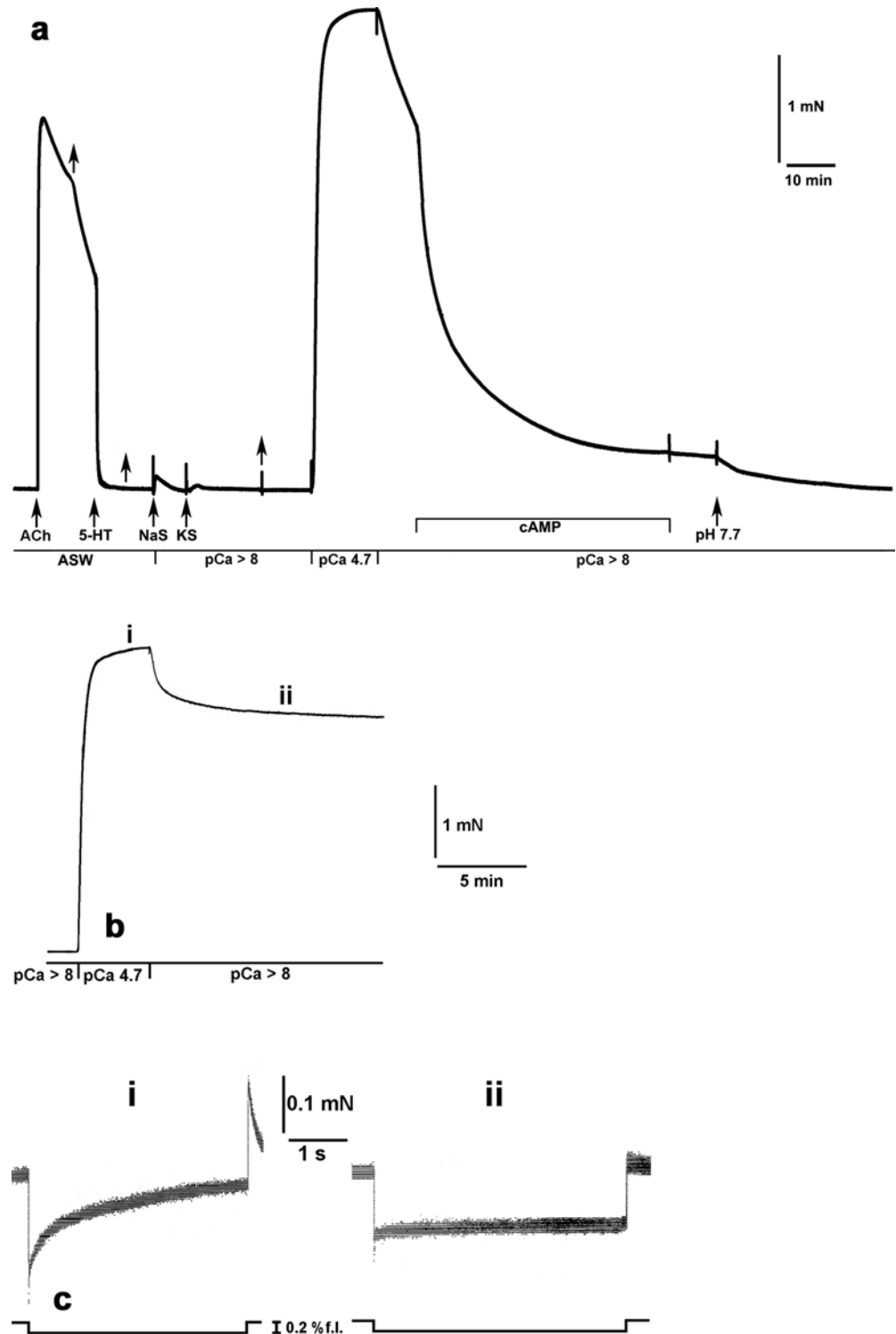
As shown in Fig. 1a our intact ABRM fibre bundles developed the catch state after activation by 20 μM ACh because washing out the ACh led to a slow relaxation that was accelerated by 20 μM 5-HT. The experiment was performed on an auxotonic apparatus like all other experiments that did not include rapid changes of the fibre length or the application of caged ATP. Only very small transient force changes were produced during skinning of the fibre bundles with 0.05% saponin in the sodium and potassium skinning solutions. Our skinned fibre bundles were activated by addition of Ca^{2+} (pCa 4.7, pH 6.7) when ATP (5 mM) was present. The force generated by skinned fibre bundles was 1.56 ± 0.36 times ($n=9$) higher than that induced by ACh in intact fibres.

Removal of Ca^{2+} (pCa > 8) resulted in slow relaxation that varied from fibre to fibre both in the amplitude and kinetics. In most cases, the faster first phase was followed by a slower phase (Fig. 1b). The force decline of the slow phase was slower than 2% of maximal force per min. In two experiments, in which this force decay was monitored for an extended period, $44.7 \pm 0.2\%$ of maximal force was still present 30 min after Ca^{2+} removal. When the fibre length was released stepwise (on the isometric apparatus, Fig. 1c), force recovered during maximal Ca^{2+} activation (time marker *i* in Fig. 1b), but

not during the slow relaxation phase after Ca^{2+} removal (time marker *ii* in Fig. 1b). Addition of cAMP (0.12 mM) resulted in acceleration of the relaxation phase (Fig. 1a). In contrast to the effect of 5-HT on intact preparations, cAMP-dependent relaxation was never complete and a steady state was reached at $8.6 \pm 3.0\%$ ($n=7$) of maximal force after approximately 1 h incubation. This was also the case when the phos-

phatase inhibitor microcystine LR (20 nM) [48] was present in the cAMP-containing solutions ($n=4$). Washing out cAMP did not affect the force, but further relaxation (to $1.7 \pm 1.0\%$ of maximum force, $n=7$, Figs. 1a, 5a) was induced by increasing pH from 6.7 to 7.7. On principle, a pH increase will be accompanied by an increase of the Ca^{2+} affinity of the Ca^{2+} buffer EGTA, thus decreasing free $[\text{Ca}^{2+}]$. However, this is not

Fig. 1 a Force recording of a fibre bundle of anterior byssus retractor muscle (ABRM) of common mussel *Mytilus edulis* in the intact and skinned state. Intact fibre bundles were activated by 20 μM acetylcholine (ACh) in artificial sea water (ASW). Removal of the ACh by washing (upwards arrowhead) resulted in a slow relaxation that was substantially accelerated by application of 20 μM 5-hydroxytryptamin (5-HT). Subsequent skinning by saponin (0.05%, w/v) in sodium (NaS) and potassium (KS) skinning solutions had very little effect on the force. In the skinned state maximal force was produced in activation solution (pCa 4.7, pH 6.7), and removal of Ca^{2+} (pCa > 8) induced a slow relaxation that was accelerated by 0.12 mM cAMP. Relaxation with cAMP was incomplete and further relaxation was induced by changing pH from 6.7 (pCa > 8) to 7.7 (pCa > 8). **b** Ca^{2+} activation of a skinned ABRM fibre bundle and its relaxation after subsequent removal of Ca^{2+} . As shown here, the Ca^{2+} removal produced an initial rapid and a subsequent slow relaxation phase in most of our experiments. *i* and *ii* indicate the times of the quick-release experiments. **c** Experiments of stepwise release (0.2% of fibre length; *f.l.*) during maximal Ca^{2+} activation (*i*) and during catch (*ii*)



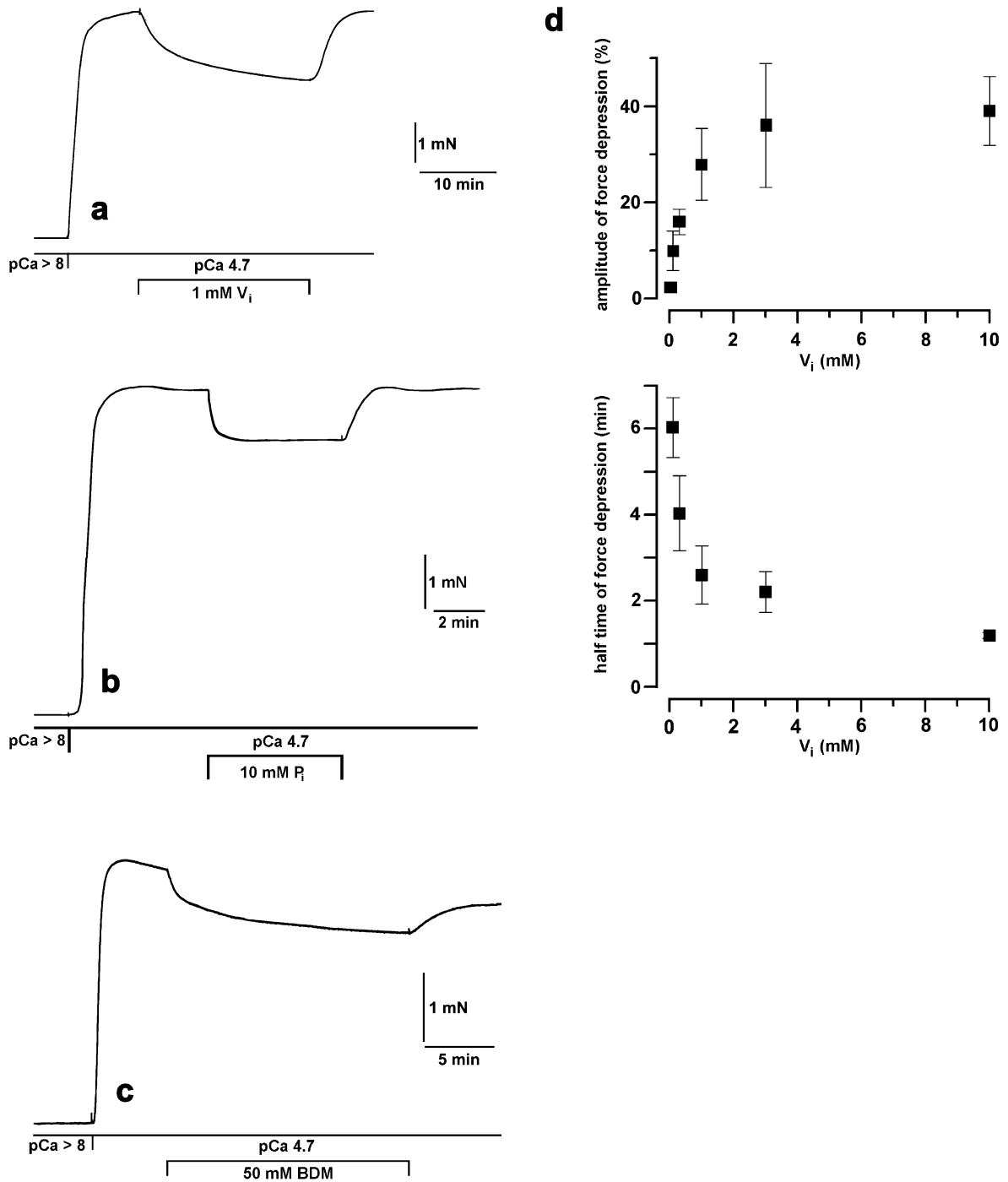


Fig. 2a–d Effects of 1 mM vanadate (V_i , **a**), 10 mM phosphate (P_i , **b**) and 50 mM 2,3-butanedione monoxime (BDM , **c**) on force generation during maximal Ca^{2+} activation of saponin-skinned ABRM fibre bundles. **d** Dependence of amplitude and half-time of force depression on $[V_i]$. Means \pm SD, $n = 21$

relevant in these types of experiments because the free $[\text{Ca}^{2+}]$ before (for more than 30 min) and after the pH change was lower than 10^{-8} M (about pCa 9 at pH 6.7 and about pCa 11 at pH 7.7), which is far below the activation threshold of the contractile machinery. As a consequence, the observed acceleration of relaxation must have been due to the pH change and not to a change in free $[\text{Ca}^{2+}]$.

Effects of V_i , P_i and BDM at maximal Ca^{2+} activation

Addition of 1 mM V_i at maximal Ca^{2+} activation reduced force to $72.1 \pm 7.4\%$ of maximum with a half-time of 2.5 ± 0.6 min (Fig. 2a). The force reduction and its kinetics were dependent on $[V_i]$ with a higher $[V_i]$ leading to larger and faster force depression. The relationships for the amplitude and half-time obtained from 21 fibre

bundles are shown in Fig. 2d. The threshold of V_i action was 10–30 μM and at 3 mM V_i around 40% reduction of maximal force was observed. This magnitude of the force reduction was not changed substantially when V_i was increased further to 10 mM ($39.0 \pm 7.1\%$, $n=3$). Both the amplitude and kinetics of force decay were half maximal at about 0.4 mM V_i . Reversible force depression to $83.0 \pm 4.1\%$ ($n=3$) of the maximum was observed when 10 mM P_i was present during maximal Ca^{2+} activation (Fig. 2b). BDM (50 mM) had a similar effect (Fig. 2c), with force falling to $68.2 \pm 9.1\%$ ($n=3$) of maximum.

Stretch activation

As indicated in Materials and Methods the experiments described below were carried out on an isometric apparatus with a resonance frequency of the force-measuring system of 7.5 kHz. Stepwise stretches (≤ 1 ms) applied during maximal Ca^{2+} activation caused a simultaneous rise in force, followed by a decrease and a subsequent delayed increase, the latter called stretch activation (Fig. 3). Stretch activation has also been observed previously with freeze-dried ABRM preparations [14]. The kinetics of this force transient were accelerated in the presence of 1 mM V_i . The following parameters of the force transients were evaluated: t_2 , the time from the beginning of stretch to the lowest force before the onset of delayed rise in force and t_3 , the time from beginning of stretch to the peak of the delayed rise in force. Within a series of stretches at different amplitudes (0.1–0.3% of fibre length) for an individual fibre, t_2 and t_3 varied with an SD of less than 9% (t_2) or 6% (t_3) of the mean. Both t_2 and t_3 were significantly different from control ($P < 0.01$) in the presence of 1 mM V_i ($n=10$, Table 1). Application of 10 mM P_i or 50 mM BDM also accelerated the kinetics of stretch activation ($n=3$, Table 1). All these effects of V_i , P_i and BDM on stretch activation kinetics were reversible. Thus, after washing out these compounds, the kinetics of stretch activation were similar to those before their application.

Force transients following rapid ATP release

To study the effect of V_i , P_i and BDM on the attachment/detachment kinetics of cross-bridges, experiments with photoliberation of ATP from caged ATP were performed. Control and test experiments (presence of V_i , P_i and BDM) were carried out on the same fibre in varying order. Student's t -test for paired samples was used for statistical analysis. Before photoliberation of ATP the fibre bundles were transferred from EGTA-rigor solution to Ca^{2+} -rigor solution (pCa 4.7) to induce low-force rigor conditions. As seen in Fig. 4a the rapid force increase induced by photoliberation of ATP was followed by a force decline (starting about 10 s after the

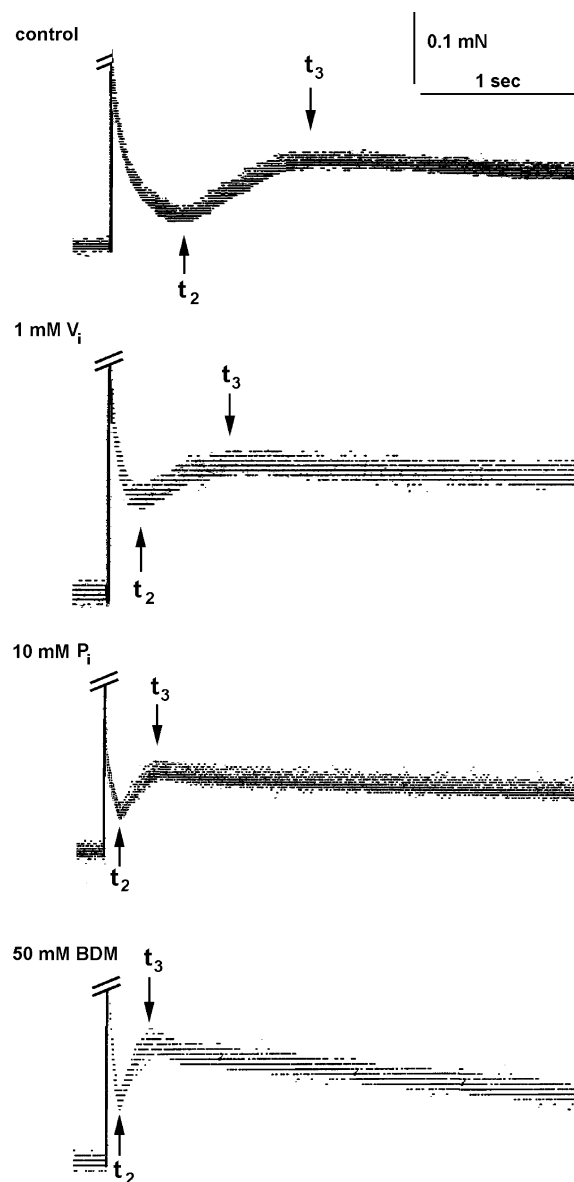


Fig. 3 Force responses of ABRM fibre bundles following stepwise stretches (≤ 1 ms, 0.1–0.3% of fibre length) applied during maximal Ca^{2+} activation showing the acceleration of the delayed force increase (time parameter t_3) and the immediate force decay following the stretch (time parameter t_2) in the presence of 1 mM V_i , 10 mM P_i and 50 mM BDM

flash) only when 1 mM V_i was present. Conversely, under control conditions a steady state was established. In the presence of V_i (1 mM) the initial force increase was faster than in control (Table 1). A slight acceleration of the force rise following quick ATP release was also observed in the presence of 10 mM P_i and 20 mM BDM (Table 1).

Figure 4b shows an experiment in which a rapid force decline was induced by flash photolysis of caged ATP in high-force rigor. In this case the fibre bundle was transferred from activation solution to Ca^{2+} -rigor (pCa 4.7) and then to EGTA-rigor (pCa > 8) solution before the photoliberation of caged ATP. The release of caged

Table 1 Effect of orthovanadate (V_i), inorganic phosphate (P_i) and 2,3-butanedione monoxime (BDM) on time parameters of stretch activation and parameters of rapid ATP release following caged-ATP photolysis. Means \pm SD; *min parentheses* (t_2 time from the beginning of the stretch to the lowest force value before the onset of delayed force increase, t_3 time from the beginning of the stretch to

the peak of the delayed force increase; $t_{1/2}$ (force rise) half-time of force rise induced by rapid ATP release in low-force rigor, $t_{1/2}$ (force decline) half-time of force decline induced by rapid ATP release in high-force rigor, T (fast relaxation) amount of force decay as a percentage of initial force during the initial fast force decline following rapid ATP release in high-force rigor)

Parameter	Control	V_i 1 mM	P_i 10 mM	BDM (20 or 50* mM)
t_2 (ms)	432 \pm 82 (16)	210 \pm 36 (10)	100 \pm 15 (3)	85.5 \pm 7.5 (3)*
t_3 (ms)	1377 \pm 254 (16)	700 \pm 72 (10)	333 \pm 26 (3)	270 \pm 30 (3)*
$t_{1/2}$ (force rise) (ms)	719 \pm 415 (10)	490 \pm 130 (6)	618 \pm 15 (3)	532 \pm 55 (3)
$t_{1/2}$ (force decline) (ms)	274.7 \pm 104.0 (6)	121.3 \pm 15.1 (3)	82.7 \pm 10.4 (3)	84.3 \pm 29.6 (3)
T (fast relaxation) (%)	58.9 \pm 5.8 (6)	64.3 \pm 2.4 (3)	60.7 \pm 4.4 (3)	68.8 \pm 6.3 (3)

All time parameters obtained in the presence of 1 mM V_i , 10 mM P_i and 20 or 50 mM BDM are significantly different from the values under control conditions ($P < 0.05$, paired Students t -test)

ATP led to a rapid force decrease followed by a slow force decline (slower than 2% of initial force per min). Compared with control, the initial force decrease was substantially faster in the presence of 1 mM V_i or 10 mM P_i (Fig. 4c) or 20 mM BDM (Table 1). After the initial rapid relaxation the force level reached around 40% of the initial force and was not significantly different from control both in presence of 1 mM V_i or 10 mM P_i (Table 1). The force decline during the slow relaxation phase was similar to that observed in the slow relaxation phase induced after Ca^{2+} removal (Fig. 1b) and was not influenced by V_i , P_i and BDM.

Effects of V_i , P_i , BDM and MgADP on the catch state

The effects of V_i , P_i and BDM on catch relaxation are shown in Fig. 1b. These compounds were added 3–60 min after Ca^{2+} removal and the duration of the exposure was 4–25 min. Addition of 1, 5 or 10 mM V_i and its subsequent removal did not affect the slow force decline during relaxation ($n = 5$) (Fig. 5a). The same was true for 10 mM P_i (Fig. 5b) and 50 mM BDM (Fig. 5c). V_i was also not effective when 0.5 or 1 mM MgADP was present during slow relaxation ($n = 4$). Addition of MgADP in the absence of V_i also did not affect force. As already shown in Fig. 1a, changing pH from 6.7 to 7.7 accelerated the relaxation considerably (Fig. 5a). It is thus clear that all investigated compounds (V_i , P_i , and BDM) substantially inhibited the force developed by skinned ABRM fibre bundles during maximal Ca^{2+} activation, but did not affect the catch state.

Discussion

The saponin method for skinning ABRM preparations was first described by Cornelius who reported that both maximum peak tension and rate of contraction are larger in saponin-treated ABRM than in Triton X-100-treated ABRM [7]. In that study the maximal Ca^{2+} -induced force after skinning was about 70% of the ACh-induced force [7]. In our experiments force after skinning was about 1.6 times higher than the ACh-induced force,

consistent with another ABRM study [6]. In the latter and in our studies a high sucrose concentration (0.7 M in [6] and 0.65 M in our study) was present during the skinning procedure. For optimal skinning of ABRM fibres it seems that adjustment of osmolarity of skinning solutions to that of artificial sea water (ASW) by sucrose is essential. The presence of a high sodium instead of a high potassium background during the first minutes of skinning and the use of BDM in the sodium skinning solution are presumably the reason why only little force generation occurred during our skinning procedure.

Effects of V_i , P_i and BDM at maximal Ca^{2+} activation

As in mammalian striated and smooth muscle [5, 9, 22, 23, 26, 35, 36, 55], V_i , P_i and BDM depressed the maximal Ca^{2+} -activated force in ABRM fibres. It is thought that V_i and P_i induce isomerisation to a stable non- or low-force-generating complex after binding to the AM.ADP state [9]. BDM is thought to act as a non-competitive inhibitor of the myosin ATPase and stabilises AM.ADP. P_i in a pre-powerstroke state. The force depression at saturating [V_i] and the V_i sensitivity are lower in ABRM than in other muscles. The force reduction elicited by V_i was about 40% of maximal force in ABRM, but is about 90% in skeletal muscle (rabbit psoas, [9]) and 80% in smooth muscle (taenia coli of guinea-pig, [26]). These differences can be explained by a smaller population of the AM.ADP state in ABRM than in other muscles. The [V_i] for half-saturation of force reduction was around 500 μ M in ABRM, but 100 μ M in rabbit psoas [9] and 10 μ M in guinea-pig taenia coli [26]. This observation can be explained by a lower affinity of the AM.ADP state for V_i in ABRM than in other muscles. The V_i -induced force depression was equally slow in ABRM and in mammalian smooth muscle [26].

The kinetics of stretch activation were accelerated by V_i , P_i , and BDM (Fig. 3). All compounds reduced both t_2 and t_3 . Based on the cross-bridge model of Kawai and colleagues [28, 29], this means that V_i , P_i , and BDM accelerate both the detachment and force-generation steps of ABRM cross-bridges. The same conclusion can be drawn from our caged ATP experiments. V_i (1 mM),

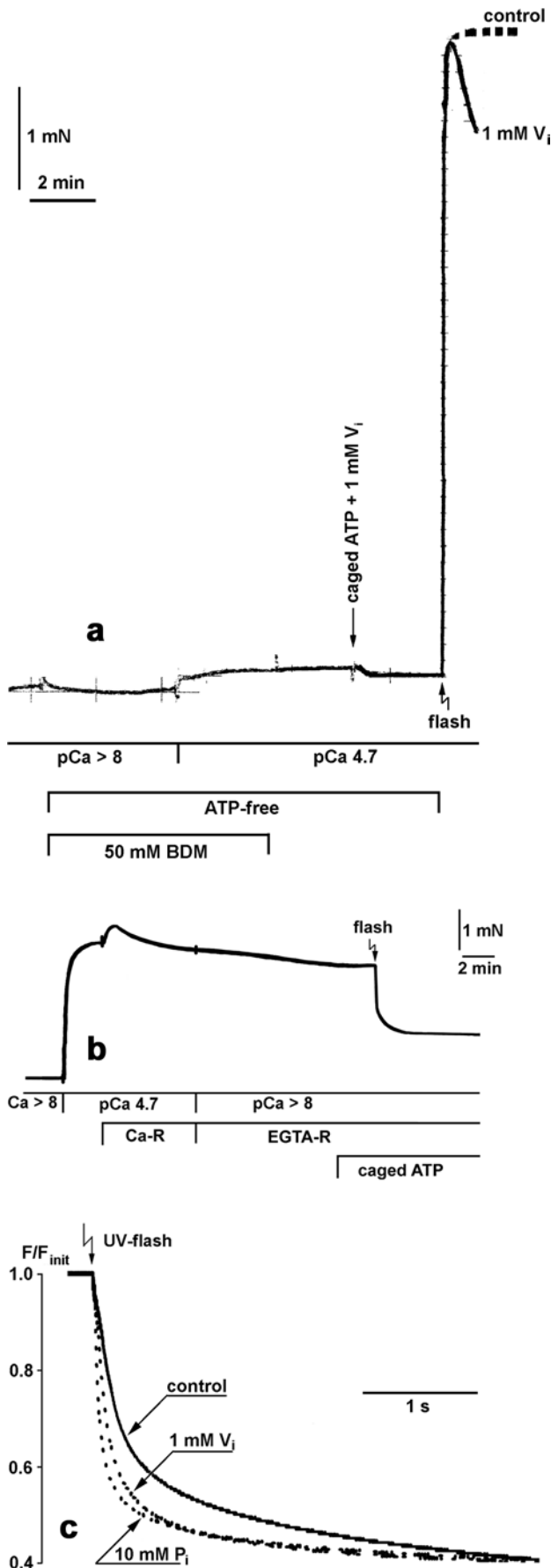


Fig. 4a–c Force recordings (at a low time resolution) from experiments in which ATP was released rapidly by UV-flash photolysis of caged ATP. In **a** ATP was released rapidly after induction of low-force rigor, in **b** after induction of a high-force rigor. In the presence of 1 mM V_i , the ATP-induced force rise was followed by a force decay (**a**). This decay was absent under control conditions (*dotted line*). **c** Superimposed, high-time-resolution force recordings of rapid ATP release experiments under control conditions and in the presence of 1 mM V_i and 10 mM P_i

10 mM P_i and 20 mM BDM significantly ($P < 0.05$) accelerated both the rise (primarily attachment) and the decline of force (detachment) from low-force rigor and high-force rigor, respectively (Table 1, Fig. 4c). Thus, V_i , P_i and BDM obviously exert qualitatively similar effects on cross-bridges in all muscles, independently of their types of Ca^{2+} regulation. Contraction is regulated by Ca^{2+} -binding to the myosin heads in ABRM [30], by Ca^{2+} -dependent phosphorylation of the regulatory myosin light chain in vertebrate smooth muscle [47] and by Ca^{2+} -binding to the thin filament protein troponin [12] in vertebrate skeletal muscle. This supports the common assumption that the basic mechanism of cross-

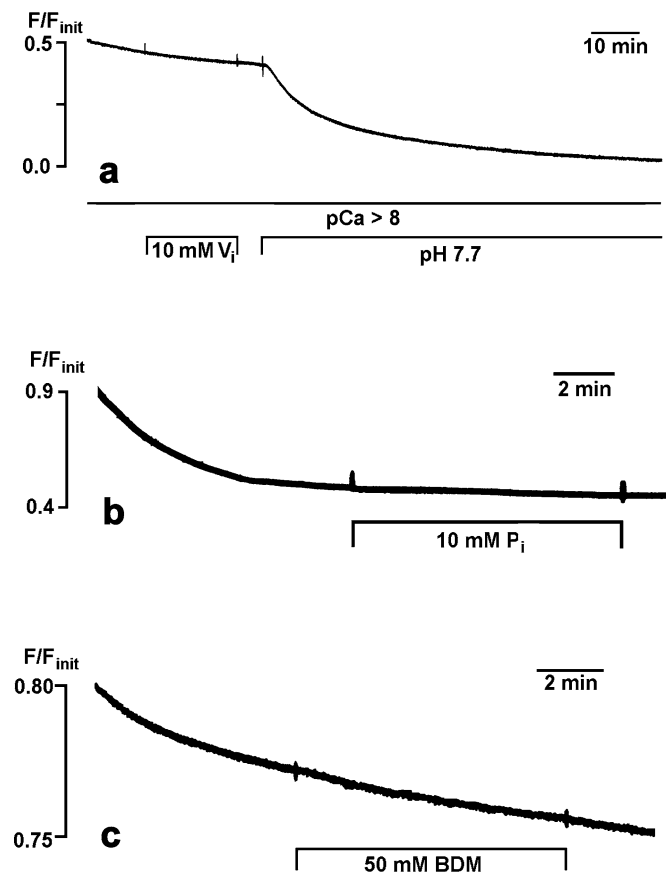


Fig. 5a–c Slow relaxation during catch induced by removal of Ca^{2+} (pCa > 8) after maximal Ca^{2+} activation (pCa 4.7). This relaxation was accelerated by changing from pH 6.7 (pCa > 8) to pH 7.7 (pCa > 8) (**a**), but it was not affected by 10 mM V_i (**a**), 10 mM P_i (**b**) or 50 mM BDM (**c**)

bridge action is the same in all muscle types independent of their regulation. Thus, there is no evidence that cross-bridges of catch muscle have any basic peculiarities that are essential in the context of the catch phenomenon.

Effects of V_i , P_i and BDM on the catch state

Although V_i , P_i and BDM exhibited remarkable effects on cross-bridge kinetics and isometric tension, these compounds had no effect on the rate of relaxation of ABRM during catch (Fig. 5). In our study, the catch state was induced in two types of experiments. First, when the preparations were transferred from maximal activation solution to relaxation solution, slow relaxation took place. Second, the catch state was also induced in experiments in which ATP was released rapidly in high-force rigor by UV flash photolysis of caged ATP. It seems that the same type of catch state is induced in both types of experiments [4, 17], because the conditions are qualitatively the same: Ca^{2+} is absent and ATP present. In both cases the slow relaxation phase appeared not to be affected by 1–10 mM V_i , 10 mM P_i or 50 mM BDM (Fig. 5). Furthermore, the amplitude of the initial fast force decay after ATP release was also not influenced by these compounds (Table 1). All these results suggest that V_i , P_i and BDM are not able to affect the catch state of ABRM.

The most popular theory for explaining the catch phenomenon is based on the effect of Ca^{2+} on the release of ADP. It is assumed that the decrease of free $[Ca^{2+}]$ after a tonic contraction [25] markedly slows ADP release so that the cross-bridges remain in a force-generating state (AM.ADP state) [3, 4, 17, 41, 45]. Whilst V_i , P_i and BDM are known to affect the AM.ADP state, our study showed that V_i , P_i and BDM did not affect force during catch. It is therefore doubtful whether catch is based on a locked AM.ADP state. V_i did not exhibit any effects, even in the presence of 0.5–1 mM MgADP, which would be expected to increase the population of the AM.ADP state. It therefore seems unlikely that catch is related to force-generating actomyosin cross-bridges at all. The conclusion that cross-bridges are not involved in the catch state is also supported by the latter's high pH sensitivity (Figs. 1a, 5a). The model for explaining catch by the force-generating actomyosin cross-bridges could only remain valid if the cross-bridges became insensitive to V_i , P_i and BDM and surprisingly highly sensitive to pH during catch.

Our conclusion concerning the molecular mechanism underlying catch seems to be contradicted by an observation of Butler et al. [4]. Using radioactively labelled ATP, those authors observed extra ADP production when catch was released by cAMP (see Fig. 6B of [4]). They concluded that this extra ADP originates from cross-bridges that continue the cycle and detach following the cAMP-induced twitchin phosphorylation. However, this extra ADP might also be produced when the PKA transfers the phosphate from ATP to twitchin

during phosphorylation. Thus, the measured ADP is not necessarily released from the myosin heads.

In 1963, Rüegg et al. [42] observed that thiourea inhibited actomyosin cross-bridges whilst the catch state was still inducible. In addition, a 5-HT-sensitive catch state (resistance against stretch) can be induced by a PCO_2 of 100 mmHg at 10 °C in the absence of any contraction [40]. The CO_2 -induced catch state is supposed to be associated with the intracellular acidification, which occurs when a muscle is exposed to bath solutions containing high PCO_2 [16]. From these observations Rüegg [40] also concluded that catch is not based on the force-generating actomyosin cross-bridges but rather on parallel structures. The present study complements and extends this experimental approach by using more recently identified agents, i.e. V_i and BDM, that affect the force-generating actomyosin cross-bridges more specifically than thiourea.

The ability to maintain tension at low energy consumption is also present, though to a lesser extent, in mammalian smooth muscle (the latch-state, [11]). BDM (7.5 mM) depresses force in electrically stimulated, intact mammalian smooth muscle without affecting the latch-state [37], implying that the slowly cycling cross-bridges, which are thought to account for the latch-state, are not affected by BDM. It is doubtful if this conclusion can be drawn from the present experiments because a subsequent study on skinned mammalian smooth muscle has shown that a much higher concentration of BDM (60 mM) is required to affect the activated contractile machinery [36]. Furthermore, BDM decreases the intracellular free $[Ca^{2+}]$ and leads to a lower level of phosphorylation of regulatory myosin light chains [36, 43]. Thus, the force depression induced by 7.5 mM BDM [37] is probably due to a lower level of activation, without the cross-bridges being affected directly in either the Ca^{2+} -activated or latch states.

Effects of cAMP and alkalisation on the catch state

Addition of 0.12 mM cAMP considerably increased the relaxation rate during catch (Fig. 1a). This is due to phosphorylation of twitchin by PKA [45]. It is noticeable that the relaxation in the presence of cAMP was never complete, even after 1 h. This seems to be in contrast to results of other laboratories [38, 44]. The discrepancy is most likely due to the fact that in our experiments an auxotonic apparatus was used, whereas other laboratories used isometric apparatuses. In contrast to isometric conditions, under auxotonic conditions the shortening (filament sliding) which occurs during contraction has to be reversed for complete relaxation. Re-stretching a shortened muscle may require a greater effort than relaxing a muscle under isometric conditions so that auxotonic conditions may favour force maintenance. In principle, the non-complete relaxation with cAMP could be due to a continuous dephosphorylation of twitchin by a phosphatase.

However, this is unlikely in view of a very recent finding that the Ca^{2+} -dependent protein phosphatase 2B is involved in dephosphorylation of twitchin [52]. In our experiments, $[\text{Ca}^{2+}]$ was below 10^{-8} M when cAMP was added so that the activity of this phosphatase is presumably very low under our experimental conditions. Furthermore, 20 nM microcystin, an inhibitor of other phosphatases (e.g. [48]), did not affect the relaxation with cAMP. Further (almost complete) relaxation could be reached when the pH of the relaxation solution ($\text{pCa} < 8$) was changed from 6.7 to 7.7. Therefore, it seems that twitchin phosphorylation alone is not sufficient to abolish the catch state completely; in addition, alkalisation seems to be required. This fits well with the observation of slight alkalisation when the catch state of intact ABRM is abolished by 5-HT [53]. As seen in Fig. 1a, 5-HT completely relaxes the muscle in the intact state. Obviously, this is due to both twitchin phosphorylation and alkalisation which occurs in the presence of 5-HT.

How cAMP-induced phosphorylation of twitchin and alkalisation abolish the catch state is not known. Butler et al. [4] have assumed that twitchin phosphorylation induces detachment of ATP-insensitive myosin heads from actin. However, the exact location of twitchin within the contractile machinery of ABRM and the possibility of an interaction with the myosin heads is not known. In principle, it appears questionable if there are sufficient twitchin molecules to regulate the large number of myosin heads. In addition to the insensitivity of the catch state to V_i , P_i and BDM, this is a further argument leading to the conclusion that catch is not based on force-generating actomyosin cross-bridges but rather on separate structures linking the myofilaments.

Aggregation of the thick filaments during catch in ABRM is a well known phenomenon [18, 21, 24, 46], although it has been argued that this may reflect an artefact caused by glutaraldehyde cross-linking during the fixation procedure for electron microscopy. On the other hand, electron micrographs show visible interconnections of adjacent thick filaments that seem to be formed by distinct projections [46, 49]. Since the interconnections are much more frequent during the catch state [49], they could be involved in force maintenance during catch. Twitchin could be the structure interconnecting the thick filaments [34]. If this were the case, the obvious corollary is that phosphorylation of twitchin and alkalisation cause a break-down of the interconnections (twitchin detachment) resulting in the abolishment of catch. However, a disconnection of myosin filaments could affect relaxation only if relative displacements of adjacent myosin filaments occur. Although this is unlikely under isometric conditions, such displacements are feasible under isotonic or auxotonic conditions (our measurements). In obliquely striated muscles of annelids such parallel movements of myosin filaments have been shown ([33, 39, reviewed in [41]). Furthermore, there are similarities in the ultrastructure

between ABRM and obliquely striated invertebrate muscles, especially concerning the shape of the dense bodies that anchor the actin filaments [46]. Thus, there is evidence to assume that also in the ABRM length changes of muscle fibres are accompanied by parallel displacements of myosin filaments. Consequently, interconnections between adjacent myosin filaments which are formed after shortening would support the passive load-bearing ability of the muscle during catch. However, these interconnections alone are not sufficient. For transmission of force along the contractile machinery of the whole muscle fibres the myosin filaments have to be connected to the dense bodies either via the actin filaments or via other structures. In vertebrate skeletal muscle titin connects the myosin filaments with the Z-disk (dense body analogue); in addition a connection of the myosin filaments with the actin filaments has been found [31]. In analogy to these findings on vertebrate skeletal muscle, in ABRM the titin-like protein twitchin could connect the myosin filaments with actin filaments and/or with the dense bodies. These connections could theoretically supply the load bearing ability of ABRM during catch.

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