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ORIGINAL ARTICLE

C. Veigel · R. D. v. Maydell · R. Wiegand-Steubing
R. Goody · R. H. A. Fink

The influence of ionic strength upon relaxation from rigor induced by flash photolysis of caged-ATP in skinned murine skeletal muscle fibres

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Abstract The influence of ionic strength upon relaxation kinetics from rigor in skinned murine extensor digitorum longus (EDL) skeletal muscle fibres was examined using photolysis of caged-ATP at low Ca^{2+}. The ionic strength was adjusted with either $KMeSO_3$ or ethylene glycol bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, dipotassium salt (K_2EGTA) in the range of $\Gamma/2$ = 65–215 mM, or I.E. 49–194 mM, where I.E. denotes ionic equivalent. Following rigor development at a $\Gamma/2$ of 165–215 mM (I.E. 144–194 mM), the liberation of approximately 0.5 mM ATP resulted in an initial 6-to 10-ms detachment phase with a decline in force of approximately 10–20% followed by a 10-to 30-ms reattachment with up to a 60% increase compared to the corresponding rigor level and a final detachment leading to complete relaxation. Interestingly, when similar ATP concentrations were liberated at lower ionic strengths between a $\Gamma/2$ of 65 mM and 110 mM (I.E. 60–100 mM), the initial detachment phase was shortened and force decreased by only approximately 5–10%, while the following reattachment phase was lengthened and led to an increased steady-state force of approximately 20–80% without final relaxation. ATP-induced detachment and subsequent reattachment were mainly determined by the currently present ionic strength and were relatively independent of the preceding rigor state which had been developed at higher or lower ionic strengths. The effects of phosphate and apyrase on the force transient suggest that reattachment of ADP- binding crossbridges may contribute to the increase in tension at high and even more at low ionic strengths. The study shows that the kinetics of initial fast relaxation and subsequent redevelopment of force following flash photolysis of similar ATP concentrations are markedly modified by the ionic strength in the narrow range of between 65 mM and 215 mM.

Key words Ionic strength · Caged-ATP · Rigor · Skeletal muscle

C. Veigel · R.D. v. Maydell · R. Wiegand-Steubing · R. H. A. Fink (✉)
II. Institute of Physiology, University of Heidelberg, Im Neuenheimer Feld 326, D-69120 Heidelberg, Germany
R. Goody
Max Planck Institute of Molecular Physiology, Dortmund, Germany

Introduction

Recent investigations into the atomic structure of myosin and actin have led to a detailed proposal of the molecular mechanisms of actin-myosin interactions [17, 25, 26]. The first step of the force-generating cycle may involve a weak electrostatic interaction between the negatively charged carboxyl groups of the actin *N*-terminus (residues 1–4, 24 and 25) and the positively charged lysine residues of the flexible loop region of myosin (residues 626–647). It has been suggested that this interaction may be responsible for the formation of weakly binding crossbridges, which may represent an essential step in the process of force generation (for reviews [3, 5]). If these interactions are electrostatic in nature, they would be expected to be dependent on ionic strength. Indeed, it has been found that lowering ionic strength greatly promotes the formation of weakly binding crossbridges and increases the steady-state force of Ca^{2+}-activated contraction and rigor tension [1, 3, 4, 9, 15, 18]. Decreasing ionic strength also affects crossbridge kinetics and increases the apparent sensitivity to Ca^{2+} of the contractile system. It is also well known that the steady-state rigor tension decreases with increasing ionic strength (e.g. [9]). However, it is still unclear whether the decrease in tension is due to a decrease in the number of attached rigor crossbridges with increasing ionic strength, as has been suggested for Ca^{2+}-activated tension (e.g. [18]), or whether the rigor crossbridges can be locked in different force-and non-force-generating states which were determined by

the ionic strength. In the present study, we have investigated the effect of ionic strength upon the relaxation kinetics from rigor using flash photolysis of caged-ATP at low Ca^{2+}.

The data show a decrease in sensitivity to ATP at low ionic strength. The relaxation kinetics seem to be determined mainly by the actual ionic strength during flash photolysis of ATP and independent of the preceding ionic-strength-dependent rigor state. Furthermore, experiments with apyrase and phosphate suggest that the decrease in sensitivity to ATP observed with lowering the ionic strength might be related to an increased ADP affinity of the acto-myosin ATPase. Some of the results have previously been published in abstract form [19].

Materials and methods

Muscle fibre preparations

Small bundles (diameter less than 100 μm) of skeletal muscle fibres were isolated from the extensor digitorum longus (EDL) of male Balb/c mice sacrificed by ether overdose. The mean diameter of the fiber bundles was estimated to be 60–80 μm, the length was 1.25–2.0 mm. The isolated fibre bundles were kept refrigerated under viscous paraffin oil before being mounted on a force transducer (model 801, SensorNor, Norway). To provide sufficientlyhigh intrinsic creatine phosphate kinase ATP-regenerating activity within the fibres, only freshly prepared fibre preparations were used.

Solutions

The ionic strength of the solutions is expressed by: (1) $I/2 = 1/2\Sigma c_i z_i^2$ according to the Debye-Hückel theory, where c_i are concentrations and z_i charges of the ith ion species, and (2) by the ionic equivalents I.E. $= 1/2\Sigma c_i|z_i|$ [9, 30]. Total and free ion concentrations were calculated using the computer programme REACT Vers. 2.03, 1991, kindly provided by Dr. G.L. Smith, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK. Absolute binding constants were taken from Fabiato and Fabiato [8] and Godt and Lindley [12], and corrected for the ionic strength or ionic equivalents of the solutions. The experiments were performed using the following types of solutions (details and compositions see below): high Ca^{2+}-activating solution, HA; ethylene glycol bis-(β-aminoethyl ether) *N,N,N'N'*-tetraacetic acid, dipotassium salt (K_2EGTA) relaxing solution, HR; low EGTA relaxing solution, LR; and ATP-free rigor solution, R. LR solutions were similar to HR solutions with EGTA replaced by the same concentration of 1, 6-diaminohexane-*N, N, N,'N'*-tetraacetic acid (HDTA). Two sets of solutions were prepared, one using K_2EGTA, the other using $KMeSO_3$ to adjust for different ionic strengths with a divalent or a monovalent anion. Both salts have previously been used for adjusting either $I/2$ or I.E. to differentiate mainly ionic-strength-dependent effects from ion-specific changes (Veigel et al. unpublished results). pH was adjusted to 6.70 ± 0.01 with KOH. In all solutions $Mg(OH)_2$ was added to give 1 mM free Mg^{2+}. All solutions were stored at −20° C. Experiments were performed at room temperature (18–22° C).

K_2EGTA solutions

The four types of solutions, HA, HR, LR and R solutions, were adjusted using K_2EGTA to the following three different ionic strengths: $I/2$ = 86 mM (10 mM EGTA), $I/2$ = 116 mM (20 mM EGTA), $I/2$ = 238 mM (60 mM EGTA), corresponding to the following I.E. (values) = 56 mM, 76 mM, 156 mM. To HA solutions, $CaCO_3$ was added to give a pCa i.e. $-\log_{10}[Ca^{2+}]$ of 4.50, HR, LR and R solutions had a pCa of ≈ 9.0. To HA, HR and LR solutions, 8 mM Na_2ATP, 10 mM disodium creatine phosphate (Na_2CP) and 152 U/ml creatine phosphate kinase were added as a ATP-regenerating system to give MgATP concentrations between 7.2 and 7.4 mM according to the ionic strength of the solutions. In R solutions, 8 mM Na_2ATP and 10 mM Na_2CP were replaced by 18 mM Na_2HDTA. All solutions contained 10 mM imidazole to buffer the pH.

$KMeSO_3$ solutions

HA, HR, LR and R solutions were adjusted using $KMeSO_3$ to the following ionic strengths: (1) the formal ionic strengths of the HA/HR solutions were: $I/2$ = 65 mM (1.1/8.9 mM $KMeSO_3$, 3.5 mM EGTA), $I/2$ = 110 mM (13.5/34.0 mM $KMeSO_3$, 10.0 mM EGTA), $I/2$ = 165 mM(18.9/58.5 mM $KMeSO_3$, 20.0 mM EGTA), $I/2$ = 215 mM (68.2/108.1 mM $KMeSO_3$, 20.0 mM EGTA), (2) the corresponding I.E. values of the HA/HR solutions were 49, 88, 132 and 182 mM. In R solutions, $I/2$ values of 65, 110, 165 and 215 mM (I.E. 60, 100, 144 and 194 mM) were adjusted by the respective to addition of 51.0, 75.6, 99.2 and 149.3 mM $KMeSO_3$. The EGTA concentrations were the same as described for the HA/HR solutions. pCa was adjusted with $CaCO_3$ to give 4.00 in HA solutions, ≈9.0 in HR and LR solutions and 7.00 in R solutions. All solutions contained 20.0 mM imidazole to buffer the pH. To HA, HR and LR solutions 5.4–5.7 mM Na_2ATP, 5.0 mM creatine phosphate and 152 U/ml creatine phosphate kinase were added to give a MgATP concentration of 5.00 mM.

Caged-ATP solutions

NPE-caged-ATP [P^3-1-(2-nitrophenyl)-ethyl ester of Na_2ATP Calbiochem, La Jolla, Calif., USA] was dissolved in R solutions to give a final concentration of 5 mM. Gluthathione (10 mM) was added to the caged-ATP solutions prior to the experiments to react with and inactivate the photolytic by-product 2-nitrosoacetophenone.

Apyrase

To lower ADP levels within the fibre in rigor state and to remove ADP contamination from caged-ATP solutions, the fibre preparation was incubated in some experiments in R solution with 18 U/ml apyrase grade VII (Sigma, Deisenhofen, Germany) for 10 min, followed by another 6-min incubation in caged-ATP solution with 10 U/ml apyrase. The ADP contamination of the untreated caged-ATP solution was estimated by HPLC to be approximately 10 μM. In caged-ATP solutions treated with apyrase the ADP contamination was lowered to less than 2.5 μM.

Phosphate

In some expriments, potassium phosphate was added to the caged-ATP solutions to test the effect of phosphate on the force transients at low and high ionic strengths. The contribution of phosphate to the ionic strength has been taken into account.

Experimental protocol

For the force measurements with a high time resolution, the output signal of the bridge amplifier was digitally recorded with an

A/D,D/A converting system (TL-1-125, Axon Instruments, Foster City, Calif., USA) which was driven by a 486 IBM-compatible computer. The data were sampled at 1–30 kHz using pClamp 5.5.1. software (Axon Instruments). The myofibre bundles were skinned for 6min using either 50 µM β-escine (Sigma) or 1% Triton X-100 (Boehringer, Mannheim, Germany) in LR solution. The sarcomere length was determined with a diode laser (670 nm) and adjusted to 2.6–2.7 µm (for details see also [10]). After skinning, the maximum Ca^{2+}-activated tension was obtained as a control procedure in HA solution. Subsequently, the preparation was relaxed in HR solution. Rigor state was induced in ATP-free R solution.

After the onset of the plateau phase, the preparation was incubated for approximately 4 min in the caged-ATP-containing solution to ensure complete diffusion of caged-ATP into the fibre preparation. Then, the ATP level was raised by ultraviolet (UV) flash photolysis of caged-ATP. If the fibre did not relax, complete relaxation was induced by HR solutions. No more than two to three rigor states were induced without controlling the maximum Ca^{2+}-activated force with HA solution. The experiment was stopped when the maximum tension decreased by more than 15% of the initial value at the respective ionic strength. Solutions were exchanged by raising the fibre bundle out of the trough before immersing it into another trough containing the new solution. Thus, the fibre was suspended in air for no more than 2–3 s.

All experiments were carried out at room temperature (20–23° C). Mean values of the data are given ± SEM.

Photolysis of caged-ATP

A high intensity xenon arc flash lamp (model JML, Rapp Optoelektronik, Hamburg, Germany) was used in our photolysis experiments [23, 24]. The pulse length of this broad range arc lamp was about 0.4 –1.0 ms and the maximum pulse energy was 120 mJ for the wavelength range between 300 nm and 400 nm. A low-pass filter with high transmittance in the UV range, but low transmittance in the spectral range above 400 nm, was placed between the focusing lens and the experimental chamber to avoid temperature changes of the preparation. For photolysis experiments 15 µl of the caged-ATP solution was placed into a small experimental chamber which was formed from two pieces of coverglass.

Quantitative determination of UV-induced ATP liberation at increasing ionic strengths

To find out whether the observed modifications in the force transients following flash photolysis of 5 mM caged-ATP at increasing ionic strengths were due to unspecific ionic strength effects or other effects, such as differences in ATP release from caged-ATP at different ionic strengths, we measured the UV flash photolysis of caged-ATP in the respective R solutions using two independent methods. After photolysis of 5 mM caged-ATP in R solutions of different ionic strengths, 15 µl of the reaction mixture was quickly frozen in liquid nitrogen and stored at −20 °C. The concentration of ATP liberated upon photolysis was determined both enzymatically according to Lamprecht and Trautschold [20] and chromatographically using HPLC.

The results of the chromatographic determination using HPLC are shown in Fig. 1. Both the chromatographic and the enzymatic determination [20] methods confirmed that there is no systematic tendency indicating an increase or decrease of ATP release with increasing ionic strength over the given range. Furthermore, the change in pH in the R solutions of $\Gamma/2$ = 65–215 mM due to the release of equimolar H^+ concentrations following photolysis of ≈ 0.5 mM ATP was estimated to be < 0.1 pH units (from pH 6.7 to pH ≈ 6.65–6.63).

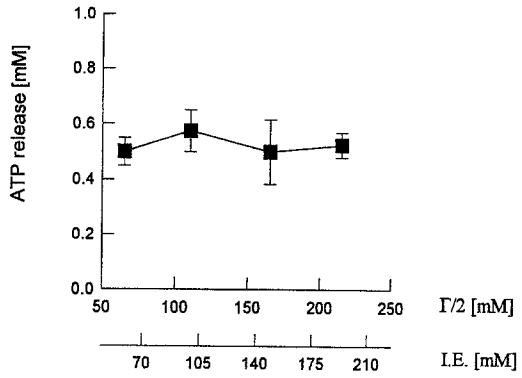


Fig. 1 ATP release following flash photolysis of 5 mM caged-ATP at increasing ionic strengths, determined using HPLC. ATP release induced by flash photolysis using a constant voltage seems to be independent of ionic strength between 65 and 238 mM. The ionic strength was adjusted using $KMeSO_3$. Each point represents the mean value ± SEM of 4–5 experiments. See Materials and methods for the definitions of $\Gamma/2$ and ionic equivalent (I.E.)

Analysis of the force transients

Following the nomenclature of Goldman and coworkers [13, 14], the complex sequence of force transients following flash photolysis of ATP can be described by an initial phase of relaxation (a_t) which is followed by a transient increase in force (b_t) before the final complete relaxation (c_t and d_t, Fig. 2, also [7, 13, 14,]). The first phase a_t following flash photolysis of ATP was defined to start immediately after the flash artefact and to end when the minimum tension was reached before the tension increased again. The subsequent phase b_t of force redevelopment was defined to end when the maximum tension was reached before the following decrease in tension. In the present paper we described and compared the phases a_t and b_t of the force transients which were observed over the whole range of ionic strengths used in this study ($\Gamma/2$ = 65–215 mM).

Determination of the detachment rate

The detachment rate of rigor crossbridges was estimated according to Goldman et al. [13]. Small stretches of 0.5–0.8% of the fibre length were applied 0.45–0.77 s (0.59 ± 0.12 s) prior to the flash photolysis of ATP. Following the idea that the time courses of tension for control and prestretch conditions converge when all prestretched rigor crossbridges have detached, the detachment rate of rigor crossbridges can be determined by the half-time for convergence of the force transients. The time of convergence is determined by arithmetic subtraction of the respective force transients.

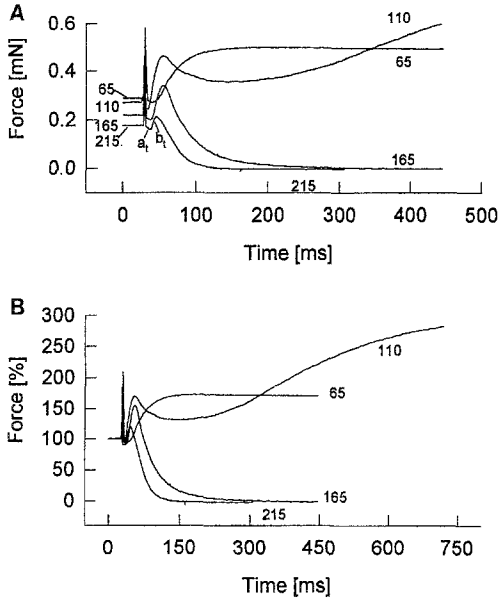
Results

Force transients from rigor following flash photolysis of caged-ATP at increasing ionic strength

The present study was designed to investigate the influence of ATP and ionic strength on the fast kinetics of relaxation from rigor in chemically "skinned" skeletal muscle fibre preparations. Therefore rigor was induced in ATP-free rigor solutions at different ionic strengths in the range of $\Gamma/2$ = 65–215 mM and I.E. = 56–194 mM. When the rigor tension had

reached its steady-state level, the fibre preparation was incubated for another 4 min in R solution of the same ionic strength containing 5 mM caged-ATP before relaxation was induced by flash photolysis of ATP. Figure 2A shows representative force transients following flash photolysis of 5 mM caged-ATP at four different ionic strengths adjusted with $KMeSO_3$. The steady-state rigor tension decreased with increasing ionic strength. At low ionic strength ($\Gamma/2$ = 65 mM) the mean rigor tension was 0.27 ± 0.03 mN (n = 10) whereas at high ionic strength ($\Gamma/2$ = 215 mM), the mean rigor tension reached only 0.18 ± 0.05 mN (n = 7). At $\Gamma/2$ = 165 mM and 215 mM, (I.E. = 144 mM and 194 mM, Fig. 2B), the initial phase a_t (see Materials and methods) of 5–10 ms with a decline to 90–80% of the preceding rigor tension was followed by the fast transient phase b_t with an increase in tension to 120–160% within 14–20 ms before the final complete relaxation, confirming the results of Goldman et al. [13] and Dantzig et al. [7]. In contrast, at lower ionic strength ($\Gamma/2$ = 110 mM and 65 mM, I.E. = 100 mM and 60 mM, Fig. 2B) the final relaxation phase was absent and a steady-state force at 160–250% of the preceding rigor tension was reached and kept constant for several minutes. The initial relaxation phase a_t was shorter (3–6 ms) and tension decreased to only 90–94% of the preceding rigor force, while the following increase in tension, phase b_t, took longer (17–100 ms) and led to greater increases in tension (165–175%).

Fig. 2 A,B Force transients following liberation of ≈0.5 mM ATP via flash photolysis at four different ionic strengths. The data were sampled at 10 kHz. **A** Absolute force values are plotted against time. The fibre dimensions were 74.5 ± 7.6 µm in diameter and 1.63 ± 0.3 mm in length. **B** The forces were normalized with respect to the steady-state rigor tension preceding the flash photolysis of caged-ATP. Note that at $\Gamma/2$ values of 165 mM and 215 mM (I.E. 144 mM and 194 mM), after the initial relaxation (a_t) and subsequent tension increase (b_t), the preparation finally relaxed (phases c_t and d_t according to Goldman et al. [12], Dantzig et al. [7], see Results and Materials and methods). In contrast, at $\Gamma/2$ values of 65 mM and 110 mM (I.E. 60 mM and 100 mM), initial relaxation (a_t) and a subsequent fast tension increase (b_t) were also observed, but the final relaxation was absent. Instead there was an increase in steady-state tension by 70–150% compared to the corresponding rigor force



Effects of increasing ionic strength upon the fast initial relaxation phase a_t and the succeeding phase b_t following photolysis of caged-ATP

To compare the effects of ionic strength upon the initial relaxation phase a_t and the following phase b_t of fast tension increase more quantitatively, the duration of the a_t and b_t phases and the decreases and increases in tension related to the preceding steady-state rigor force were evaluated for eight to ten experiments. The results are summarized in Fig. 3A-D. We characterized the effects of ionic strength on the force transients by evaluation of the duration and magnitude of the change in tension in the initial relaxation phase a_t and the following phase of tension increase b_t. Figure 3A shows that the maximum decrease in tension at the end of the a_t phase reached to 92 ± 1.5% of the preceding rigor force at $\Gamma/2$ = 65 mM, whereas at $\Gamma/2$ = 215 mM tension decreased somewhat more to 80.4 ± 4.5% related to the preceding rigor level when $KMeSO_3$ was used to increase the ionic strength (open circles). Furthermore, the duration of the a_t phase was prolonged from 4.3 ± 1 ms to 8.6 ± 2.4 ms when the ionic strength was increased from $\Gamma/2$ = 65 mM to 215 mM (Fig. 3B). Interestingly, the effects of increasing ionic strength within this relatively small range upon the succeeding phase b_t were even more pronounced. The peak increase in tension during the b_t phase was reduced from 164 ± 10% to 107 ± 8% relative to the preceding rigor tension (Fig. 3C). In addition, the duration of the b_t phase markedly decreased from 80 ± 17 ms to 14 ± 6 ms (Fig. 3D) when the ionic strength was raised. When increasing the ionic strength with the divalent cation $EGTA^{2-}$ (filled circles) very similar effects were observed (n = 3–4) compared to those obtained when using $KMeSO_3$ to adjust the ionic strength, indicating that the effects of increasing ionic strength upon relaxation from rigor could not be explained by specific effects of either ion used.

ATP-dependent force transients at low and high ionic strength

It is known that at high ionic strength (e.g. 200–300 mM) an increase in steady-state tension following rigor can be induced by addition of micromolar ATP concentrations in the absence of Ca^{2+} (e.g. [13, 27]) whereas higher ATP concentrations induce relaxation.

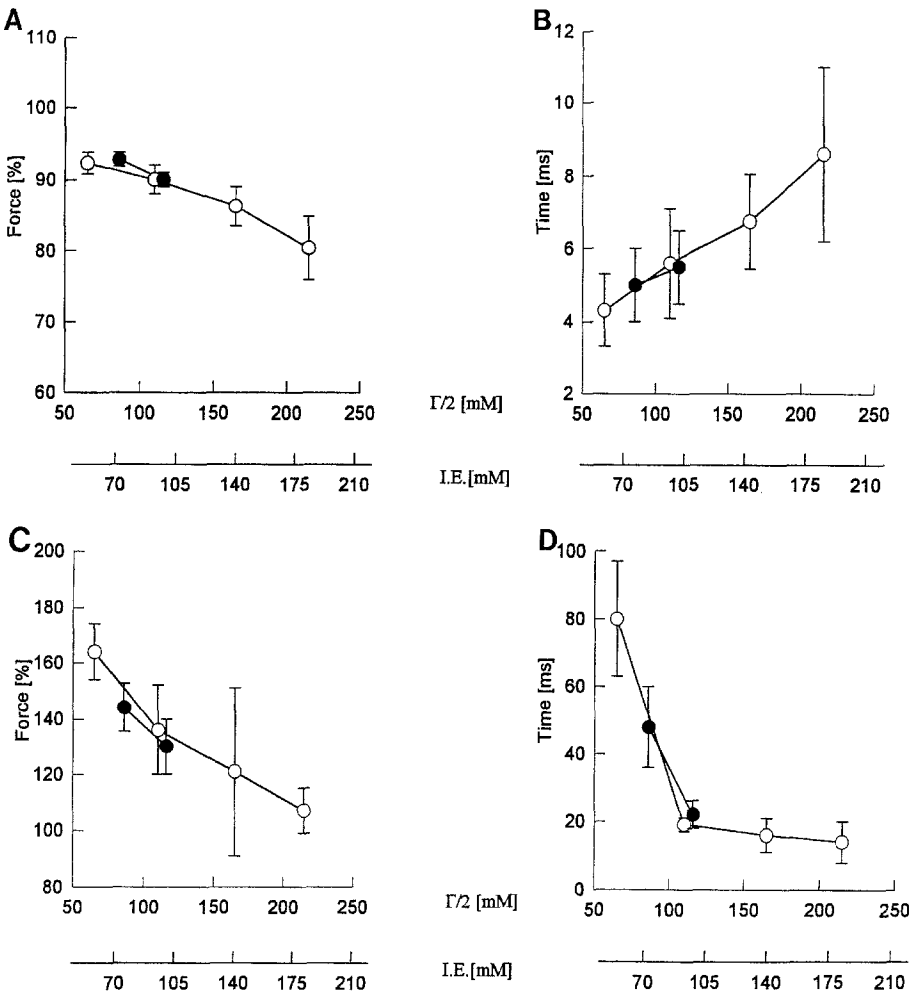


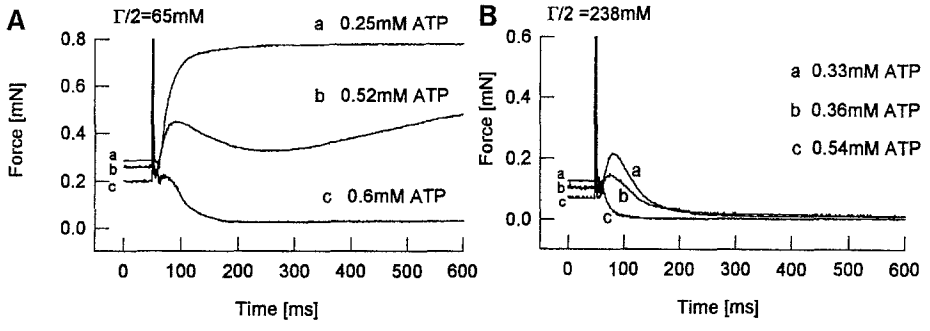
Fig. 3 A–D Effects of increasing ionic strength on initial relaxation (a_t) and the subsequent tension increase (b_t) (see Materials and methods). **A** The maximum decrease in tension during phase a_t with increasing ionic strength. **B** The duration of phase a_t has been evaluated. **C** The decrease in the maximum tension of b_t phase is shown. **D** The duration of phase b_t (time-to-peak) with increasing ionic strength is shown. Each data point represents the mean value ± SEM obtained from 8–10 experiments. The absolute values for the steady-state rigor tension preceding photolysis of caged-ATP were 0.27 ± 0.03 mN at $\Gamma/2$ = 65 mM and 0.18 ± 0.05 mN at $\Gamma/2$ = 215 mM. Ionic strength was increased with either $KMeSO_3$ (*open symbols*) or K_2EGTA (*closed symbols*). The ionic strength is expressed by $\Gamma/2$ according to the Debye-Hückel Limiting Law [*upper scale*, valid for K_2EGTA (●) and $KMeSO_3$ (○) data] and by the ionic equivalents I.E. (*lower scale*, valid only for the $KMeSO_3$ data; for the K_2EGTA data, I.E. was calculated to be 56 mM and 76 mM)

Figure 4 shows ATP concentration-dependent force transients following rigor state at low ($\Gamma/2$ = 65 mM) and high ($\Gamma/2$ = 238 mM) ionic strengths. The data show that lowering the ionic strength from $\Gamma/2$ = 238 mM to $\Gamma/2$ = 65 mM strongly reduced the sensitivity to ATP such that photolysis of 0.6–0.7 mM ATP was required to induce relaxation (Fig. 4B).

Fig. 4 A,B ATP concentration-dependent force transients at low ($\Gamma/2$ = 65 mM, **A**) and high ($\Gamma/2$ = 238 mM, **B**) ionic strength. Different ATP levels were obtained by varying the voltage of the flash lamp. Note the lower sensitivity to ATP at $\Gamma/2$ = 65 mM where photolysis of > 0.5 mM ATP is required to achieve steady-state relaxation, whereas at $\Gamma/2$ = 238 mM relaxation can be achieved with less than 0.33 mM ATP. Fibre dimensions: 72 ± 7.5 μm diameter, 1.75 ± 0.2 mm length; sarcomere length 2.6–2.7 μm

Effect of phosphate and apyrase on ATP-dependent force transients at low and high ionic strength

ATP-induced relaxation from rigor can be accelerated by addition of millimolar concentrations of phosphate to the caged-ATP solution at high ionic strength (e.g. [21], $\Gamma/2$ = 200 mM; [33], $\Gamma/2$ = 150 mM). The effect of phosphate on the time course of relaxation has been explained by promotion of reversed steps in



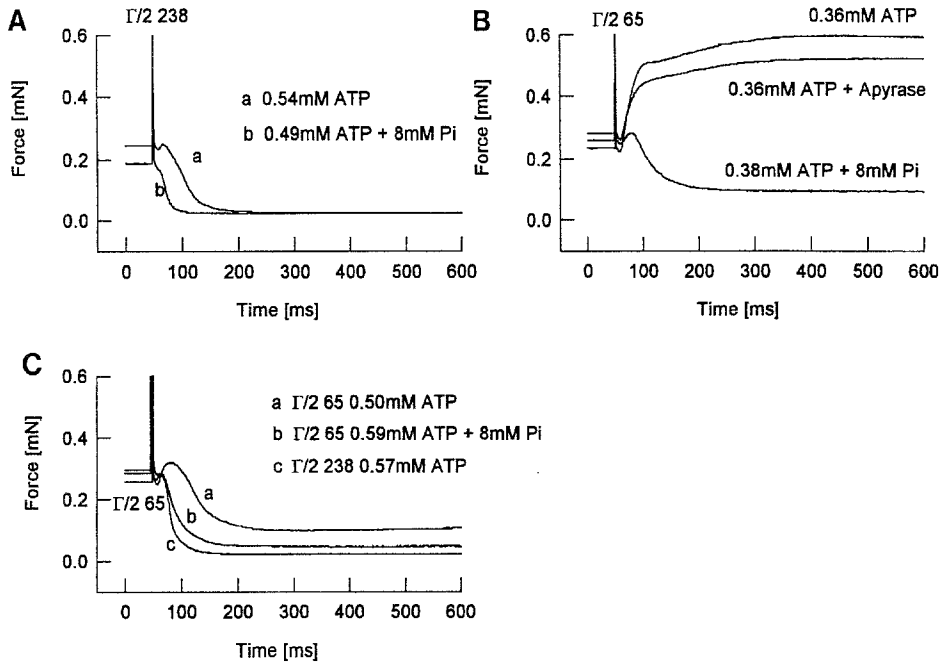


Fig. 5 A–C Effects of phosphate and apyrase on ATP-dependent force transients at low and high ionic strengths. **A** Addition of 8 mM phosphate (*Pi*) accelerates the relaxation process at $\Gamma/2 = 238$ mM. **B** At $\Gamma/2 = 65$ mM, relaxation instead of an increase in steady-state tension can be achieved with 0.38 mM ATP if 8 mM Pi is added (ionic strength kept constant). **C** The effect of phosphate on relaxation at low ionic strength is even stronger than the effect caused by increasing the ionic strength from a $\Gamma/2$ of 65 mM to a $\Gamma/2$ of 238 mM. **B** Apyrase reduces the increase in steady-state tension by 0.36 mM ATP at low ionic strength by $\approx 15\%$. Before photolysis of 0.36 mM ATP, the preparation was incubated in rigor solution with 18 U/ml apyrase grade VII for 10 min followed by another 6-min incubation in caged-ATP solution with 10 U/ml apyrase. Fibre dimensions: 73 ± 10 μm diameter, 1.6 ± 0.3 mm length; sarcomere length 2.6–2.7 μm

the crossbridge cycle which results in actomyosin dissociation and inhibition of reattachment:

$$\text{A. M.ADP}_i + \text{P} \rightleftharpoons \text{A. M. ADP.P}_i \rightleftharpoons \text{M. ADP .P}_i + \text{A} \quad (1)$$

Where A, M and Pi denote actin, myosin and phosphate respectively. Thus, addition of phosphate can be used to study the effect of ADP- binding crossbridge states on the force transients at low and high ionic strengths.

Addition of 8 mM phosphate accelerated relaxation at high ionic strength (Fig. 5A). There was still a lag in the time course of relaxation which might be explained by some reattachment even in the presence of phosphate. At low ionic strength, however, (Fig. 5B) relaxation instead of an increase in steady-state tension was observed in the presence of 0.38 mM ATP if 8 mM phosphate was added to the caged-ATP solution. This observation would suggest that ADP-binding crossbridge states are mainly responsible for reattachment and force generation at low ionic strength, and that in the crossbridge cycle ADP binding itself might be very sensitive towards changes in the ionic strength. This hypothesis is further supported by the data in Fig. 5C which show that the time course of relaxation with 8 mM phosphate at low ionic strength became at least as fast as or even faster than the time course obtained with similar ATP concentrations at high ionic strength.

Apyrase has been used to remove free ADP (and ATP) contamination in skinned fibre preparations and in caged-ATP solutions (e.g. [21, 29, 33]). As shown in Fig. 5B, the effect of apyrase on the force transients was tested at low ionic strength. The fibre in rigor state was incubated with apyrase for 10 min before being incubated for another 6 min in caged-ATP solution which also contained apyrase (see also Materials and methods). The effect on the force transient following photolysis of 0.36 mM ATP was quite small. Due to the treatment with apyrase the ADP concentration in the caged-ATP solution was reduced from ≈ 10 μM to less than the resolution limit of our HPLC protocol, which was 2.5 μM. One possible explanation of the relatively small effect of apyrase compared to the effect of phosphate might be that ADP is strongly bound at low ionic strength and therefore inaccessible to the cleaving enzyme.

Effects of changing the ionic strength at steady-state rigor tension before photolysis of caged-ATP

Finally we wanted to test whether the force transients following photolysis of caged-ATP were mainly determined by the actual ionic strength during ATP release, or whether the ionic strength during the development of rigor tension, which determined the ionic-strength-dependent rigor tension (see Fig. 2A and e.g. [9]), could influence the kinetics of the force transients. Therefore

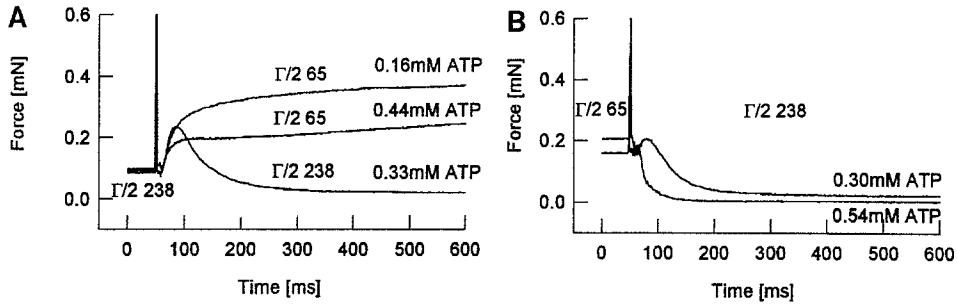


Fig. 6 A,B Effects of changing the ionic strength at steady-state rigor before photolysis of caged-ATP. **A** Rigor tension was developed at $\Gamma/2$ = 238 mM (I.E. 156 mM). When steady-state rigor tension was reached, the solution was changed and the preparation was incubated for ≈ 4 min in a rigor solution including 5 mM caged-ATP at a lower ionic strength of $\Gamma/2$ = 65 mM (I.E. = 49 mM). The force transients following flash photolysis of ATP were similar to the force transients obtained at a $\Gamma/2$ of 65 mM without changing the ionic strength, as shown in Fig. 4A. B Steady-state rigor was developed at a $\Gamma/2$ of 65 mM before the solution was changed to a rigor solution containing 5 mM caged-ATP and a higher ionic strength of $\Gamma/2$ = 238 mM. Again, photolysis of 0.3 and 0.5 mM ATP cause relaxation with similar time courses as observed at a $\Gamma/2$ of 238 mM without a change in the ionic strength, as shown in Fig. 4B. Fibre dimensions: 64 ± 5.5 μm diameter, 1.4 ± 0.2 mm length; sarcomere length = 2.6–2.7 μm

we induced rigor tension at $\Gamma/2$ values of 238 mM and 65 mM and subsequently changed to lower ($\Gamma/2$ = 65 mM) and higher ($\Gamma/2$ = 238 mM) ionic strengths before ATP was liberated by flash photolysis. Interestingly, during these changes of ionic strength, the initially established rigor force was not changed (see also [9]). When rigor tension was developed at a $\Gamma/2$ of 238 mM, but flash photolysis of ATP was performed at a $\Gamma/2$ of 65 mM, a steady-state increase in tension was observed with 0.16 mM and 0.44 mM ATP (Fig. 6A). A similar sensitivity to ATP has been observed in the experiments at low ionic strength ($\Gamma/2$ = 65 mM) without a change in the ionic strength (Fig. 4A). In Fig. 4B rigor was induced at a $\Gamma/2$ of 65 mM before the ionic strength was increased to a $\Gamma/2$ of 238 mM followed by photolysis of ATP. Relaxation was observed with photolysis of 0.3 mM and 0.54 mM ATP with similar ATP concentration-dependent time courses as observed in the experiments at a $\Gamma/2$ of 238 mM without a change in the ionic strength (Fig. 4B).

For a more detailed analysis of possible differences in the force transients due to different preceding rigor states, the detachment rate was determined from the half-time for convergence of force transients following small stretches according to Goldman et al. [13]. The data are shown in Fig. 7. Figure 7A shows typical force transients which were used to estimate the detachment rates and their dependence on the ATP concentration at low and high ionic strength. The results are summarized in Fig. 7B. At low and high ionic strengths, the detachment rate increased with increasing ATP concentration. However, the mean detachment rates were significantly higher at high ionic strength ($\Gamma/2$ = 238 mM). The addition of 8 mM phosphate to the caged- ATP solution promoted relaxation at low and high ionic strengths, but we could not observe an effect on the respective detachment rates.

If the ionic strength was increased from low to high values during rigor (diamonds) the detachment rates were as fast as those values at high ionic strength without changing $\Gamma/2$ (open circles and triangles). Thus, the detachment rate seems to be mainly determined by the actual ionic strength during photolysis of ATP and no influence of the preceding rigor state on the force transients following photolysis of ATP could be detected.

Discussion

Ionic strength effects on ATP-dependent force transients

In recent models of the molecular details of the actin and myosin interaction in the crossbridge cycle it has been suggested that ionic interactions are involved in the docking process of myosin to actin, but also in the following stereospecific interaction step of the two molecules [17, 25, 26]. In the present study we show that the ATP-dependent kinetics of relaxation and activation of force reflecting detachment and reattachment of crossbridges are markedly modified by changes in the ionic strength in the narrow range of $\Gamma/2$ from 65 mM to 215 mM (I.E. 60–194 mM). In earlier studies it has been shown that at high ionic strength ($\Gamma/2$ = 200–300 mM) an increase in steady-state tension following rigor can be induced by addition of micromolar ATP concentrations in the absence of Ca^{2+} whereas higher ATP concentrations induce transient increases in tension and final relaxation [13, 27, 28]. Our data obtained from murine EDL fibres show that the sensitivity to ATP of the force transients following photolysis of caged-ATP is reduced with lowering ionic strength. Thus, a marked increase in steady-state tension instead of relaxation is observed with up to 0.5 mM ATP when the ionic strength is lowered to less than 110 mM. This result is consistent with the data of Yamada et al. [34], who described an increase in steady-state tension in partially crosslinked rabbit

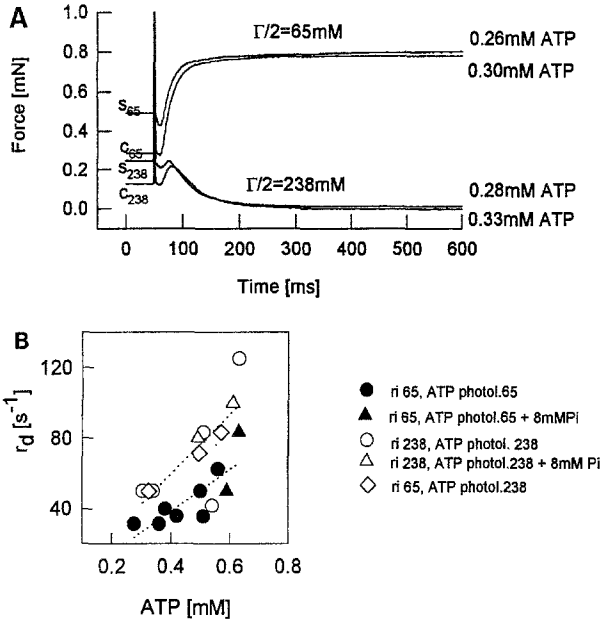


Fig. 7 A,B Superimposed records from successive rigor-photolysis trials with application of small stretches to determine the detachment rate (r_d) at high ($\Gamma/2$ = 238 mM) and low ($\Gamma/2$ = 65 mM) ionic strength. Records are labelled C_{65} and C_{238} for control conditions at $\Gamma/2$ 65 and 238 mM and S_{65} and S_{238} for (0.6% at $\Gamma/2$ = 65 mM and 0.8% at $\Gamma/2$ = 238 mM) prestretch of the fibres 0.46 ± 0.06 s before photolysis of caged-ATP. **B** r_d is estimated from the decay of the difference in tension arising from a 0.6–0.8% prestretch of the fibres before photolysis. The *symbols* represent the exprimental data points obtained from 20 pairs of trials. The *dotted lines* are linear regression fits of the data points from photolysis at low ($\Gamma/2$ 65 mM) and high ($\Gamma/2$ 238 mM) ionic strength. The r_d values obtained at $\Gamma/2$ = 65 mM are significantly smaller than the r_d values at $\Gamma/2$ = 238 mM (for details see text). The fibre dimensions were 73 ± 8.4 μm diameter, 1.6 ± 0.3 mm length. The mean rigor tension in control conditions (c) and after prestretch (s) were c: 0.27 ± 0.03 mN and s: 0.41 ± 0.06 mN (n = 10) at $\Gamma/2$ = 65 mM (●, ▲); c: 0.15 ± 0.06 mN and s: 0.33 ± 0.12 mN (n = 7) at $\Gamma/2$ = 238 mM (○, △); c: 0.22 ± 0.06 mN and s: 0.3 ± 0.06 mN (n = 3) when $\Gamma/2$ was changed during rigor (◇). The relative changes of the fibres due to the stretches were 0.63 ± 0.13% at $\Gamma/2$ = 65 mM; 0.57 ± 0.1% at $\Gamma/2$ = 238 mM; and 0.66 ± 0.1% when $\Gamma/2$ was changed. The reciprocal of the half-time for the decay (r_d [s^{-1}]) is plotted against the final ATP concentration

psoas fibres following flash photolysis of ≈1 mM caged-ATP at a $\Gamma/2$ of 100 mM. The sensitivity to ATP of the force transients at high ionic strength in our experiments was similar to the sensitivity to ATP observed when studying rabbit psoas fibres (Goldman et al. [13]).

The increase in tension in phase b_t following photolysis of caged-ATP at a low Ca^{2+} concentration has been explained by a cooperative phenomenon which allows reattachment in the presence of a critical number of attached crossbridges (e.g. [2, 13, 33]). As the initial phase of relaxation a_t is dominated by crossbridge detachment and the following phase of tension increase b_t is dominated by reattachment, we have investigated the changes in amplitude and duration of phases a_t and b_t with increasing ionic strength. The data suggest that both processes, detachment and reattachment, are modified by changes in the ionic strength, in general resulting in a reduction of detachment and in an enhancement of reattachment with a decrease in ionic strength. This conclusion is derived from the observed decrease in the ATP-dependent detachment rates and the strong increase in amplitude and duration of the tension recovery in phase b_t with lowering ionic strength.

The ionic-strength-induced modifications of ATP-dependent force transients do not seem to depend on the anion species

Under the given experimental conditions, no differences were found in the effects on phases a_t and b_t whether the monovalent anion $MeSO_3^-$ or the divalent anion $EGTA^{2-}$ was used to increase the ionic strength (expressed as $\Gamma/2$ or I.E.). This suggests that the observed modifications of phases a_t and b_t are due to effects of the ionic strength. The modifications of phases a_t and b_t have been related to both parameters used in the literature to characterize ionic strength—i.e $\Gamma/2$ according to the Debye-Hückel Limiting Law, and I.E., which only considers the simple valency of the ions in solution – as it has been shown that in some cases $\Gamma/2$ (e.g. [1]) whereas in others I.E. (e.g. [9, 30]), seemed to be more appropriate to describe non-specific ionic strength effects, depending on the ion species and the ion concentration range used. In our study the sensitivity of both parameters, $\Gamma/2$ and I.E., to reveal ionic-strength-dependent changes in phases a_t and b_t was similar (see Fig. 3).

ATP, strain and ionic strength effects on crossbridge detachment rate

For further analysis of the ionic strength effects on the crossbridge detachment process we determined the ATP-dependent detachment rates at high and low ionic strengths. The detachment rates increased with rising ATP concentrations both at high and low ionic strengths, but the detachment rates at low ionic strength were slightly lower. From other studies [13, 33, 6] it has been concluded that the detachment rate depends on both the ATP concentration and the initial rigor tension (crossbridge tension) and that the detachment rate increases with increasing initial rigor tension. Within the given narrow range of initial rigor tension following stretch at low or high ionic strength in our experiments (0.41 ± 0.06 mN at $\Gamma/2$ = 65 mM; 0.33 ± 0.12 mN at $\Gamma/2$ = 238 mM) we could not resolve a strain-dependent change of the detachment rate at either ionic strength. Therefore the increase in detachment rate shown in Fig. 6 should be mainly due to the increase in ATP concentration at both ionic strengths.

Assuming that ATP was released from NPE-caged-ATP following flash photolysis with a rate constant of $\approx 100\ s^{-1}$ the second-order rate constant of ATP-induced crossbridge detachment, k_d, is estimated to be $\approx 4 \cdot 10^5\ M^{-1}s^{-1}$ at high ionic strength, which is within the range of second-order rate constants determined by Goldman et al. [13] for a similar ionic strength ($I/2$ = 200 mM) in their study of fast twitch rabbit psoas fibres. Lowering the ionic strength to 65 mM caused a reduction in the rate constant of crossbridge detachment to about $2 \cdot 10^5\ M^{-1}s^{-1}$ under our experimental conditions. The similarity of the k_d values from rabbit psoas and murine EDL fibres at higher ionic strengths indicates that the ATP-induced crossbridge detachment is similar in fast twitch mammalian fibres of both species (see also [31]).

The effects of phosphate and apyrase upon relaxation at high and low ionic strengths

The reattachment process was investigated by addition of phosphate to the caged-ATP solutions. Addition of 8 mM phosphate accelerated relaxation at high and low ionic strengths, suggesting that ADP-binding crossbridges are involved in reattachment and redevelopment of force in the absence of Ca^{2+} at high but also at low ionic strengths. The detachment rates did not seem to be affected by phosphate. Following a recent model, which requires ADP-binding crossbridges for cooperative reattachment during relaxation [33], the slightly reduced detachment rates in our study at low compared to high ionic strength and the strong inhibiting effect of phosphate on redevelopment of tension might indicate that lowering ionic strength increases ADP binding in the crossbridge cycle following photolysis of ATP. Following this idea we tested the effect of apyrase which has been used previously to remove ADP contamination in the fibre and in the caged-ATP solution and to prevent crossbridge reattachment during relaxation [21, 29, 33]. Interestingly, under our experimental conditions at low ionic strength, the increase in tension following flash photolysis of caged-ATP was only slightly reduced after treatment with apyrase. One explanation of this result could be that apyrase for some reason is not able to effectively remove ADP from the fibre at low ionic strength, or that the affinity for ADP of actomyosin is enhanced at low ionic strength so that ADP effects are observed at ADP concentrations at which apyrase is ineffective in removing ADP. Otherwise the data would indicate reattachment in the absence of ADP at low ionic strength. The ADP binding constant for apyrase with a low ATPase: ADPase ratio has been estimated to be $\approx 4 \cdot 10^4\ M^{-1}$ at a $I/2$ of $\approx$ 80 mM (pH 6.7; 30° C; [22]). A somewhat lower value of $5 \cdot 10^3\ M^{-1}$ has been determined for the ADP affinity constant of the rabbit acto · S1 ATPase at an ionic strength of $\approx$ 110 mM (pH 7.0, 20° C; [11]). However, very little is known about the ADP affinity of acto.S1 and apyrase at lower ionic strengths.

Influence of changes in ionic strengths once rigor has established upon ATP-dependent force transients

In other studies we have shown that the kinetics of rigor tension development and the steady-state rigor tension depend on the currently present ionic strength, whereas the rigor steady-state reached at any $I/2$ value itself is very resistant and stable against subsequent changes in the ionic strength [9, Veigel et al. unpublished results]. Because of this particular stability and resistance of the steady-state rigor, one could expect that the kinetics of the detachment and reattachment would be influenced by the preceding rigor state. However, within the given resolution of our experimental conditions, an influence of the preceding rigor state on the time course and the detachment rates could not be identified, which might indicate a fast equilibration of interacting actin and myosin to the new ionic strength conditions after photolysis of ATP. Interestingly, it has also been shown in recent time-resolved electronmicroscope studies that, following photolysis of caged-ATP, a fast transition of crossbridges from the oriented rigor state to the disoriented state precedes Ca^{2+} -activated force generation [16, 32].

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