The Initial Contraction of Skinned Muscle Fibers on Photorelease of ATP in the Presence of ADP

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Abstract 1) We photoreleased ATP in skinned muscle fibers from rat psoas, and examined the effects of ADP on the ATP-induced isometric transient. 2) On release of ATP, the muscle stiffness initially falls at any $[Ca^{2+}]$. ADP slowed this stiffness decrease, suggesting competition between ADP and ATP. Even in the presence of ADP, the rate of stiffness decrease was proportional to the amount of released ATP, but was not affected by Ca^{2+} , P_i , or the fiber tension. 3) In the absence of ADP, the Ca^{2+} -sensitive contraction occurs only after the initial phase. However, in the presence of ADP, the fiber developed tension during the initial phase before the late contraction. The initial contraction was insensitive to Ca^{2+} , resistant to P_i and BDM, larger on releasing more ATP, but not markedly influenced by the fiber tension. 4) Our results seem to suggest that, in the physiological reaction cycle, the cross-bridges develop significant force immediately after the binding of ATP, before releasing P_i .

Key words: skeletal muscle, cross-bridge, caged ATP, photolysis, Ca^{2+} .

The cross-bridge, i.e. the complex of the myosin head and actin in muscle fiber, produces contractile force by hydrolyzing ATP into ADP plus P_i . Physiological and biochemical studies so far generally indicate the following cycle of cross-bridge reaction (see introduction of [1] for refs.):

In the absence of ATP, myosin heads (M) are tightly bound to actin (A) to form the rigor cross-bridges (R). On binding ATP, the cross-bridges enter the weak-

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binding state (W), where the interaction between myosin and actin is rapid but so weak that they are substantially dissociated from each other. ATP is quickly hydrolyzed into ADP + P_i on myosin in this state. The strong-binding, force-generating state (F) lies next to state W, and P_i release is closely related with the W-to-F transition (see [2, 3] for details of P_i release). The regulatory action of Ca²⁺ is assumed to be mainly from the Ca²⁺-sensitivity of this weak-to-strong transition [4]. After force generation, the cross-bridges are slowly isomerized to rigor cross-bridges that still hold ADP (ADP·R). The ADP·R cross-bridges soon release ADP to return to the nucleotide-free rigor state (R). The contractile force induced at the previous W-to-F transition is kept until this stage, and is lost at the following R-to-W transition in the next cycle. In this scheme, when one adds ADP in the system, the ADP acts as a competitive inhibitor for the binding of ATP.

By photolysis of caged ATP in skinned muscle fibers, one can examine the ATP-induced mechanical response of the cross-bridges [5, 6]. Much evidence for the above scheme was obtained in such studies with caged ATP (see [1]). Dantzig and colleagues [1] studied the effects of ADP on the ATP-induced reaction in muscle fibers. Recently, we have conducted a similar study of ADP, but at a low temperature and at various [Ca²⁺]. We have made several new findings and obtained some conclusions which are different from those by the previous authors [1]. We suggest that the replacement of ADP with ATP ($\text{ADP} \cdot \text{R} \xrightarrow{-\text{ADP}} \text{R} \xrightarrow{+\text{ATP}} \text{W}$) is a physiological force-generating step, which is insensitive to Ca²⁺ and P_i. An abstract has appeared elsewhere [7].

METHODS

The materials, setup, and procedures were the same as previously described [8]. The skinned fibers were prepared from rat psoas muscle at 20°C, stored in the relaxing solution at 4°C, and used within 36 h. A segment of a muscle fiber was mounted on the setup for experiment. At each trial in the experiment, the fiber segment was first put into the rigor state by washing it with an ATP-free (rigor) solution, and then transferred to a trough filled with a solution containing caged ATP. After equilibration for 30–60 s, a UV flash was applied to the fiber to release ATP from caged ATP, and the mechanical transient on photolysis was recorded for later analysis. The photolysis trials were carried out at 7–9°C, pH 7.0, and an ionic strength of 0.20 M. Ca²⁺ was buffered with 10 mM EGTA. ADP and other agents were added in the photolysis solution when required. The muscle fibers were kept isometric during photolysis transient, but in some cases they were stretched by about 1% several seconds before photolysis, to increase the prephotolysis rigor tension. The muscle stiffness was monitored throughout the experiments by applying a length oscillation (500 Hz, 0.1–0.15% [5, 9]). The “maximum active tension” in RESULTS refers to the steady-state tension obtained by ATP release in the presence of 10 mM Ca·EGTA (pCa ≈ 4.5) without ADP and P_i.

The amounts of ATP photoreleased from caged ATP was examined by

chromatographic analyses. When the flash energy of the xenon lamp for photolysis was the maximum (200 J), the photolysis efficiency was 17–25% in this study. The efficiency was reduced, when required, by decreasing the flash energy to a half or a quarter. The rate of ATP release was estimated to be about 40 s^{-1} at our experimental temperature, $\approx 8^\circ\text{C}$: it has been reported [5] that the rate is 118 s^{-1} at 21°C with $Q_{10}=2.3$. Caged ATP used in this study was the NPE-caged ATP (P^3 -1-(2-nitro) phenylethyl adenosine-5'-triphosphate) purchased from Dojindo Laboratory (Japan), and contaminant ATP in this batch was reported to be 0.026% of caged ATP. $\text{K}\cdot\text{ADP}$ (type [II]) and AP_5A (P^1, P^5 -di(adenosine-5')-pentaphosphate) were from Boehringer-Mannheim-Yamanouchi (Japan).

In RESULTS, the figures with $\pm$ signs after the mean values represent SEMs, and the n values in parentheses indicate the numbers of the examined fibers, unless otherwise stated.

RESULTS

ATP-induced transient in stiffness and effects of ADP

Upper panel of Fig. 1 shows the stiffness transients induced by photorelease of 0.84 mM ATP at three different $[\text{Ca}^{2+}]$: a supramaximal ($\text{pCa} \approx 4.5$), an intermediate ($\text{pCa} 6.6$), and an extremely low $[\text{Ca}^{2+}]$ ($\text{pCa} > 8$). We added 12 mM P_i in the low $[\text{Ca}^{2+}]$ solution to suppress the contractile activity [10]. On release of ATP, stiffness initially decreased, the time course of which was almost independent of $[\text{Ca}^{2+}]$ (Table 1) as in the previous study [10]. In the presence of Ca^{2+} , stiffness increased later showing Ca^{2+} -dependent contraction. The late contraction at the intermediate $[\text{Ca}^{2+}]$ was transient as has been reported [8].

The stiffness transients in the presence of 0.63 mM ADP are illustrated in the lower panel of the same figure (Fig. 1). Two effects of ADP could be seen in the ATP-induced transients of stiffness. Firstly, ADP slowed the rate of decrease in stiffness immediately after the release of ATP. As shown in Table 1, the extent of this effect was independent of $[\text{Ca}^{2+}]$, and thus the rate of initial stiffness decay was still independent of $[\text{Ca}^{2+}]$ even in the presence of ADP. Because ADP competes

Table 1. Effect of ADP at various $[\text{Ca}^{2+}]$ on the rate of the ATP-induced initial decrease in stiffness.*

pCa	$t_{70\%}^{**}$ (ms) at 0.84 mM ATP	
	Control	+0.63 mM ADP
≈ 4.5	15.8 (± 0.4)	33.5 (± 1.3)
6.6	14.8 (± 0.4)	30.2 (± 0.6)
> 8 (+ P_i)***	17.2 (± 0.6)	32.9 (± 1.2)

*The means ($\pm$ SEM) of 5 observations are presented. The experiment was the same as that shown in Fig. 1. **Time for stiffness to decrease to 70% of the rigor level.

***The solution for the lowest $[\text{Ca}^{2+}]$ contained 12 mM P_i .

Fig. 1. The ATP-induced transients of stiffness at three different $[Ca^{2+}]$, in the absence (upper panel) and presence (lower) of 0.63 mM ADP. ATP (0.84 mM) was photoreleased at time zero. All the photolysis solution contained 10 mM EGTA, and the amounts of added $CaCl_2$ for $pCa \approx 4.5$, 6.6, and > 8 were 10, 3.16 and 0 mM, respectively. The $pCa > 8$ solution contained 12 mM P_i . The experiment was made on 5 muscle fibers, changing the order of trials, and the data shown were obtained by averaging.

with ATP in the ATP-induced mechanical transient [1, 11], this effect of ADP was probably due to such competition.

In a Ca^{2+} -free (10 mM EGTA) experiment with three muscle fibers, we varied the amount of released ATP and measured the halftime of the monotonic stiffness decrease. On average, the halftime in the absence of ADP was 27, 42, and 79 ms when 0.96, 0.48, and 0.24 mM ATP was photoreleased, respectively. In the presence of 0.60 mM ADP, the corresponding values were 53, 90, and 182 ms. Thus, in both cases $[halftime]^{-1}$ ($\approx$ rate) was approximately proportional to $[ATP]$, being consistent with the idea of competition between ADP and ATP. (We added 4.7 mM P_i in all the Ca^{2+} -free solutions for the same purpose as described above.)

Another effect of ADP was enhancement of the Ca^{2+} -sensitive, late contraction after the initial phase (Fig. 1). This effect was examined in our previous study [8], and was characterized as a sensitization of the late contraction to Ca^{2+} . This effect could be also explained by the competition of ADP with ATP, because the late contraction was also enhanced when the amount of photoreleased ATP was reduced, which will be described later.

Initial contraction in the presence of ADP

The third effect of ADP was found in the tension transients. This effect was spectacular when $[Ca^{2+}]$ was intermediate and the rigor tension before ATP release was set low, and the experiment in Fig. 2 was carried out under these conditions. In

the control transient (without ADP) at pCa 6.4 (Fig. 2, a), both tension and stiffness decreased immediately after release of 0.86 mM ATP. In the presence of 0.63 mM ADP (Fig. 2, b₁ and b₂), the initial decrease in stiffness was slower and the late contraction was larger than the controls like in Fig. 1 (and Table 1).

The remarkable finding in the experiment in Fig. 2 was that, in the presence of ADP (Fig. 2, b₁ and b₂), tension increased immediately after release of ATP in the phase where stiffness was rapidly decreasing. A slight tension depression separated the initial tension development from the late one.

The initial and late contractions, which were distinguished in the above tension time courses, had different sensitivities to P_i (orthophosphate) and BDM (2,3-butanedione monoxime [12]). As shown in Fig. 2 (c), when we added 10 mM P_i to the Ca + ADP solution, the time course of stiffness lost its late bump and became almost monotonic. In accordance with this, the late tension development was also abolished in the presence of P_i. On the other hand, the initial tension development, at least its early part, was not affected by P_i. The initial time course of stiffness was also insensitive to P_i. Suppression of the contraction with 12 mM BDM (4 sets of data from 2 fibers) resulted in a situation (not shown) similar to that with P_i, although this concentration of BDM seemed to be less effective than 10 mM P_i, leaving a small but distinct late contraction.

Fig. 2. Paired records of tension (solid line) and stiffness (broken line) on the release of ATP (0.86 mM). All the pairs (a-c) were obtained at pCa 6.4 (4.23 mM CaCl₂ + 10 mM EGTA). Pairs b₁ and b₂ were taken in the presence of 0.63 mM ADP, and c in the presence of 10 mM P_i with the same amounts of ADP. The peak tensions in b₁ and b₂ were about 44% of the maximum active tension. The data were taken from one fiber in order from top to bottom. The effects of ADP were verified with 5 fibers in total, and the effects of P_i with 3 out of the five.

The difference in Ca^{2+} -sensitivity between the initial and late contractions was obvious in the experiment in Fig. 3, which was performed in the presence of 0.78 mM ADP. At $\text{pCa} \approx 4.5$, the late contraction rose to a plateau after the initial contraction. However, in the absence of Ca^{2+} (10 mM EGTA; $\text{pCa} > 8$), the late contraction was abolished while the initial contraction was left unaffected. Note that both photolysis trials were carried out in the presence of a low concentration of P_i (3.8 mM) to make the initial contraction clear. Similar results were obtained with four other fibers.

The magnitudes of the two contractions were affected differently by varying the amount of photoreleased ATP: Reduction in the released ATP suppressed the initial contraction and enhanced the late contraction [8]. All the photolysis trials were performed in the presence of 0.60 mM ADP. When we reduced the released ATP from 0.96 to 0.24 mM, the peak of the late tension at pCa 6.4 with no P_i increased by 24%, while the peak of the initial tension at $\text{pCa} > 8$ and 4.7 mM P_i decreased by 20% (average of three fibers). Similar but smaller effects of 0.48 mM ATP were also observed in another experiment (two muscle fibers). The peak size of the initial tension was measured as will be described later.

Test of strain-dependence of the mechanical transients

Dantzig and colleagues [1] suggested that the initial tension development might be caused by possible strain-dependence of the ATP-induced cross-bridge detachment (see DISCUSSION). Thus, we examined whether or not the size of rigor tension before ATP release influenced the mechanical transient immediately

Fig. 3. Minimal effect of Ca^{2+} on the initial tension development. ATP 1.0 mM was released at $\text{pCa} \approx 4.5$ (10 mM Ca-EGTA) and > 8 (10 mM EGTA). In both cases, the photolysis solution contained 0.78 mM ADP and 3.8 mM P_i . The two tension records were obtained in successive trials with one fiber.

after the release of ATP. We could detect no great strain-dependence as follows.

The experiment in Fig. 4 was made on 5 muscle fibers releasing 0.65 mM ATP in the presence of 1.43 mM ADP and 7.0 mM P_i in the absence of Ca^{2+} (10 mM EGTA). The high concentration of ADP was to saturate the cross-bridges with ADP. To change the cross-bridge strain, the muscle in rigor was stretched before release of ATP in one trial and kept isometric in the other trial. The isometric rigor tension was about 35%, on average, of the maximum active tension, and stretching increased this to 92% of the maximum. There was a shoulder or a bump on both tension time courses, indicating the initial contraction in the presence of ADP.

The time courses of stiffness change in the two trials (Fig. 4, *stif*) were almost identical, with no sign of strain-dependence of the rate of the initial reaction. It should be noted that the rigor stiffness was $\approx 30\%$ higher in the trial with stretch than in the isometric one, probably because of the presence of the non-linear end-compliance (see DISCUSSION). The halftime for the stiffness decrease was $113(\pm 7)$ ms ($n=5$) in the isometric trial, and this was only $4(\pm 5)\%$ larger than

Fig. 4. Time courses of tension (solid line) and stiffness (broken line) on release of 0.65 mM ATP in the absence of Ca^{2+} (10 mM EGTA). ADP 1.43 mM and 7.0 mM P_i were added in the caged ATP solution. In one trial (top) the muscle fiber was stretched by about 1% 2 s before ATP release, while in the other trial (middle) the fiber was kept isometric. In the upper two panels, each stiffness record was scaled so that before release of ATP its height is the same as that of the corresponding tension record. In the bottom panel, the two stiffness records were scaled so as to have the same amplitude (*stif*). The original rigor stiffness with stretch was 28% larger than the isometric one. The rigor tension with stretch was 92% of the maximum active tension. The data were obtained by averaging 5 sets of records from different fibers. See the legend of Fig. 7 for Δten .

in the trial with stretch. Note that the halftime was as short as $39(\pm 1.2)$ ms in the absence of ADP (not shown in Fig. 4), regardless of whether the fiber was stretched or kept isometric.

We evaluated the magnitude of the developed tension assuming that the stiffness decrease represented the time course of degradation of the rigor tension with ATP binding. We superimposed the stiffness trace on the tension record scaling the former as in Fig. 4 (top and middle), and measured the height of tension above the stiffness trace. The peak of the tension above stiffness in the stretch trial was $96(\pm 3)\%$ ($n=5$) of that in the isometric trial.

ADP-dependence of the initial mechanical transient

The halftime for the stiffness decrease and the magnitude of the initial contraction were dependent on [ADP], as shown in Fig. 5. The amount of released ATP was 0.76 mM. The halftime (Fig. 5A) increased monotonically with increasing [ADP], and at 1.81 mM ADP this was about 3 times longer than the control. The presence of 8.9 mM P_i in the photolysis solutions slightly shortened the stiffness halftime (by about 10%; Fig. 5A, $\circ$ vs. $\bullet$), which could be due to suppression of the ADP-enhanced late contraction. We analyzed these data based on the following simplified Scheme 2, taking account of the finite rate ($\approx 40 \text{ s}^{-1}$) of ATP release in photolysis [5]:

The rate constants estimated from the analysis were as follows: $k_{+T} = 5.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $k_{-D} = 29 \text{ s}^{-1}$, $k_{+D} = 5.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, and thus $K_D (=k_{-D}/k_{+D}) = 0.51 \text{ mM}$. According to Sleep and colleagues [13], caged ATP binds to the cross-bridges with $K_i \approx 1.6 \text{ mM}$. We released 0.76 mM ATP from 4.5 mM caged ATP in these particular experiments, so that in the above (k_{+T} and k_{+D}) and K_D would be under- and overestimated, respectively, by a factor of $\approx 3.3 (=1 + (4.5 - 0.76)/1.6)$.

The peak size of the initial contraction increased with the increase in [ADP] as in Fig. 5B, and the shape of the concentration-response curve was not greatly affected by 8.9 mM P_i ($\square$ vs. $\bullet$). The peak size at 1.81 mM ADP (with 0.76 mM ATP) was $15(\pm 1.5)\%$ ($n=12$) of the maximum active tension. The fitting of a simple hyperbolic curve to the data resulted in the apparent K_m of 0.08 mM for ADP.

Possible effect of contaminant ATP in the solutions

One may suspect that the initial contraction in the presence of ADP might be somehow due to contamination of ATP in the ADP solution. The cross-bridge reaction may be cycling very slowly with the small amount of ATP even before photorelease of ATP. A significant amount of cross-bridges might be in state F (Scheme 1) dragging other cross-bridges in state $\text{ADP} \cdot \text{R}$. On photorelease of ATP, the passive force of the $\text{ADP} \cdot \text{R}$ cross-bridges disappears, so that the active

Fig. 5. The half-time of stiffness decay (A) and the magnitude of initial contraction (B) as functions of [ADP]. ATP 0.76 mM was released from 4.5 mM of caged ATP in the absence of Ca^{2+} (10 mM EGTA). The different symbols represent different experiments, each being with 5–6 muscle fibers. Two experiments (+, ●) were carried out without P_i and two (○, □) in the presence of 8.9 mM P_i . The muscle fibers in the experiments with ● and ○ were the same. In A, the broken line represents the data calculated in the kinetic model described in the text (Scheme 2). In B, the peak size was normalized so that it is 100% at 1.81 mM ADP and 0% without ADP. The broken line is $\text{peak} = \text{peak}_{\text{max}} / (1 + K_m / \text{ADP})$, fitted to the data.

force of the F cross-bridges would be uncovered. In the experiment shown in Fig. 6, we added a small amount of ATP to the ADP solution to examine such possibilities.

The experiment was carried out under the Ca^{2+} -free conditions in the presence of P_i . In Fig. 6, the obtained tension transients on release of ATP are superimposed with appropriately scaled stiffness traces to inspect the sizes of initial contractions. In the control without ADP (Fig. 6, a), tension and stiffness decreased with almost the same time course, showing little sign of the initial contraction. When we added 60 μM ADP in the caged ATP solution (Fig. 6, b), the initial contraction appeared. This initial contraction did not seem to be due to conversion of two ADPs into ATP plus AMP by the myokinase intrinsic in the muscle fibers, because the inhibitor of the kinase, AP_5A [14], had no effect on the result even at 0.1 mM (Fig. 6, c).

Fig. 6. Minimal effect of contaminant ATP on the initial contraction. All the photolysis solutions contained 4.5 mM caged ATP, 8.9 mM P_i , and 10 mM EGTA with no added Ca^{2+} , and 0.75 mM ATP was released at time zero. The solid lines are the tension records. The broken lines are the stiffness traces which were scaled so that their pre-photolysis levels are the same as the corresponding tension records. The caged ATP solution contained no added nucleotides in a, 60 μ M ADP in b, 60 μ M ADP plus 0.1 mM AP_5A in c, 60 μ M ADP plus 6 μ M ATP in d, 60 μ M ATP in e, and 0.2 mM ADP in f. The incubation time in the caged ATP solutions was 30 s in all the cases. In c, AP_5A was introduced 30 s before introduction of ADP. Each pair of records was obtained by averaging 5 pairs from 5 fibers. The muscle fibers were stretched by 1%, 5 s before ATP release.

The addition of 6 μ M ATP to the ADP solution (Fig. 6, d) did not remarkably enhance the initial contraction. Probably the cross-bridge ATPase in the fiber hydrolyzed ATP into ADP during the incubation before photolysis. When we replaced the 60 μ M ADP with the same amount of ATP (Fig. 6, e), the fiber developed a significant force during the incubation time, but the size of the initial contraction was almost the same as the control with 60 μ M ADP. Therefore, the small amount of ATP contaminating our ADP ($ATP/ADP < 0.2\%$) did not seem to be the cause of the initial contraction. It should be noted that the size of the initial contraction was several times larger at 0.2 mM ADP (Fig. 6, f) than at 60 μ M as has been shown in Fig. 5.

Time course of "tension difference"

In the previous studies [1, 11] the method of tension difference was employed to estimate the time course of cross-bridge "detachment" ($ADP \cdot R + ATP \rightarrow W + ADP$) on release of ATP. In the stretching experiments, such as shown in Figs. 4

and 7, the two records with the different rigor tensions approached each other in time after ATP release. It is likely that cross-bridges lose the initial rigor force on detachment. Thus, it is reasonable to assume that the time course of the tension difference (Δten), i.e. the algebraic difference between the two records, represented the time course of the rigor degradation. However, it turned out that Δten was not always reliable:

The time courses of stiffness, tension and Δten were compared in the experiment in Fig. 7. $[Ca^{2+}]$ was set at an intermediate concentration (pCa 6.4) so as to attenuate the late contraction. Because of the presence of 0.63 mM ADP, the initial contraction preceded the late contraction. The time course of stiffness change (Fig. 7, lower) was not greatly affected by the rigor tension before ATP release, like in the absence of Ca^{2+} (Fig. 4), and consisted of three phases: (i) the fast decrease during the initial contraction, (ii) the following upward deflection due to the late contraction, and (iii) the final decay to the baseline due to the transient nature of the contraction.

The time course of Δten (Fig. 7) was smoother than that of stiffness, but not ideally monotonic. Its initial time course was roughly the same as that of stiffness (i), which is consistent with the theoretical idea. However, the later time course of Δten had a significant bump which temporally coincided with the late phases (ii–iii)

Fig. 7. Tension (solid line) and stiffness (broken line) on release of 0.86 mM ATP at pCa 6.4 in the presence of 0.63 mM ADP. The fiber was stretched by 1% before ATP release in one trial (str), and kept isometric in the other trial (iso). The tension difference ($\Delta ten = str - iso$) is superimposed on the stiffness records. Note that stiffness and Δten traces are scaled so as to have the same amplitudes. The rigor tension in the trial with stretching was 90% of the maximum active tension. The rigor stiffness in the stretching trial was 33% higher than that in the isometric trial.

in the stiffness records. This deviation of Δt_{ten} from the ideal time course was confirmed with other three fibers. These time courses of Δt_{ten} at the intermediate $[\text{Ca}^{2+}]$ were similar to those obtained by Dantzig and colleagues [1] in the absence of Ca^{2+} .

The shape of the Δt_{ten} time course under the Ca^{2+} -free conditions (Fig. 4) was different from the above. Its later half was convex downward (Fig. 4), and showed large fiber-to-fiber variation (not shown). We also examined Δt_{ten} 's at the supra-maximal $[\text{Ca}^{2+}]$ ($\text{pCa} \approx 4.5$) with two fibers, and confirmed that they were relatively smooth as in the previous studies [1, 11].

As mentioned earlier, the initial phase of the ATP-induced reaction was not significantly affected by Ca^{2+} , judging from the time courses of tension and stiffness. The initial time course of Δt_{ten} was also insensitive to Ca^{2+} . The experiment was performed with two muscle fibers at three different $[\text{Ca}^{2+}]$: $\text{pCa} > 8$, 6.4, and ≈ 4.5 . The amount of released ATP was relatively small (0.41 mM) so as to slow the binding of ATP. The halftime of Δt_{ten} in the absence of ADP was 50–60 ms irrespective of $[\text{Ca}^{2+}]$, and similar to the stiffness halftime (68 ± 3 ms; mean $\pm$ SD, 6 trials). The Δt_{ten} halftime in the presence of 0.63 mM ADP were about 215, 200, and 185–200 ms at $\text{pCa} > 8$, 6.4, and ≈ 4.5 , respectively, and again similar to the stiffness halftime at $\text{pCa} > 8$, 200–205 ms. Thus, we saw no substantial Ca^{2+} -dependence of the cross-bridge "detachment."

DISCUSSION

In the present and previous [8] studies, we examined the effects of ADP on the mechanical transient initiated by photorelease of ATP in rigor muscle fibers. There were three effects of ADP on the transient: 1) ADP slowed the initial decrease in stiffness immediately after the release of ATP. This was probably from competition between ADP and ATP [1, 11] in the reaction of nucleotide replacement ($\text{ADP} \cdot \text{R} \xrightleftharpoons[-\text{ADP}]{+\text{ATP}} \text{R} \xrightleftharpoons[-\text{ADP}]{+\text{ATP}} \text{W}$). The rate of this reaction was not greatly affected by P_i , BDM, Ca^{2+} , or the pre-photolysis rigor tension. 2) ADP enhanced the late, Ca^{2+} -sensitive development of tension and stiffness that has been considered [15] to occur with the release of P_i after ATP hydrolysis ($\text{W} \rightarrow \text{F} + \text{P}_i$). This effect of ADP was discussed in our previous study [8]. 3) Only in the presence of ADP did the muscle fiber develop tension during the initial phase of the ATP-induced reaction ($\text{ADP} \cdot \text{R} \xrightleftharpoons[-\text{ADP}]{+\text{ATP}} \text{R} \xrightleftharpoons[-\text{ADP}]{+\text{ATP}} \text{W}$). Unlike the late contraction with P_i release, this "initial contraction" was not sensitive to Ca^{2+} , P_i , or BDM. It was also independent of the pre-photolysis rigor tension.

Cross-bridge "detachment" induced by ATP. It has been implied [1, 5, 6] that the cross-bridges are substantially detached in the weak-binding state (W) in the initial reaction after the binding of ATP (Scheme 1). This "detachment" monitored with stiffness in the absence of Ca^{2+} was slowed by ADP. Although the tension difference (Δt_{ten}) was not always reliable, it also suggested the same ADP effect that stiffness indicated.

The ADP-dependence of the halftime of the detachment-like reaction (Fig. 5A) was simpler than that obtained by Dantzig and colleagues (Fig. 6A of Dantzig *et al.* [1]). We carried out the experiments at 8°C and took the stiffness records for analysis of the effect of ADP. In the absence of Ca^{2+} , stiffness showed monotonic time courses under our conditions, so that we measured the halftime of the overall time course. On the other hand, the previous authors [1] performed experiments at 21°C and took Δten for analysis. The time courses of Δten under their conditions were similar to or more complex than ours (Figs. 4 and 7), and they measured the halftime of the late phase of the complex time course.

In this study, the initial time course of stiffness indicated that Ca^{2+} does not affect the detachment-like reaction even in the presence, as well as the absence [10], of ADP (Fig. 1 and Table 1). Ca^{2+} seemed to be a simple inducer of the late contraction. Dantzig and colleagues [1], from their analysis of Δten , suggested that Ca^{2+} doubled the rate of “etachment” in the presence of ADP (Fig. 6A of Dantzig *et al.* [1]). In this study, the initial time course of Δten was independent of $[\text{Ca}^{2+}]$, like that of stiffness, even in the presence of 0.6 mM ADP.

Strain-dependence of “detachment”. Dantzig and colleagues [1] inferred the strain-dependent detachment of ADP-rigor cross-bridges from the result of their “negative tension” experiment (Fig. 11 of Dantzig *et al.* [1]), which was similar to the one in Fig. 4 but was carried out at a long sarcomere length. Because of the high resting tension in this situation, they could set the rigor tension lower than the resting level, by releasing rigor fibers. When ATP was photoreleased in the presence of ADP, tension rapidly rose to the resting level in the negative-tension trials, while it slowly fell to this level in the normal, isometric trials. The difference between the two rates was interpreted to mean that the ADP·R cross-bridges reached the detached state (W) faster when the cross-bridge strain was lower or more negative.

Danzig and colleagues added P_i or BDM in the photolysis solutions to inhibit the contraction ($\text{W} \rightarrow \text{F} + \text{P}_i$). However, the initial contraction probably remained in their transient. Thus, the “relaxation” in their negative-tension trials could be the rising phase of the initial contraction in this paper, while that in the isometric trials could be its falling phase. Therefore, the result of their negative tension experiment does not seem to evidence the strain-dependent behavior of the ADP·R cross-bridges.

To test the strain-dependence, we compared the time courses of stiffness in the two photolysis trials: the fiber was kept isometric in one trial and stretched in the other (Figs. 4 and 7). The two time courses were almost identical to each other. Because of the presence of the non-linear end-compliance, which is more compliant at lower tension, it is likely that the muscle stiffness at lower tension was underestimated in our experiments [6]. Therefore, the identical time courses of our stiffness in the two trials above implies that the actual muscle stiffness decreased faster when the muscle was more strained. This strain-dependence is in the direction opposite to what was suggested in the negative tension experiment. The

opposite strain-dependence has been observed in other studies of Δ ten [5, 11].

Initial contraction during "detachment". In the presence of ADP, the initial reaction on release of ATP was a contraction where tension per stiffness (500 Hz) appeared to be relatively high. When we released 0.8 mM ATP in the presence of 1.8 mM ADP, the size of the tension which developed during the initial contraction was about 15% of the maximum steady tension. The size became small as the amount of photoreleased ATP was reduced. Probably, the magnitude of the initial contraction is larger when the rate at which the ADPs on the cross-bridges are replaced with ATPs is faster. This rate-dependence may be the reason why K_m for ADP in Fig. 5B (0.08 mM) was lower than K_D in Fig. 5A (0.5 mM): When we increased [ADP] keeping [ATP] constant, the ADP-ATP replacement was slowed and this, in turn, reduced the size of the initial contraction.

Dantzig and colleagues [1] found in their Ca^{2+} -experiments (Fig. 17 of Dantzig *et al.* [1]) that the initial tension decrease in the presence of ADP was much smaller than expected from the simple model (Scheme 1). Judging from our result in Fig. 3, the previous authors' observation seems to be closely related with the initial contraction we examined in this study. They proposed several possible mechanisms to explain their results. In the following, we consider if the proposed mechanisms or others can explain our results without assuming that the nucleotide replacement induces actual force generation before the release of P_i .

(1) The mechanism that the previous authors [1] mentioned first was the strain-dependent ADP-release from the $\text{ADP}\cdot\text{R}$ cross-bridges ($\text{ADP}\cdot\text{R} \rightarrow \text{ADP} + \text{R}$). The $\text{ADP}\cdot\text{R}$ cross-bridges might release ADP faster when they are negatively strained than when they are positively strained. If so, in the early phase of the ATP-induced detachment, the breakdown of the negatively strained cross-bridges results in an increase in the fiber tension.

There is, however, no clear evidence for the fast detachment of the negatively strained rigor cross-bridges, as discussed above. Furthermore, we could not detect such a strain-dependent detachment, but could observe the initial contraction that is not strain-dependent. Because the low rigor tension in our experiment was not negative, it was still possible that the amount of the negatively strained cross-bridges was too small to be detected in the time course of stiffness. Even if this was the case, the initial contraction detected in the tension time course would have been strain-dependent under the hypothesis. An analysis of our results with a quantitative model in APPENDIX also failed to support the strain-dependence mechanism.

(2a) Another mechanism suggested previously [1] was the cooperative interaction between the cross-bridges. When one of the two myosin heads is of $\text{ADP}\cdot\text{R}$ and the other one W, the force-generating transition of the W head ($\text{W} \rightarrow \text{F} + \text{P}_i$) might be accelerated by the $\text{ADP}\cdot\text{R}$ partner. However, this cooperation does not seem compatible with our observation that the initial contraction is resistant to P_i and BDM. (2b) Another type of cooperation was also considered previously [1]. The pairs of W and $\text{ADP}\cdot\text{R}$ cross-bridges might keep the same tension they had

before detachment of the W head. This mechanism explains the maintenance of tension but not the increase in tension on detachment.

(3) The addition of ADP to the rigor fiber reduces its tension [1]. The apparent K_m of ADP for this effect was about 0.1 mM under our experimental conditions (Horiuti, unpublished observation) and 0.02 mM in the study by Dantzig and colleagues [1]. However, its simple reversal ($\text{ADP} \cdot \text{R} \xrightleftharpoons{-\text{ADP}} \text{R}$) in the ATP-initiated reaction ($\text{ADP} \cdot \text{R} \xrightleftharpoons{-\text{ADP}} \text{R} \xrightleftharpoons{+\text{ATP}} \text{W}$) would not be the cause of the initial contraction. Firstly, the decrease in tension by ADP is only 10–20% of the rigor tension ([1]; Horiuti, unpublished observation). Secondly, the initial contraction became larger when the R cross-bridges disappeared faster in the presence of larger amounts of ATP (this study).

In the above, we considered several possible mechanisms (1–3) for the initial contraction, but none of them were satisfactory. Instead, our experimental results with the Ca^{2+} - and P_i -insensitive, rapid tension development on release of ATP seem to suggest that the individual cross-bridges generate contractile force during or immediately after the replacement of ADP with ATP before the other process of force generation starts with the release of P_i . Both processes of force generation should be investigated to understand the contractile mechanism of the cross-bridge reaction.

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APPENDIX

In this section, we assume that the initial contractions in the experiment in Fig. 4 were caused by the mechanism that the negatively strained rigor cross-bridges detach much more rapidly than the positively strained ones. Based on this assumption, we estimate the amount of the negatively strained cross-bridges in the isometric trial and how much of this should have remained in the stretch trial in order to determine the expected change in the size of the initial contraction.

We take a Gaussian for the population density of the cross-bridges as regards the strain, and assume that the stress is proportional to the strain. The mean stress measured by the standard deviation is denoted by μ . If the value of μ is given, we can calculate the fraction (f^-) of the negatively strained cross-bridges and the mean of the negative stress (μ^-), as shown in Fig. 8. In the isometric trial in Fig. 4, the size of the initial contraction was about one-half of the rigor tension. Thus, under the strain-dependence hypothesis, $\mu^- \approx 0.5 \cdot \mu$, so that $\mu \approx 0.44$, $\mu^- \approx 0.22$, and $f^- \approx 0.33$ (Fig. 8, α and a). In other words, in the isometric trial, one-third of the total cross-bridges seemed to be negatively strained and they must have detached faster than the other, positively strained ones. Why was the decay of stiffness monotonic?

In the stretching trial (Fig. 4), the rigor tension was 2.6 times higher than in the isometric trial. Thus, in the model calculation (Fig. 8, β and b), μ is increased

Fig 8. The fraction (f^-) of the cross-bridges negatively strained, and the mean negative stress (μ^-), both as functions of the mean stress (μ). The data were calculated as follows: $f^- = \int_{\mu^-}^{\infty} g(x)dx$ and $\mu^- = \int_{\mu^-}^{\infty} (x - \mu^-) \cdot g(x)dx$, where $g(x) = \exp(-x^2/2)/\sqrt{2\pi}$.

to $1.16 (=0.44 \times 2.6)$, which results in a decrease in f^- to 0.13, where μ^- is only 0.06. Therefore, the size of the tension developed by the negatively strained cross-bridges could have been reduced to one-quarter ($=0.06/0.22$) by stretching. Why did this not happen in our experiment?

The results of the calculations did not seem to be greatly dependent on the type of the distribution function of the cross-bridge strain. If we take a uniform distribution ($g(x) = 0.287, |x| < 1.745$), instead of the Gaussian; $\mu \approx 0.47$, $\mu^- \approx 0.23$, and $f^- \approx 0.37$ in the isometric trial; and $\mu \approx 1.22 (=0.47 \times 2.6)$, $\mu^- \approx 0.04$, and $f^- \approx 0.15$ in the stretch trial.