Relaxation Kinetics Following Sudden Ca^{2+} Reduction in Single Myofibrils from Skeletal Muscle

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ABSTRACT To investigate the roles of cross-bridge dissociation and cross-bridge-induced thin filament activation in the time course of muscle relaxation, we initiated force relaxation in single myofibrils from skeletal muscles by rapidly (~10 ms) switching from high to low [Ca^{2+}] solutions. Full force decay from maximal activation occurs in two phases: a slow one followed by a rapid one. The latter is initiated by sarcomere "give" and dominated by inter-sarcomere dynamics (see the companion paper, Stehle, R., M. Krueger, and G. Pfitzer. 2002. *Biophys. J.* 83:2152–2161), while the former occurs under nearly isometric conditions and is sensitive to mechanical perturbations. Decreasing the Ca^{2+}-activated force preceding the start of relaxation does not increase the rate of the slow isometric phase, suggesting that *cycling* force-generating cross-bridges do not significantly sustain activation during relaxation. This conclusion is strengthened by the finding that the rate of isometric relaxation from maximum force to any given Ca^{2+}-activated force level is similar to that of Ca^{2+}-activation from rest to that given force. It is likely, therefore, that the slow rate of force decay in full relaxation simply reflects the rate at which cross-bridges leave force-generating states. Because increasing [P_i] accelerates relaxation while increasing [MgADP] slows relaxation, both forward and backward transitions of cross-bridges from force-generating to non-force-generating states contribute to muscle relaxation.

INTRODUCTION

Striated muscle fibers relax when myoplasmic [Ca^{2+}] falls and the cation dissociates from troponin C (TnC). Switching off the regulatory system inhibits formation of new force-generating cross-bridges while attached cross-bridges progressively dissociate causing force decay. A. F. Huxley (1957) suggested that the kinetics of force relaxation could provide information about the kinetics of cross-bridge detachment. This argument presumes that no new cross-bridges are recruited in the absence of Ca^{2+}. At the sarcomere level, however, feedback mechanisms between attached cross-bridges and thin filament activation may promote cross-bridge formation and delay force relaxation (for a review see Gordon et al., 2000). Strongly attached rigor cross-bridges can sustain thin filament activation by cooperative mechanisms and can also enhance Ca^{2+} binding to TnC. However, there is no compelling evidence that *cycling* cross-bridges produce these effects after Ca^{2+} removal (Gordon et al., 2000).

In living muscle the roles of cross-bridge dissociation and cross-bridge-induced thin filament activation during relaxation from maximal tetanic force are obscured by the relatively slow removal of Ca^{2+} from CaTnC by the sarcoplasmic reticulum (e.g., Caputo et al., 1994; Jiang and Julian, 1999). Rapid photogeneration of Ca^{2+} chelators from caged moieties may circumvent this problem (Patel et al., 1998; Wahr et al., 1998; Palmer and Kentish, 1998; Hoskins et al., 1999). One major limitation of this approach is that the increased Ca^{2+} buffering capacity following photolysis of caged Ca^{2+} chelators is too limited to produce complete force relaxation from maximal activation (Wahr et al., 1998).

Because myofibrils equilibrate with solutions in <1 ms, rapid solution switching in single myofibrils (Colomo et al., 1998; Tesi et al., 1999, 2000) can be used to abruptly drop the [Ca^{2+}] and thus constrain the factors that alter relaxation kinetics. We use this approach in single myofibrils from fast and slow skeletal muscles to investigate the roles of regulatory mechanisms and cross-bridge dissociation in the time course of force decay. A definite advantage of rapid solution switching is that it provides information on the kinetics of force relaxation and on the kinetics of force activation. We find that when Ca^{2+} is reduced to subthreshold levels relaxation proceeds both in the forward and reversed direction in the cross-bridge cycle, and that cycling cross-bridges probably do not contribute to sustained maintenance of force during relaxation.

MATERIALS AND METHODS

In the present experiments we used techniques to measure and control the force and length of single myofibrils activated and relaxed by rapid solution switching (Colomo et al., 1998; Tesi et al., 1999, 2000). Briefly, single myofibrils or thin bundles of two to four myofibrils were prepared by homogenization of glycerinated samples of frog tibialis anterior, rabbit psoas, and soleus muscles (Tesi et al., 1999, 2000). For the experiments, a small volume of the myofibril suspension was transferred to a temperature-controlled trough (5 or 15°C) filled with relaxing solution (pCa 8.00). Selected myofibrils (40–90 μm long, 1–3 μm wide) were mounted horizontally between two glass microtools in a force recording apparatus. One tool was a cantilever force probe of known compliance (1–3 nm nN^{-1}; frequency response 2–5 kHz). The second tool was connected to the lever arm of a length-control motor that could produce rapid (1 ms) length changes (Colomo et al., 1994). The initial length (l_0) of the preparation was

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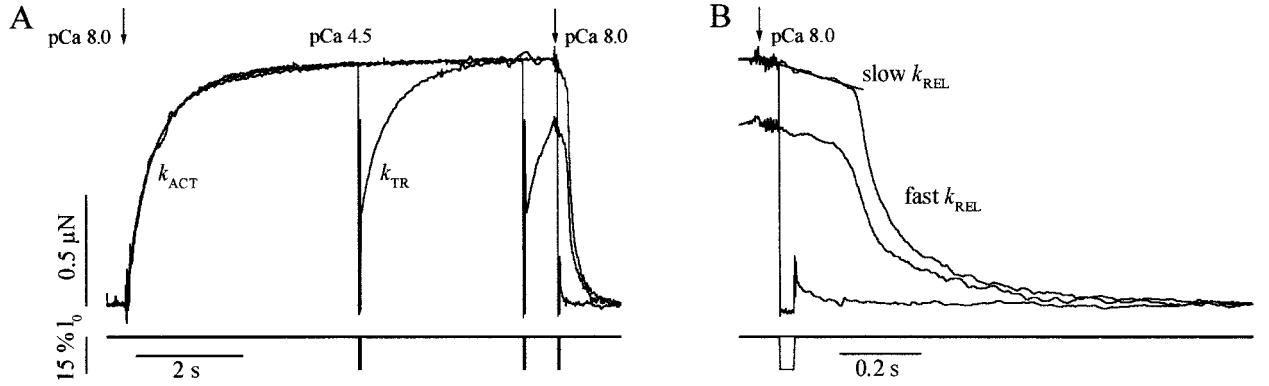

FIGURE 1 Kinetics of full force generation and relaxation following rapid pCa switch in rabbit psoas myofibril at 5°C. The arrows mark the start of the solution change in the preparation. (*A*) Three tension responses to activation-relaxation cycles are superimposed (*top traces*). In each cycle a large release-restretch (*bottom traces*) is applied to the preparation at different times. When applied under steady-state conditions of activation the maneuver results in full tension redevelopment, while there is no significant force redevelopment when the release-restretch is applied early (50 ms) after switching pCa from 4.5 to 8.0. (*B*) Same traces as in *A* on expanded time scale to better show the kinetics of force relaxation. The rate constant of the early slow force decline (slow k_{REL}) is estimated from the slope of the regression line fitted to the force trace normalized to the entire amplitude of force relaxation. Rate constants for the final fast phase of tension decline (fast k_{REL}), for tension development after Ca^{2+}-activation (k_{ACT}), and for tension redevelopment following release-restretch (k_{TR}) are estimated from monoexponential fits.

set 5–10% above the slack myofibril length. Initial sarcomere lengths were (means ± SE) 2.11 ± 0.02 μm (n = 35), 2.54 ± 0.01 μm (n = 120), and 2.42 ± 0.02 μm (n = 46) in frog tibialis, rabbit psoas, and soleus myofibrils, respectively. Mounted myofibrils were activated and relaxed by rapidly translating the interface between two flowing streams of solution of different pCa across the length of the preparation. The solution change occurred with a time constant of 2–3 ms and was complete in ~10 ms (Colomo et al., 1998; Tesi et al., 2000).

Standard solutions, calculated as previously described (Tesi et al., 2000), were at pH 7.0 and contained 10 mM total EGTA (CaEGTA/EGTA ratio set to obtain different pCa values in the range 8.0–4.5), 5 mM MgATP, 1 mM free Mg^{2+}, 10 mM MOPS, propionate and sulfate to adjust the final solution to an ionic strength of 200 mM and monovalent cation concentration of 155 mM. Creatine phosphate (10 mM) and creatine kinase (200 units ml^{-1}) were added to all solutions but those containing 3 mM MgADP. Standard solutions contained 170 ± 30 μM (n = 17), contaminating inorganic phosphate (P_i) from spontaneous breakdown of MgATP and CP (Tesi *et al.,* 2000). Contaminant P_i was reduced in some experiments to <5 μM (P_i-free solutions) by a P_i scavenging enzyme system (purine-nucleoside-phosphorylase with substrate 7-methyl-guanosine; Tesi et al., 2000).

RESULTS

Fig. 1 shows the force responses of a rabbit psoas myofibril to full activation-relaxation cycles at 5°C. Average maximum tension (P_0) and kinetic parameters for full force generation and relaxation are given in Table 1.

In all myofibril types examined, the time course of Ca^{2+}-activated force development was approximately monoexponential (Fig. 1 *A*); the activation rate constant (k_{ACT}) was the same as k_{TR} (Table 1), the rate constant of tension redevelopment following a release-restretch applied to the myofibril under conditions of steady activation (Brenner, 1988). As shown in Fig. 2, for rabbit psoas and frog tibialis

TABLE 1 Means (±SE) of maximum tension (P_0) and kinetic parameters for full force generation and relaxation in different myofibril preparations and experimental conditions

Preparations and Conditions	Force Generation			Relaxation		
				Slow Phase		Fast Phase
	P_0 (mN mm^{-2})	k_{ACT} (s^{-1})	k_{TR} (s^{-1})	Duration (ms)	k_{REL} (s^{-1})	k_{REL} (s^{-1})
Frog Tibialis (5°C)	290 ± 30 (19)	9.1 ± 0.4 (19)	9.1 ± 0.7 (11)	160 ± 5 (13)	1.4 ± 0.10 (13)	11.5 ± 1.1 (13)
Rabbit Psoas (5°C)	223 ± 11 (59)	2.6 ± 0.1 (46)	2.7 ± 0.1 (25)	180 ± 5 (51)	0.6 ± 0.03 (51)	17.7 ± 1.1 (51)
3 mM MgADP	282 ± 33 (14)*	2.1 ± 0.1 (14)†	2.1 ± 0.1 (14)†	252 ± 13 (14)†	0.4 ± 0.03 (14)†	8.4 ± 1.0 (14)†
P_i-free (<5 μM)	265 ± 14 (36)†	1.9 ± 0.1 (10)†	2.0 ± 0.1 (10)†	232 ± 8 (10)†	0.4 ± 0.07 (10)†	17.5 ± 1.8 (10)
5 mM P_i	112 ± 10 (19)†	6.3 ± 0.3 (16)†	6.4 ± 0.4 (12)†	86 ± 7 (15)†	3.4 ± 0.36 (15)†	13.9 ± 1.4 (15)
60 μM MgATP	318 ± 32 (19)†	1.1 ± 0.1 (17)†	—	367 ± 23 (17)†	0.2 ± 0.02 (17)†	2.4 ± 0.2 (17)†
Rabbit Psoas (15°C)	300 ± 12 (54)	7.8 ± 0.2 (36)	8.0 ± 0.4 (14)	68 ± 3 (36)	1.6 ± 0.07 (36)	46.6 ± 2.3 (36)
Rabbit Soleus (15°C)	220 ± 30 (36)	2.0 ± 0.2 (22)	2.1 ± 0.3 (12)	413 ± 51 (13)	0.3 ± 0.04 (13)	2.1 ± 0.2 (13)

pCa of relaxing and activating solution, 8.00 and 4.50 respectively. Numbers in parentheses = n; *$p < 0.05$; †$p < 0.01$ (Student's t-test) versus the same parameter measured in rabbit psoas myofibrils at 5°C with standard solutions (no MgADP added, contaminant [P_i] ~170 μM, [MgATP] 5 mM).

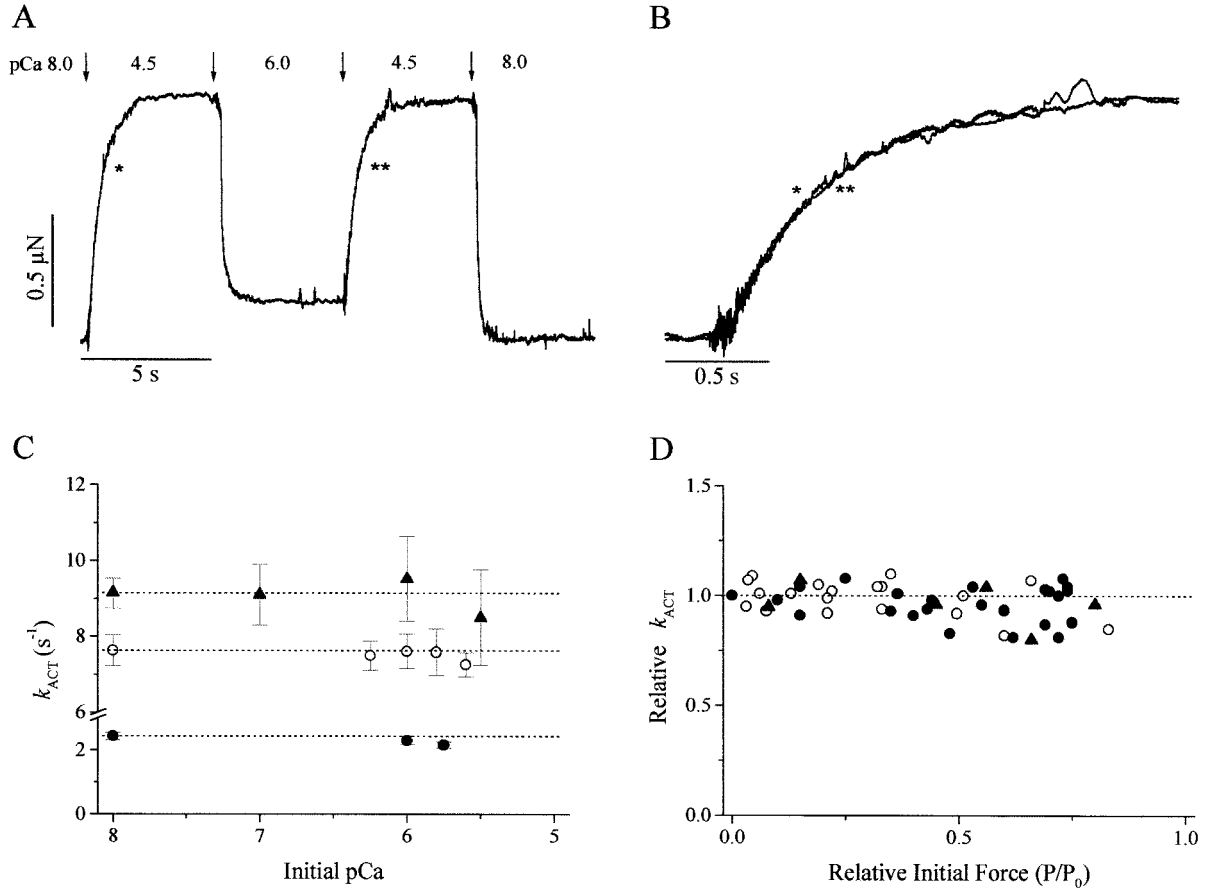

FIGURE 2 Effects of initial $[Ca^{2+}]$ on the kinetics of force development following maximal activation. (*A*) The force response of rabbit psoas myofibril (5°C) to maximal activation (pCa 4.5) starting from submaximal activation (pCa 6.0) is compared with that starting from fully relaxed conditions (pCa 8.0). (*B*) Same traces as in *A* on expanded time base and normalized for the amplitude of the force change. (*C*) k_{ACT} values in response to maximal activation (pCa 4.5) measured from different initial pCa values in rabbit psoas myofibrils at 5°C (*closed circles*) and 15°C (*open circles*) and in frog tibialis myofibrils at 5°C (*closed triangles*); data points are means ± SE of 4 to 15 myofibrils. (*D*) k_{ACT} values in response to maximal activation (pCa 4.5) are plotted versus the relative Ca^{2+}-activated force preceding the start of activation. k_{ACT} values are normalized for the values measured from fully relaxed conditions (pCa 8.0, relative initial force = 0). Symbols as in panel *C*.

myofibrils, k_{ACT} was not increased by increasing Ca^{2+} and Ca^{2+}-activated force levels preceding the switch to maximally activating solution (pCa 4.5). The similarity between k_{ACT} and k_{TR} values (Table 1) and the lack of significant effects of the initial $[Ca^{2+}]$ on k_{ACT} (Fig. 2) indicate that thin filament activation processes are much more rapid than those underlying force generation.

The time course of full force relaxation following Ca^{2+} removal to subthreshold level (pCa 8.0, see Fig. 1 *B*) was biphasic, starting with a slow, seemingly linear, phase followed, after a "shoulder," by a fast, exponential, relaxation phase. The rate constant of the linear phase (slow k_{REL}, estimated by normalizing the average slope of the linear force change against the amplitude of the total force decay) was more than four times slower than k_{ACT} while the rate constant of the final fast phase of relaxation (fast k_{REL}) was usually faster than k_{ACT} (Table 1). [The early slow force decay (linear phase of relaxation) is assumed to be the initial part of an exponential process that, if it lasted for the whole relaxation transient, would lead force to its final steady-state value with a rate constant equal to the initial slope of force decay divided by the amplitude of the overall force decay. Evidence for the consistency of this assumption is given by the monoexponential force decay observed in partial relaxation (see Fig 4 *A*).]

Slow k_{REL} and the duration of the slow force decay were very sensitive to temperature, myofibril type, mechanical perturbations, and chemical interventions that affect cross-bridge kinetics (see Table 1 and below). These data indicate that the slow force decay component of relaxation is not an artifact of the method used to reduce the $[Ca^{2+}]$ in the myofibril. No significant force was redeveloped on restretch following release-restretch maneuvers to detach force-generating cross-bridges at the beginning of the slow relaxation phase (e.g., Fig. 1 *B*). This finding shows that, soon after the

solution change, [Ca^{2+}] was effectively reduced below the threshold for new cross-bridge formation.

Effects of mechanical perturbations

The time course for complete force relaxation in myofibrils corresponds to that previously described for intact fibers relaxing from maximum tetanic force (Huxley and Simmons, 1970, 1973; Cleworth and Edman, 1972). Huxley and Simmons (1970) found that in living fibers the initial linear phase of relaxation is isometric while the final exponential force decay, invariably, begins when one end of the fiber suddenly starts to lengthen. To learn to what extent mechanical factors are associated with the transition from slow to fast relaxation, small (0.2–2% l_0) and rapid (1 ms) length changes were applied to myofibrils soon after Ca^{2+} removal. In all myofibril types, stretch (Fig. 3 *A* representative data from rabbit soleus myofibrils at 15°C) abbreviated the slow force decay and accelerated the transition to the fast relaxation phase, while release (Fig. 3 *B*) had slight but significant opposite effects on the duration of the linear phase. The average dependence of the duration of the slow phase of relaxation on the size of the length changes applied to seven soleus myofibrils is shown in Fig. 3 *C*. Stretches just larger than 0.5% l_0 halved the duration of the slow relaxation phase also in rabbit psoas and frog tibialis myofibrils (data not shown). The results are consistent with the idea that the slow relaxation requires isometric sarcomeres and that the transition from slow to fast relaxation is initiated by sarcomere elongation that breaks the isometric conditions (Huxley and Simmons, 1970, 1973; Stehle et al., 2002). We conclude, therefore, that only slow k_{REL} provides reliable information about cross-bridge kinetics during sarcomere isometric relaxation.

Effects of initial and final [Ca^{2+}] and force levels on relaxation

Slow k_{REL} may represent either only the rate of detachment of force generating cross-bridges under nearly isometric conditions or it may also include cooperative thin filament activation by force-generating cross-bridges. If activation by attached cross-bridges dominates slow k_{REL}, one expects it would be more sensitive to the force level preceding relaxation than to that at the end of relaxation.

To learn of the effects of initial Ca^{2+}-activated force on slow k_{REL}, rabbit psoas and frog tibialis myofibrils were activated at a pCa between 4.5 and 6.0 and then fully relaxed at pCa 8.0. Plots of slow k_{REL} versus initial force level are shown in Fig. 4 *D* for both preparations at 5°C, while experimental tracings obtained from a frog myofibril in maximum and submaximum activation-relaxation cycles are compared in Fig. 4, *A-C*. The normalized relaxation transients in Fig. 4 *C* show that the time course of the overall force decline was little affected by decreasing the Ca^{2+}-activated force at the start of relaxation. In frog myofibrils, slow k_{REL} was not significantly modified by changes in the initial force level (Fig. 4 *D*, *open symbols*). In rabbit

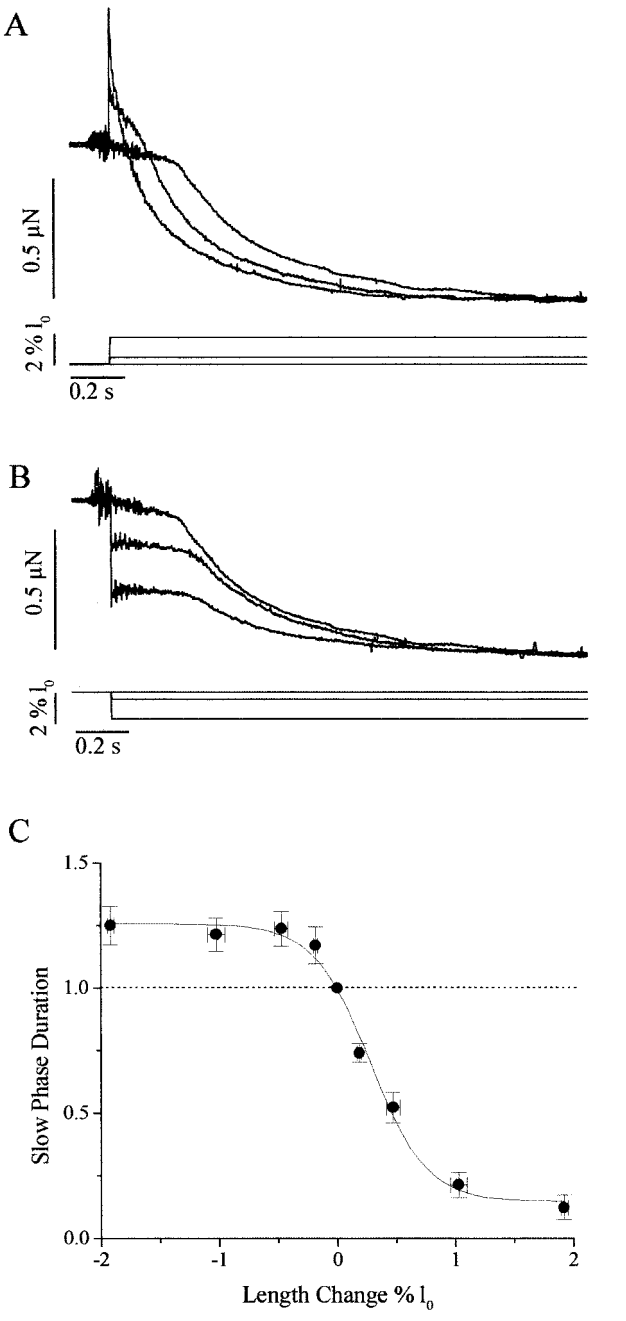

FIGURE 3 Effects of length changes on full force relaxation of rabbit soleus myofibril at 15°C. Small and rapid stretches (*A*) and releases (*B*) are applied to the maximally activated myofibril (pCa 4.5) 50 ms after the start of full relaxation (pCa 8.0). The resulting force traces are compared with those of a control relaxation. (*C*) Dependence of the duration of the slow phase of relaxation (normalized for the control value) on the amplitude of the applied length change (stretches, positive values; releases, negative values). Data points are means ± SE ($n = 7$).

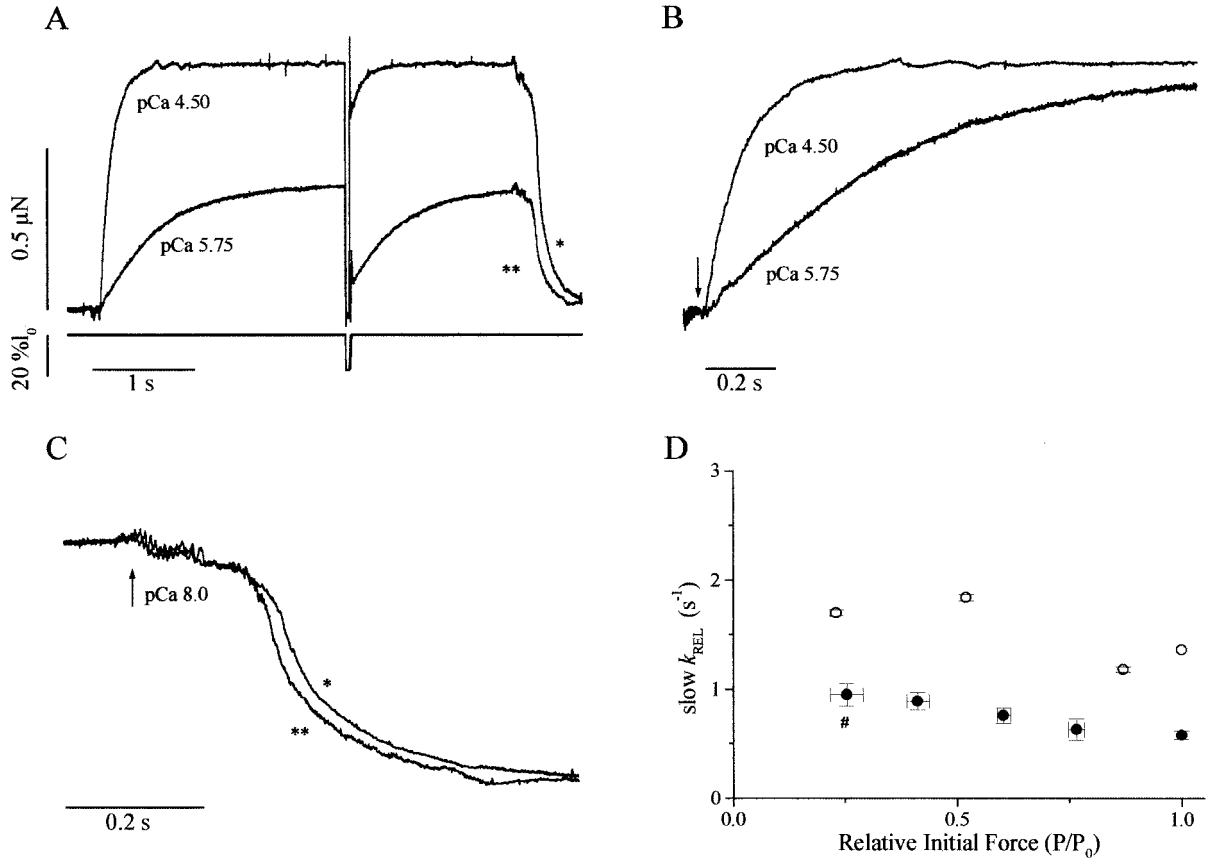

FIGURE 4 Effects of initial $[Ca^{2+}]$ on full force relaxation in frog tibialis myofibril at 5°C. (*A*) Maximal (pCa 4.5, *) and submaximal (pCa 5.75, **) activation-relaxation cycles are compared. (*B*) Same traces as in *A* on expanded time base and normalized to illustrate the effect of final Ca^{2+}-activated force on the time course of force activation. (*C*) Same traces as in *A* on expanded time base and normalized to illustrate the effect of decreasing initial $[Ca^{2+}]$-activated force on the time course of force relaxation upon Ca^{2+} removal. (*D*) Rate constants of the early slow force decline (slow k_{REL}) measured in frog tibialis (*open symbols*) and rabbit psoas (*closed symbols*) myofibrils at 5°C are plotted versus relative Ca^{2+}-activated force preceding the start of relaxation. Each point represents means ± SE of 5–13 frog tibialis myofibrils and 5–39 rabbit psoas myofibrils; #$p < 0.01$ versus slow k_{REL} measured from maximal activation.

psoas myofibrils, however, some acceleration of slow k_{REL} occurred following the reduction of the initial Ca^{2+}-activated force (Fig. 4 *D*, *closed symbols*). The change, which was accompanied by some shortening of the linear phase of force decay, only became significant ($p < 0.01$) when relaxation started at low Ca^{2+}-activated force (~0.25 P_0).

In contrast to the slow k_{REL}, analysis of the kinetics of force development from the experiments described above clearly showed that k_{ACT} (as well as k_{TR}) was strongly dependent on $[Ca^{2+}]$ in the activating solution (e.g., Fig. 4 *B*), as previously reported in caged-Ca^{2+} experiments on rabbit psoas and frog fibers (Ashley et al., 1991; Araujo and Walker, 1994; Patel et al., 1996; Wahr and Rall, 1997). An example of the dependence of k_{ACT} on final Ca^{2+}-activated force is shown in Fig. 5 *D* (*closed squares with errors bars*) for rabbit psoas myofibrils at 15°C.

To determine the effects of final force on the relaxation, myofibrils were maximally activated at pCa 4.5 and then partially relaxed to a given submaximum pCa between 5.5 and 6.0. As shown in Fig. 5, *A* and *C* for rabbit psoas myofibrils at 15°C, relaxation kinetics were dependent on the amount of residual Ca^{2+}-activated force. Partial relaxation from maximum force to forces above 50% of maximum was usually monophasic, and its time course was nearly the same as that of force development following activation to the same final $[Ca^{2+}]$ and force level (Fig. 5, *A* and *B*). The time course of such monophasic relaxation could be described by a single rate constant (k_{REL}) that, like k_{ACT}, increased with increasing final Ca^{2+}-activated force levels (Fig. 5 *D*, *closed circles*). Partial relaxation from maximum force to forces below 50% of maximum was biphasic, as in full relaxation, but the initial, linear force decay lasted longer and fast k_{REL} was slower than for full relaxation (Fig. 5 *C*). There was a slight but significant trend for the slow k_{REL} to increase with increasing final Ca^{2+}-activated force levels (Fig. 5 *D, closed circles*). Together, slow k_{REL} measured in biphasic relaxation and k_{REL} of monophasic relaxation displayed the same dependence on

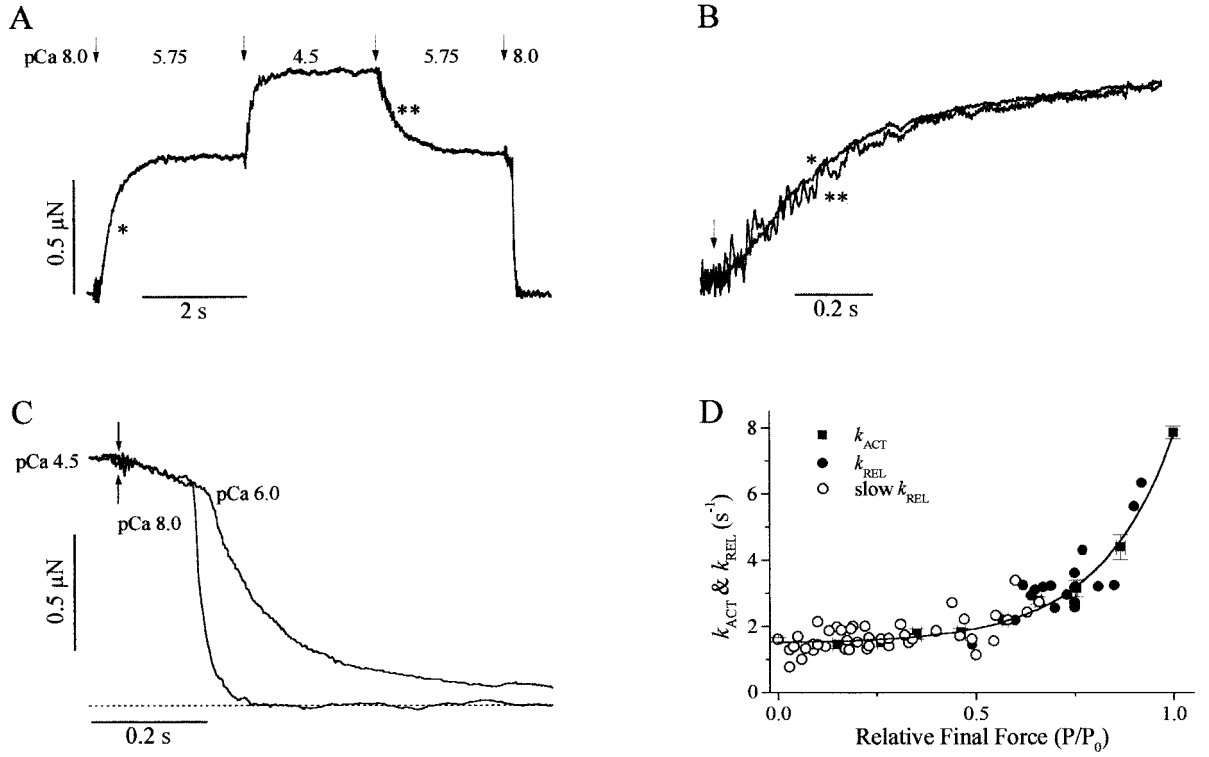

FIGURE 5 Effects of final [Ca^{2+}] on relaxation from maximal force in rabbit psoas myofibril at 15°C. (*A*) Force responses of the myofibril to step decreases and increases in pCa; with this experimental protocol the kinetics of force development and partial force relaxation can be compared at the same final [Ca^{2+}]. Note the monophasic behavior of the partial force decline from maximum to submaximum Ca^{2+}-activated force (**). (*B*) Comparison of the time course of partial force relaxation from maximal to submaximal activation (pCa 4.5–5.75; **) with that of force development from relaxing conditions to submaximal activation (pCa 8.0–5.75; *); same trace as in *A* on expanded time base and after normalization and reversal of the relaxation trace. (*C*) Comparison of the time course of full-force relaxation from maximal activation with the time course of partial force relaxation to a low final level of Ca^{2+}-activated force. Under this condition partial relaxation is biphasic. (*D*) Rate constants for force activation and relaxation are plotted versus final Ca^{2+}-activated force levels. Closed squares are means ± SE of k_{ACT} values measured from 8–35 rabbit psoas myofibrils; closed circles are scattered k_{REL} values estimated from exponential fits of monophasic partial relaxation of the kind shown in panel *A*; open circles are scattered slow k_{REL} values estimated from the early slow force decline of the kind shown in panel *C;* the open circle at zero force is the mean (±SE) of slow k_{REL} in full relaxation.

the final Ca^{2+}-activated force level as k_{ACT} (Fig. 5 *D*). Qualitatively similar results were obtained for rabbit psoas and frog tibialis myofibrils at 5°C.

The results suggest that the deactivation, following sudden Ca^{2+} removal, is rapid. Sustained activation, either arising from cycling force-generating cross-bridges or from residual Ca^{2+} bound to TnC, does not seem to significantly limit slow k_{REL}. We conclude that slow k_{REL} in full relaxation is predominantly the apparent rate with which attached cross-bridges leave force-generating states.

Effects of ADP, P_i, and ATP on full relaxation

The effects of substrate and products of myosin ATPase were investigated in maximally activated rabbit psoas myofibrils at 5°C to identify the cross-bridge transitions contributing to slow k_{REL}. The kinetics of relaxation could be significantly limited by steps in the cross-bridge cycle associated with ADP release from strong actomyosin complexes. Although addition of 3 mM MgADP produced only a modest increase in active force generation, it significantly prolonged relaxation (Table 1 and Fig. 6 *A*). It did so by increasing the duration of the slow, linear phase of force decay and reducing both slow and fast k_{REL}.

As previously shown (Tesi et al., 2000), P_i addition had large effects on force generation in myofibrils that were consistent with the idea that P_i causes dissociation of force-generating cross-bridges to non-force-generating states by reversal of the power stroke. The effects of 5 mM P_i on full force relaxation of rabbit psoas myofibrils are shown in Fig. 6 *B* and Table 1. Increases in [P_i] caused a faster onset of the rapid relaxation by accelerating and shortening the initial, linear force decline. However, the final rapid force decay was not significantly affected by P_i. Early relaxation was, instead, very sensitive to P_i because slowing and prolongation of the slow phase was observed by reduction of the P_i contamination of nominally P_i-free standard solutions (~170 μM) to below 5 μM (see Table 1). The large effects of P_i on the slow relaxation phase suggest that the reversal of the power stroke contributes to the slow k_{REL}. Results

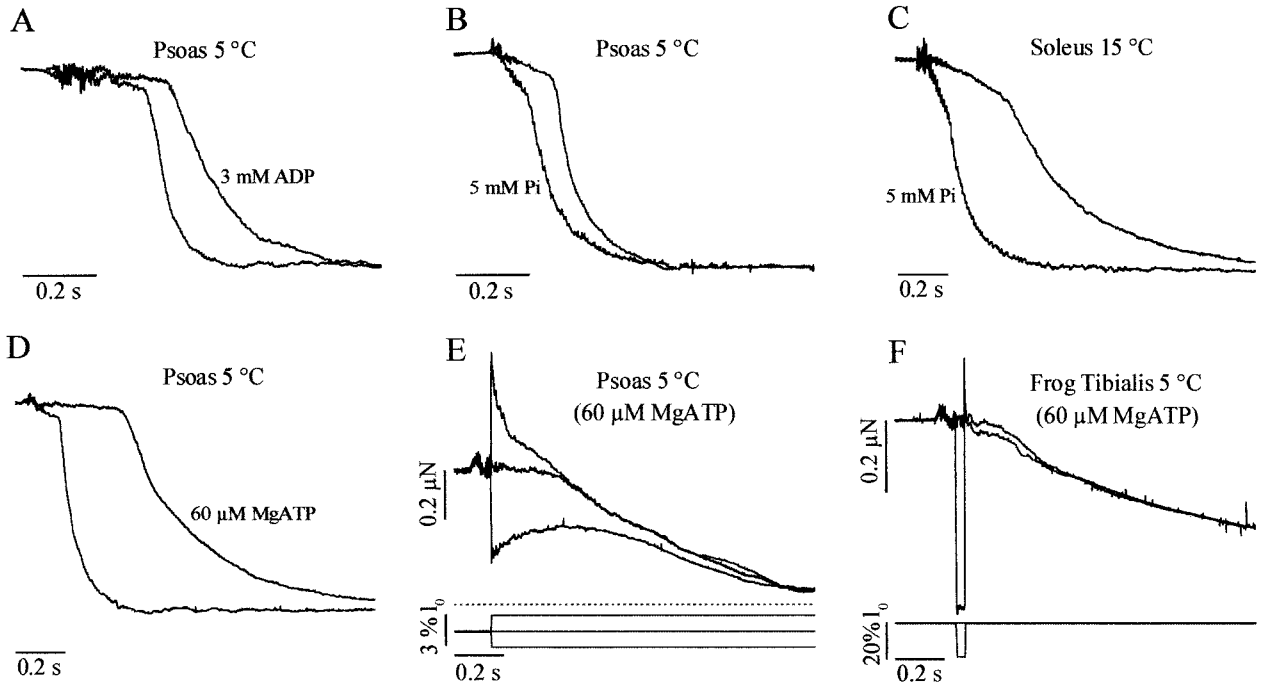

FIGURE 6 Effects of changes in product and substrate concentrations on the kinetics of full force relaxation. In all cases pCa was suddenly changed from 4.5 to 8.0, and control and test force traces were normalized to better show the change induced by each intervention on the time course of relaxation. (*A*) Rabbit psoas myofibril, 5°C; maximum force in 3 mM MgADP was 1.20 the control force. (*B*) Rabbit psoas myofibril, 5°C; maximum force in 5 mM P_i was 0.47 the control force. (*C*) Rabbit soleus myofibril, 15°C; maximum force in 5 mM P_i was 0.68 the control force. (*D*) Rabbit psoas myofibril, 5°C; maximum force in 60 μM MgATP was 1.05 the control force. (*E*) Effects on force relaxation of relatively small and rapid length perturbations applied 50 ms after the solution change to rabbit psoas myofibril in low MgATP solutions (5°C). *Dotted line,* zero force level. (*F*) Effect on force relaxation of large release-restretch applied 50 ms after the solution change to frog tibialis myofibril in low MgATP solutions (5°C).

similar to those shown for rabbit psoas myofibrils were also obtained in rabbit soleus myofibrils (Fig. 6 *C*) and in frog tibialis (data not shown). In soleus myofibrils, besides the usual large effect on the slow relaxation phase, P_i also accelerated the final exponential phase; 5 mM P_i increased fast k_{REL} of soleus myofibrils from 2.1 $\pm$ 0.2 s^{-1} (see Table 1) to 7.2 $\pm$ 1.4 s^{-1} ($n = 6$).

Lowering [MgATP] prolonged relaxation of rabbit psoas myofibrils. While the effect was significant below 400 μM MgATP, it was very large below 100 μM. Comparison of relaxation transients at 5 mM and 60 μM MgATP are shown in Fig. 6 *D,* while average kinetic parameters for full relaxation at 60 μM MgATP are given in Table 1. The effects of [MgATP] on force generation in myofibrils have been previously described (Tesi et al., 1999). The observed decrease of slow k_{REL} was consistent with the effect of ATP on the dissociation of strongly bound actomyosin complexes. The large effects of low [MgATP] on the overall relaxation kinetics, however, suggest that an additional mechanism, besides reduction in the cross-bridge dissociation rate, delays force decline. At low [MgATP] relatively small stretches applied to the myofibril following Ca^{2+} removal failed to shorten relaxation, whereas releases produced force redevelopment transients (Fig. 6 *E*). Even large release-restretches applied to frog tibialis myofibrils after Ca^{2+} removal did not modify relaxation (Fig. 6 *F*). These observations support the idea that accumulation of a small number of rigor-like actomyosin complexes, in contrast to strongly attached cycling cross-bridges, can significantly sustain thin filament activation and limit the rate of relaxation.

DISCUSSION

Summary

Rapid increases or decreases of [Ca^{2+}] in myofibrils by solution switching initiate force development and force relaxation transients with [Ca^{2+}]-dependent kinetics. The kinetics of both transient types are dictated primarily by the final [Ca^{2+}] (Fig. 5 *D*), while the effects of the initial [Ca^{2+}] are minimal (Figs. 2 and 4). When compared at the same final [Ca^{2+}], force activation and relaxation transients have similar kinetics (Fig. 5 *D*). Fast, symmetric "on" and "off" regulation by [Ca^{2+}] is consistent with models in which the weak to strong cross-bridge transition is modulated by final [Ca^{2+}] with no feedback from cycling cross-bridges (Brenner, 1988; Millar and Homsher, 1990; Regnier et al., 1995; Brenner and Chalovich, 1999). During full force relaxation, when [Ca^{2+}] is reduced below the thresh-

old for force activation, the regulatory system is rapidly and completely turned off, preventing recruitment of new force-generating cross-bridges (e.g., Fig. 1 *B*). Attached cross-bridges slowly decay through both forward (ADP release) and backward (P_i binding) transitions from force-generating to non-force-generating states. Sudden collapse of isometric sarcomere conditions accelerates force decay through increased rates of cross-bridge detachment.

Kinetics of force activation

Rapid elevation of $[Ca^{2+}]$ in myofibrils by solution switching initiates tension development processes with properties similar to those seen in tension transients in skinned fibers initiated by caged Ca^{2+} photolysis (Ashley et al., 1991; Araujo and Walker, 1994; Patel et al., 1996; Wahr and Rall, 1997) or by release-restretch (Brenner, 1988; Metzger et al., 1989). However, the maximum first-order rate constants for psoas fibers ($\sim$5 and 15 s^{-1} at 5 and 15°C, respectively) are twofold higher than those recorded here (2.6 and 7.8 s^{-1}, see Table 1). In the present experiments sarcomere length was not controlled and the time course of force development may be slowed by sarcomere shortening. Although compliance is likely to influence tension transient kinetics, we do not think it represents a major determinant of the difference between myofibril and fiber kinetic data. The overall series compliance of psoas myofibrils ($\sim$5% l_0 at maximum force (Tesi et al., 1999)) is not larger than that usually found in skinned fibers and clamp of sarcomere length during tension development in psoas fibers increases k_{TR} by $<$20% (Chase et al., 1994). P_i accumulation in skinned fibers during contraction (Pate et al., 1998) and the large effects of $[P_i]$ on k_{ACT} and k_{TR} in psoas muscle offer a more likely explanation for the slower k_{ACT} and k_{TR} found in psoas myofibrils (in which $[P_i]$ matches that of the perfusing solution). When P_i accumulation in rabbit psoas fibers is prevented by a fast P_i "mop," the rate constant of force development following caged Ca^{2+} photolysis is slower (2.2 s^{-1} at 5°C (He et al., 1997)) than k_{ACT} found here. Consistent with this explanation, k_{ACT} and k_{TR} found in soleus myofibrils are close to k_{TR} previously reported for rabbit soleus fibers (Millar and Homsher, 1992). In general, slow muscle fibers are less affected by P_i accumulation during contraction because of their lower ATPase rate. Moreover, soleus k_{TR} is less influenced by $[P_i]$ than psoas muscle (Millar and Homsher, 1992; Tesi et al., 2000).

As previously shown in psoas fibers (Araujo and Walker, 1994) and in cardiac preparations (Palmer and Kentish, 1998; Stehle et al., 2002) in all myofibril types, k_{ACT} is strikingly similar to k_{TR} (Table 1) and does not increase by increasing the initial $[Ca^{2+}]$ (Fig. 2; see also Wahr and Rall, 1997). This provides evidence that k_{ACT} is neither rate-limited by the speed of the solution change nor by the speed with which thin filaments bind Ca^{2+} and activate. Instead, it is likely that k_{ACT}, as well as k_{TR}, reflect isometric cross-bridge turnover rates ($f_{app} + f'_{app} + g_{app}$) that, at high activation levels, are dominated by the apparent forward rate of the force-generating transition (f_{app}) (Brenner, 1988).

In agreement with a number of studies in fibers (reviewed in Gordon et al., 2000), both k_{ACT} and k_{TR} in myofibrils strongly depend on the final $[Ca^{2+}]$ (Figs. 4 *C* and 5 *D*). Since the kinetics of P_i release were shown to be independent of $[Ca^{2+}]$ (Millar and Homsher, 1990; Walker et al., 1992; Tesi et al., 2000), it is likely that modulation of the force-generating transition in the cross-bridge cycle by $[Ca^{2+}]$ is an indirect effect. A rapidly established and $[Ca^{2+}]$-dependent equilibration between inactive and active states of regulated actin may provide the mechanism by which the transition from weakly to strongly bound cross-bridge states becomes much more likely when $[Ca^{2+}]$ is high and actin is in its active form (Brenner and Chalovich, 1999; see also Landesberg and Sideman, 1994). The increased probability that cross-bridges effectively enter the force-generating states will increase the rate of force development so that the process does behave as if it is a kinetic regulation.

Kinetics of force relaxation

Full relaxation is markedly biphasic with a slow, linear initial phase that is terminated when isometric sarcomere conditions collapse (Stehle et al., 2002). Monophasic relaxation transients, like those in Fig. 5 *A,* suggest that nearly isometric sarcomeres can be maintained throughout force decay if significant force is left at the end of relaxation. The properties of k_{REL}, measured under nearly isometric conditions (Fig. 5 *D*), suggest that deactivation of the regulatory system (like activation) involves fast Ca^{2+} equilibration, whereas k_{REL} (like k_{ACT}) reflects isometric cross-bridge turnover that is switched to a new $[Ca^{2+}]$-dependent level soon after solution change. When $[Ca^{2+}]$ is reduced below the threshold for force activation, the probability that cross-bridges undergo the weak to strong transition rapidly approaches zero and cross-bridge turnover is dominated by the apparent forward (g_{app}) and backward (f'_{app}) rates with which cross-bridges leave force-generating states. The effects of P_i and ADP on slow k_{REL} (Fig. 6 and Table 1) are consistent with two pathways for cross-bridge detachment.

Because P_i decreases maximum force while ADP increases it (Table 1), the effects of P_i and ADP on slow k_{REL} could be taken as an indication that the number of force-generating cross-bridges contributes to the relaxation kinetics. However, reducing cross-bridge number by decreasing Ca^{2+}-activated force levels preceding the start of relaxation has no effects (or only minor effects) on slow k_{REL} (Fig. 4), whereas P_i causes a huge acceleration of the slow force decay (Table 1). Moreover, stiffness measurements (Lu et al., 2001) indicate that the increase in force induced by ADP in maximally activated psoas fibers may not be due to an increase in cross-bridge numbers, but rather it may be

attributed to an increase in mean force per cross-bridge. Finally, any feedback from cycling cross-bridges on the rate of the weak to strong cross-bridge transition is expected to sustain activation following sudden decrease in $[Ca^{2+}]$ and introduce asymmetries between force relaxation and activation kinetics when they are compared at the same final $[Ca^{2+}]$. As shown in Fig. 5 *D,* this was not the case, thus it appears that the effect of strong attachment of cycling cross-bridges on thin filament activation in Ca^{2+}-activated myofibrils under our standard experimental conditions is rather small. The present results, however, cannot exclude that thin filament activation by some ADP-bound cross-bridge states (Lu et al., 2001) contributes to the prolongation of relaxation observed in rabbit psoas myofibrils in the presence of ADP (Fig. 6 *A* and Table 1).

Although relaxation from maximum force in myofibrils closely resembles that described for intact frog fibers after tetanic stimulation (Huxley and Simmons, 1970, 1973; Cleworth and Edman, 1972), slow k_{REL} is significantly faster in frog tibialis myofibrils (1.4 s^{-1} at 5°C, see Table 1) than in intact fibers (well below 1 s^{-1} at 4°C, as referenced in Gordon et al., 2000). The difference is further evidence that in myofibrils, activation rapidly disappears upon Ca^{2+} removal. In the intact fiber Ca^{2+} removal is relatively slow (Caputo et al., 1994; Jiang and Julian, 1999) and force decay is delayed by cross-bridge reattachment. Consistent with this explanation, intact fibers, unlike myofibrils (e.g., see Fig. 1), can significantly redevelop force after small releases (Jiang and Julian, 1999) or large release-restretches (data not shown) applied during the linear relaxation phase. Residual thin filament activation that allows recruitment of some new cross-bridges may also account for the large decrease of slow k_{REL} seen in myofibrils at low ATP (Fig. 6 *D* and Table 1). The effect is too large to be justified by decrease of actomyosin dissociation: with a second-order rate constant $\geq 10^5$ M^{-1} s^{-1} at 5°C (Goldman et al., 1984), actomyosin detachment at 60 μM ATP is too fast to limit slow k_{REL}. A few rigor-like cross-bridges sustaining activation offers a more reasonable explanation for the observed deceleration of force decay (see also Fig. 6, *E* and *F*).

Force relaxation induced by photolysis of caged Ca^{2+} chelators in skinned muscle preparations usually lacks the slow phase (Patel et al., 1996; Palmer and Kentish, 1998) or exhibits a short linear relaxation phase that is only slightly slower than the subsequent exponential force decay (Patel et al., 1998; Hoskins et al., 1999). However, a pronounced biphasic shape of diazo-2-induced force relaxation, closer to that observed here, has been described 1) in skinned rabbit psoas fibers after reducing $[P_i]$ in the myofilament lattice by a fast P_i-"mop" (Luo et al., 2001), and 2) in skinned frog fibers after care was taken to reduce fiber end compliance and improve sarcomere homogeneity (Wahr et al., 1998). These observations suggest that P_i accumulation and early breakdown of isometric conditions contribute to the lack of a clear slow phase of relaxation in most photolysis studies. We show here that the slow phase of relaxation is very sensitive to $[P_i]$ and small length perturbations. The opposing effects of release and stretch on the slow relaxation phase (Fig. 3) indicate that transition from slow to fast relaxation is favored by increasing mechanical strain on the individual cross-bridges. As the maximum strain a cross-bridge can bear is limited, it may be that during a large force decay, in the absence of significant recruitment of new cross-bridges, even slight nonuniformities in the force-generating capabilities of sarcomeres in series will inevitably end with the collapse of isometric conditions. At the time of the tension shoulder, remaining cross-bridges in the weakest sarcomere(s) are under steadily increasing strain. P_i may preferentially bind to highly strained cross-bridges as the threshold is reached to an extent that the force-bearing capacity of individual cross-bridges is exceeded. These events may favor rapid cross-bridge detachment and "give" of the weakest sarcomere(s). Although we cannot measure sarcomere length simultaneously with force measurements, Stehle et al. (2002) have demonstrated in cardiac myofibrils that a sudden elongation of just one to a few sarcomeres coincides with the tension "shoulder" in the relaxation transient following Ca^{2+} removal.

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