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Phosphate Release and Force Generation in Skeletal Muscle Fibers

Abstract. *Rapid laser pulse–induced photolysis of an adenosine triphosphate precursor in muscle fibers abruptly initiated cycling of the cross-bridges. The accompanying changes in tension and stiffness were related to elementary mechanochemical events of the energy-transducing mechanism. When inorganic phosphate was present at millimolar concentrations during liberation of adenosine triphosphate in the absence of calcium, relaxation was accelerated. Steady active tension in the presence of calcium was decreased but the approach to final tension was more rapid. These results suggest that, during energy transduction, formation of the dominant force-generating cross-bridge state is coupled to release of inorganic phosphate in a reaction that is readily reversible.*

Inorganic phosphate (P_i) is present in muscle cells at millimolar concentrations, and during activity or fatigue the P_i concentration increases severalfold (*1*). Inorganic phosphate reduces the steady tension (*2, 3*) and stiffness (*2*) of isometrically contracting skinned muscle fibers. We report here experiments involving a photochemical technique that abruptly initiates the actomyosin cross-bridge cycle in skinned fibers. The results suggest an important role for P_i release in the cross-bridge cycle.

Single skinned fibers from rabbit psoas muscle were equilibrated in the rigor state with 10 m*M* "caged" ATP [P^3-1-(2-nitro)phenylethyladenosine 5′-triphosphate] (*4*), a photolabile precursor of ATP. Tension and stiffness transients were monitored as ATP (500 to 800 μ*M*) was rapidly liberated from caged ATP in the fiber by a pulse of radiation (wavelength, 347 nm) from a frequency-doubled ruby laser (*5*). A piezoelectric element applied a 500-Hz, 1-μm sinusoidal length oscillation to the fiber for stiffness measurements and the tension was detected by a high-bandwidth transducer (*5, 6*). A phase-sensitive lock-in amplifier separated the sinusoidal portion of the transducer signal into components in phase and 90° out of phase with the change in length. The amplitudes of these sinusoidal components are qualitatively related to the mechanical state of the cross-bridges. If we assume that the elasticity of an attached cross-bridge is fixed and neglect artifacts due to end compliance, then the in-phase sinusoidal component is the stiffness proportional to the number of attachments (*5*). The 90° out-of-phase component (quadrature stiffness) indicates viscoelasticity of the attached cross-bridges. After a small abrupt change in length, during an active contraction, the tension recovers within a few milliseconds to almost its value before the change in length (*7*). This quick recovery is diminished or absent in rigor (*8*). With sinusoidal changes in length, the quick recovery of active cross-bridges results in a phase shift of the sinusoidal tension response (*9*). In our experiments a positive deflection of the quadrature stiffness traces resulted from this phase shift and thus indicated the viscoelasticity of cross-bridges actively producing force (*5, 10*).

Figure 1 (A and B) shows superimposed tension recordings from successive rigor-photolysis-relaxation trials in the absence of Ca^{2+}. In one trial the fiber was held isometric and in the other it was stretched 0.53 percent 1 second before photolysis. After the liberation of ATP there was a rapid drop in tension due to cross-bridge detachment. The two tension traces then converged and remained superimposed during the final relaxation.

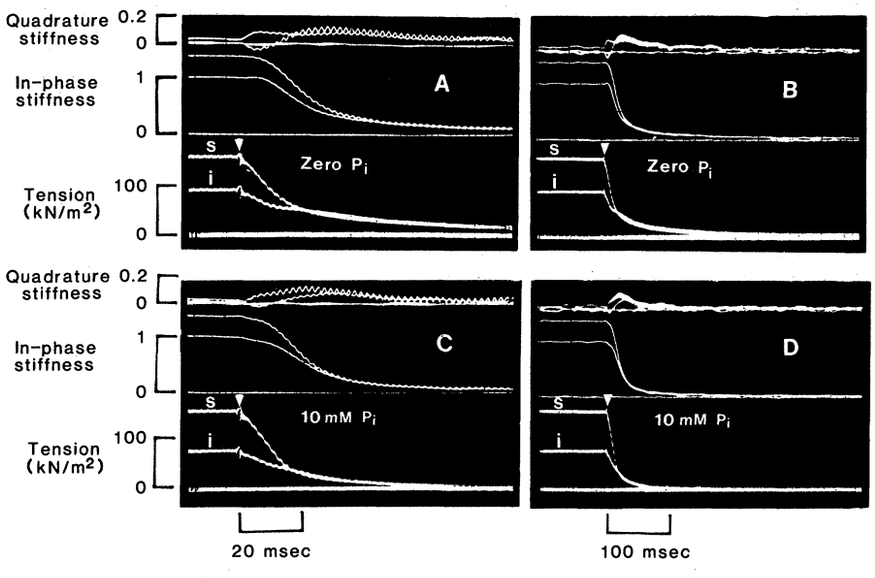

Fig. 1. (A to D) Tension and stiffness transients recorded during photolysis of caged ATP in a single glycerol-extracted fiber held isometric (i) or stretched (s). Shown are fast (A and C) and slow (B and D) time-base recordings obtained during trials without (A and B) and with (C and D) 10 m*M* orthophosphate (P_i). The fiber was equilibrated with a rigor-inducing solution containing 20 m*M* EGTA, 100 m*M* trimethylaminoethanesulfonic acid, 1 m*M* Mg^{2+}, $<10^{-8}$ *M* Ca^{2+}, 10 m*M* reduced glutathione, 0 to 10 m*M* P_i, 10 m*M* caged ATP, and 15 to 25 m*M* 1,6-diaminohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (final ionic strength, 200 m*M*; *p*H 7.1; 22°C). The oscilloscope sweeps were triggered before photolysis, and the mechanical transients induced by the liberation of 700 μ*M* ATP (arrowhead) were recorded (*5, 6*). Stiffness was monitored at 500 Hz. Stiffness traces have been normalized to the in-phase rigor stiffness. Baseline stiffness and tension measurements are those of the fully relaxed fiber. The first 10 msec of the stiffness records contain artifacts associated with the laser pulse.

We previously presented evidence indicating that (i) most cross-bridges from the original rigor state detach by the time the tension traces converge; (ii) transient tension redevelopment is due to reattaching cross-bridges, as suggested by the positive quadrature stiffness (upper traces in Fig. 1, A and B); and (iii) the final relaxation indicates subsequent detachment (*5, 6*).

In further experiments, we found that equilibration of the fiber with a constant 10 m*M* concentration of P_i before photolysis reduced the amplitude of transient redevelopment of tension and markedly increased the rate of final relaxation (Fig. 1, C and D, and Fig. 2, inset). Inorganic phosphate had no appreciable effect on rigor tension and little effect on the rate of tension convergence upon liberation of ATP, suggesting that the rate of cross-bridge detachment from rigor was not altered. Active cross-bridge reattachment still occurred, as indicated by a positive quadrature stiffness (Fig. 1, C and D) and a net increase in tension when initial tension was low. However, the final rate of relaxation increased as P_i concentration was increased to 25 m*M* (Fig. 2).

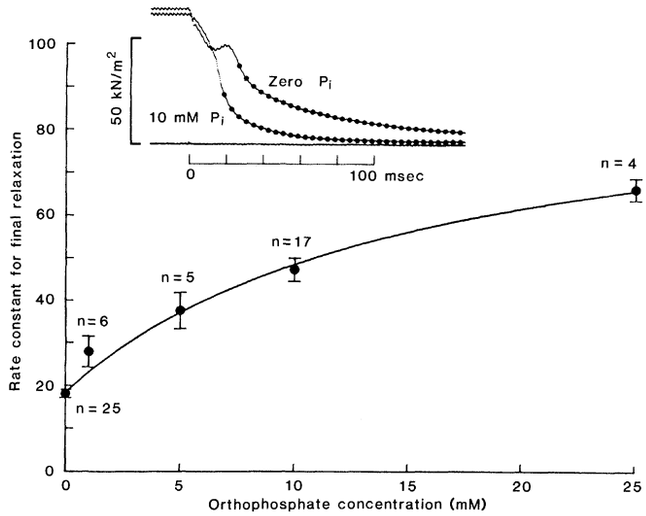

Fig. 2. Phosphate dependence of the rate of final tension relaxation from rigor induced by photogeneration of ~600 μ*M* ATP. Inset: two superimposed isometric tension transients recorded in photolysis trials as described in the legend to Fig. 1, with 0 or 10 m*M* P_i in the solution. The final relaxation phases in each trial were fitted (●) by the sum of two exponentials with rate constants of 261 sec^{-1} and 46.3 sec^{-1} at 10 m*M* P_i and 159 sec^{-1} and 17.9 sec^{-1} in the absence of P_i. These rates depended strongly on the concentration of P_i, as illustrated in the graph for the component with the lower rate. The biphasic nature of the final relaxation phase is not yet explained by our simulations (*6, 10*). The mean ± standard error for a series of experiments is shown at each P_i concentration.

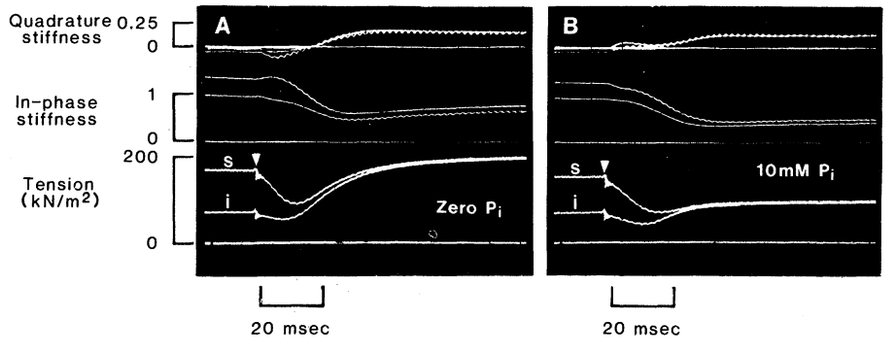

Fig. 3. Initiation of active contraction from the rigor state by liberation of ATP under conditions similar to those described in the legend to Fig. 1, but with ~100 μ*M* free Ca^{2+}. (A) Tension and stiffness with no added P_i and 700 μ*M* ATP liberated. (B) Tension and stiffness with 10 m*M* added P_i and 870 μ*M* ATP liberated. Baselines in each trace record stiffness or tension of the relaxed fiber.

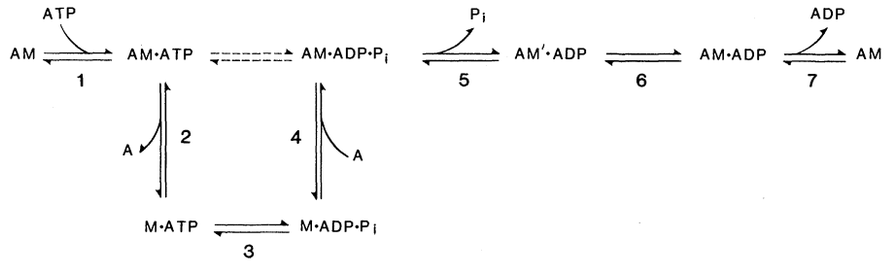

Fig. 4. Proposed biochemical reaction scheme for ATP hydrolysis during muscle contraction (*11–15, 17*). AM′ · ADP is formed only during ATP hydrolysis and can bind P_i. Abbreviations: A, actin; and M, myosin.

Our results may be interpreted on the basis of the scheme shown in Fig. 4 for the actomyosin-adenosinetriphosphatase reaction derived from solution studies in several laboratories (*11, 12*). Important features of this biochemical mechanism are that ATP induces rapid dissociation of actomyosin and that the actin-binding and ATP hydrolysis steps are readily reversible (*12–15*). The rate-limiting step of the actomyosin-adenosinetriphosphatase reaction is open to question at physiological ionic strength but appears to be associated with reaction steps 5 or 6 or both in muscle fibers (*16*). AM · ADP (actomyosin–adenosine diphosphate) is the ternary complex formed by addition of ADP to actomyosin. Sleep and Hutton (*17*) postulated that an AM′ · ADP state (Fig. 4) capable of binding P_i was formed during ATP hydrolysis catalyzed by actomyosin. AM · ADP may be on the pathway during ATP hydrolysis (Fig. 4), or it may be on a side branch.

In the photolysis experiments described above, the cross-bridges begin in the rigor (AM) state. Photochemical release of ATP induces cross-bridge detachment through reactions 1 and 2 (Fig. 4). Reattachment and force generation (reactions 4 to 7) then occur in both the presence and absence of Ca^{2+} (*5, 6, 10*). In the experiments without added Ca^{2+} the increased rate of final relaxation in

the presence of P_i can be explained by reversal of reactions 5 and 4. A reversible attachment step was also included in our recently published kinetic simulation (*10*) for two reasons: (i) it allows for current estimates of the number of cross-bridges attached during active contraction and (ii) it explains the observation that the final relaxation rate after caged ATP photolysis (Fig. 1B) is more rapid than the overall rate of ATP hydrolysis per myosin head (<2 sec^{-1}) (*16*).

If attachment and force generation are indeed reversible, then the observed rate constant for tension redevelopment in the presence of Ca^{2+} would be given by the sum of the forward and reverse reaction rates. An increase in P_i concentration would then increase the rate constant for tension redevelopment by increasing the rate of reversal of reaction 5 and would decrease steady active tension by shifting the distribution of cross-bridge states toward AM · ADP · P_i and M · ADP · P_i.

Our interpretation is based on the assumption that the mechanical measurements reflect P_i binding to the myosin active site (reversal of reaction 5). Alternatively, P_i might increase the rate of the forward reactions 6 and 7. This seems unlikely, however, because the rate of ATP hydrolysis by an actively contracting fiber would then be expected to increase markedly in the presence of P_i. However, other effects of P_i on reactions 6 and 7 have not been ruled out.

Addition of ADP and then P_i to a muscle fiber in the rigor state does not decrease the tension. Thus the results of our photolysis experiments suggest that P_i binds to the AM′ · ADP formed only through reactions 3, 4, and 5 during ATP hydrolysis, as proposed for isolated actomyosin (*17*). The lifetime of AM′ · ADP has not been quantitatively determined in isolated actomyosin or in fibers. However, mechanical strain on the cross-bridge might shift the equilibrium between AM′ · ADP and AM · ADP · P_i toward AM · ADP · P_i. In that case AM′ · ADP would produce more mechanical force than AM · ADP · P_i and the hypothesis would imply that P_i release is intimately coupled to formation of the dominant force-generating state (*18*).

Our results may help to explain other mechanical phenomena in muscles. Inorganic phosphate reduces the amplitude of stretch-induced activation in insect fibrillar flight muscle (*19*) and increases the relaxation rate of skinned smooth muscle (*20*). These effects are consistent with a reversal of step 5, although other factors may be involved. During fatigue of intact vertebrate skeletal muscle, tension decreases as P_i, ADP, and H^+ concentrations increase (*1*). If the accumulation of P_i contributes to this decrease in tension (*21*), then our results provide a qualitative explanation for fatigue development in terms of cross-bridge kinetics.

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Defect in Vitamin B_{12} Release from Lysosomes: Newly Described Inborn Error of Vitamin B_{12} Metabolism

Abstract. *Cultured diploid fibroblasts from a patient with a previously undescribed inborn error of cobalamin metabolism accumulate unmetabolized, nonprotein-bound vitamin B_{12} in lysosomes. These cells are able to endocytose the transcobalamin II-B_{12} complex and to release B_{12} from transcobalamin II. The freed vitamin B_{12} is not released from lysosomes into the cytoplasm of the cell. This suggests that there is a specific lysosomal transport mechanism for vitamin B_{12} in the human.*

Vitamin B_{12} (cobalamin) coenzymes in mammalian cells mediate three apoenzyme-catalyzed reactions in mammalian cells: (i) synthesis of methionine from homocysteine, which is mediated by methyl cobalamin (CH_3-B_{12}); (ii) interconversion of methylmalonyl coenzyme A and succinyl coenzyme A; (iii) and interconversion of α- and β-leucine. The latter two reactions are mediated by 5′ deoxyadenosyl cobalamin (Ado-B_{12}). The importance in human metabolism of the first two reactions has been revealed by inherited defects in these reactions; cells from patients with vitamin B_{12}–responsive forms of these disorders are defective in accumulation of the appropriate coenzyme form of cobalamin (*1*). The entry of physiological concentrations of cobalamin into mammalian cells is dependent on the presence of a transport protein that binds the cobalamin [transcobalamin II (TCII)]; interaction of the protein-cobalamin complex with specific receptors on the cell surface; endocytosis of the complex; degradation of the binding protein by lysosomal proteases; and release of the free vitamin B_{12} into the cytoplasm (*2*). Although a complex transport system for the entry of vitamin B_{12} into bacteria and several mutants has been described (*3*), the only inherited defect reported to affect the entry of cobalamin into mammalian cells is the absence of biologically active TCII. We now describe a defect in the transport of cobalamin from lysosome to cytoplasm.

Skin fibroblasts were obtained from an infant girl with developmental delay, minimal methylmalonic aciduria (50 mg/100 ml) responsive to vitamin B_{12}, and no megaloblastic anemia or homocystinuria. Control and proband fibroblasts, free from mycoplasma contamination, were grown in Eagle's minimum essential medium containing 10 percent fetal bovine serum. Cobalamin uptake was studied by replacing the fetal bovine serum with 10 percent human serum