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Effect of MgATP on stiffness measured at two frequencies in Ca^{2+}-activated muscle fibers

(cross-bridge/tension/rate constant/rigor/substrate)

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ABSTRACT **The stiffness of skinned crayfish single muscle fibers was continuously monitored at two frequencies. The length of the fibers was oscillated by the sum of two sine waves (5 Hz and 100 Hz) of small amplitudes. In saline containing saturating amounts of Ca^{2+}, the stiffness ratio (5 Hz:100 Hz) was constant as the MgATP (substrate) concentration was raised from 0 to 2 μM, then it decreased with a further increment in MgATP. The systematic decrease in the stiffness ratio in MgATP above 2 μM indicates the presence of faster transitions in the cross-bridge cycle. This dependence of the stiffness ratio on MgATP is predictable if we use the two-state model of A. F. Huxley (1957) with a modification, in which MgATP promotes the dissociation of the attached cross-bridges.**

In our previous report on the stiffness of skinned muscle fibers (1) we described the elastic properties of fibers in rigor, i.e., in the absence of MgATP (substrate). We chose to begin with a study of rigor because it is a relatively well-defined state (2–4), without the complexity of cycling cross-bridges, in which we can quantify the basic viscoelastic properties of the contractile machinery.

We demonstrated that at least two forms of rigor can be developed dependent on the path by which rigor is induced. The qualitative properties of the two rigor forms are similar in that stiffness is not constant but a function of the strain in the fibers. Furthermore, the stiffness of both rigor forms is essentially constant when measured with sinusoidal length changes with a range of 0.25–133 Hz (1, 5). Based on this observation we concluded that there are no transitions in rigor cross-bridges. A similar conclusion was reached by others (6, 7).

The work reported here is a study of cross-bridge stiffness in fibers that were first brought to low rigor, and then exposed to increasing concentrations of MgATP in the presence of sufficient Ca^{2+} to induce maximal activation. Our purpose was to observe the effect of actively cycling cross-bridges on relative stiffness without net shortening. The results are more complex to interpret than those obtained in rigor because multiple transitions between cross-bridge states are possible (8–11), and because the rate constants for these transitions are believed to depend on both the chemical environment (12, 13) and the size of the length changes imposed (8, 9, 14, 15).

MATERIALS AND METHODS

Details on the muscle preparation, solutions, and experimental apparatus have been described (1). In brief, single muscle fibers were dissected from the walking leg of crayfish in control saline (200 mM NaCl/5 mM KCl/13.5 mM $CaCl_2$/5 mM Tris·HCl buffer, pH 7.4), and the sarcolemma was removed ("skinned") mechanically (16) in skinning saline [1 mM $Mg(OAc)_2$/2 mM ATP/5 mM ethylene glycol-bis(p-aminoethyl ether)-*N,N'*-tetraacetate (EGTA)/170 mM potassium propionate/5 mM imidazole buffer, pH 7.00]. The skinned muscle fiber was mounted between two clamps, one of which was connected to a servo-controlled length driver and the other to a strain gauge for tension detection (Fig. 1). The sarcomere length was measured by the optical diffraction technique (17, 18) and adjusted to 7.0–8.2 μm, where the length-tension diagram has a plateau (19). The muscle length was driven by the sum of two sine waves (5 Hz and 100 Hz) of equal amplitudes (each causing a change of 0.1–0.3% in the muscle length). The two sine wave components induced in the tension were separated by appropriate band-pass filters and their root mean square (RMS) values were detected by rectifying the filtered signals. The stiffness of the muscle fiber is defined as RMS (tension)/RMS (length) at each frequency.

All the activating solutions contained 3 mM free ATP (not bound to Ca^{2+} or Mg^{2+}) and 5 mM CaEGTA, and were buffered with 5 mM imidazole at pH 7.00; the ionic strength was adjusted to 210 mM by addition or deletion of potassium propionate. The saline surrounding the skinned fiber was constantly stirred to avoid depletion of ATP inside and around it.

RESULTS

A typical experiment is shown in Fig. 2. The muscle fiber was initially at rest in relaxing saline (R, same composition as the skinning saline) and then at RIG it was brought into the "low rigor" condition (1) by replacing the saline with one containing ethylenediamine-*N,N,N',N'*-tetraacetate (EDTA) (10 mM) but no Mg or ATP. Within a few minutes the low-rigor stiffness attains a stable value. At C this saline was replaced by one containing 5 mM CaEGTA (pCa = $-\log [Ca^{2+}] = 5.2$). This treatment does not alter either tension or stiffness in the rigor condition (1). The pCa (ranging between 5.5 and 5.0 in different experiments) was chosen so that the tension was at a maximum for all MgATP concentrations tested in this study (20).

Upon addition of 3 mM ATP to the CaEGTA bathing medium there was a slow but marked rise in tension and stiffness. This response is due to the formation of MgATP (substrate) complex because of the presence of Mg in the ATP as a contaminant (0.5–0.85 μM per mM ATP; ref. 16). The pS ($-\log [MgATP^{-2}]$) of this solution was estimated to be 5.7 ± 0.1, which is sufficient to generate about 70% of maximum tension when pCa is 5.0–5.5 (Fig. 3, curve T). The Mg contamination was insignificant at higher MgATP concentrations.

With subsequent stepwise increase in MgATP concentration, the stiffness at both 5 and 100 Hz peaked at pS ~5 and then

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Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetate; EDTA, ethylenediamine-*N,N,N',N'*-tetraacetate.

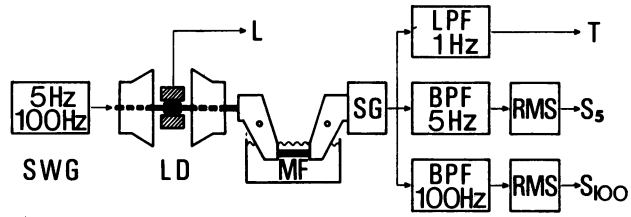

FIG. 1. Experimental arrangement and the method of data analysis. The length of the muscle fiber (MF) is controlled by the length driver (LD) made of two speakers and a length detector (L is the length signal, see ref. 5 for details). LD is commanded by the sum of two sine waves (5 Hz and 100 Hz) from two generators (SWG, Wavetek 110). The tension signal of MF is sensed by a strain gauge (SG, Bionix F-100) and amplified by a Clevite-Brush Carrier amplifier. For tension detection (T) the signal is sent to a low pass filter (LPF; 2 pole Butterworth filter; cut off frequency, 1 Hz). For stiffness detection at 5 Hz (S_5) and at 100 Hz (S_{100}) the signal is first sent to band pass filters (BPF: 4 poles, $Q = 3.5$) with resonance frequencies of 5 Hz and 100 Hz, respectively, and the outputs are then sent to RMS (root mean square) detector circuits. The resulting signals are further filtered by 1-Hz Butterworth filters before they are recorded on a multichannel pen recorder as in Fig. 2. The total compliance of LD, two muscle clamps, and SG is 8 nm/dyn. The bathing solution (5 ml) surrounding MF is constantly stirred and the temperature is controlled to 20.0 ± 0.5°.

declined. The peak stiffness measured at 100 Hz was used to normalize the stiffness data from 13 experiments (Fig. 3). This peak stiffness was comparable to that of muscle in high rigor (1). The ratio S_5/S_{100} was essentially constant for substrate concentration ranging from rigor to pS 5. The ratio declined as the substrate concentration was increased (Fig. 3). The tension, however, remained approximately constant (*cf.* ref. 20).

It is possible that we observed an effect of increasing MgATP concentration on the stiffness ratio because increasing ATP reduces a depletion of ATP in the core of the muscle fiber. However, this possibility is remote because, in separate experiments, the concentration of free ATP (not bound to Ca or Mg) was changed between 3 and 12 mM at constant MgATP concentration with no significant change in the results.

DISCUSSION

At low concentrations of MgATP the actin units are "turned on" (21, 22) and the muscle is insensitive to Ca^{2+} (23). Thus, the

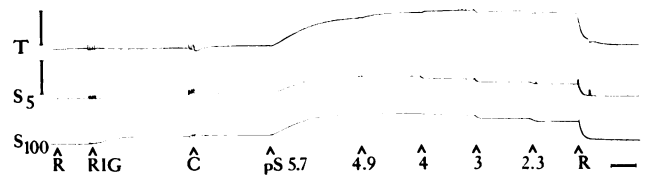

FIG. 2. Simultaneous time course of tension (T), 5-Hz stiffness (S_5), and 100-Hz stiffness (S_{100}). The muscle fiber was first placed in the relaxing saline (R), then brought into low rigor by four repetitive washes with 10 mM EDTA solution (RIG). At C, CaEGTA (pCa 5.2) was introduced (three washes) with subsequent incremental concentrations of MgATP (pS values are shown). The time, needed for equilibration of both the tension and the stiffness, decreases continuously with increase in substrate concentration. Free ATP was constant at 3 mM, and total EGTA was constant at 5 mM. At the end of the experiment the fiber was relaxed in R. The stiffness at 5 Hz or 100 Hz is virtually zero in R. In this preparation the low-rigor stiffness is smaller than the average (56 ± 5%, SEM, $n = 13$; Fig. 3). Solution changes at RIG, C, and R involve repetitive substitution of the full volume, which causes large wash artifacts. These were partially blocked out in the records. The artifacts were negligible with incremental changes in substrate concentration. Calibration bar for tension is 100 dyn; for stiffness, 2×10^5 dyn/cm; and for time, 1 min.

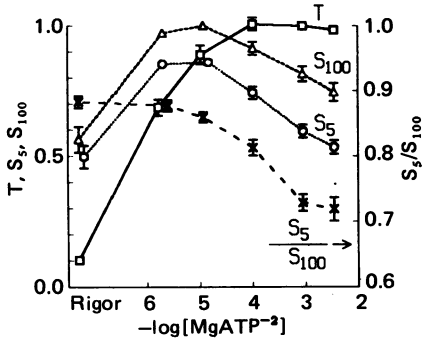

FIG. 3. Mean effect of MgATP on tension (T, □) and stiffness at two frequencies (S_5, ○; S_{100}, △). Abscissa is in pS units. Tension is normalized to that at pS = 3.0, and S_5 and S_{100} are normalized to S_{100} at pS = 4.9 before values were averaged (left ordinate). The stiffness (S_5/S_{100}, ×) is plotted on the right ordinate. This summary includes 13 experiments similar to the one in Fig. 2. SEM are represented by vertical bars. Those smaller than the symbol are not shown.

mechanism for the rise in tension and stiffness at high pS should be the same whether Ca^{2+} is present or not. In the absence of Ca^{2+} we can assume (1, 16) that cross-bridges shift from a low-rigor (little tension) state to a tension state by occasional detachment and re-attachment transitions which are limited by MgATP concentration.

Stiffness should be nearly constant (~100%) in this range because most of the cross-bridges are assumed to be attached to actin (2–4), i.e., detachment is rate limiting. However, the stiffness in low rigor is 56% of the maximum on the average, and it increases toward the maximum at pS ~5 (Fig. 3). The explanation for the increase in the stiffness is that some cross-bridges or other in-series elements are slack in low rigor, and thus little tension and low stiffness are registered (1). If this slack is removed by stretching the low-rigor muscle, an increase in stiffness is observed (figure 3 of ref. 1). The slack is also removed in trace MgATP concentrations. In the latter condition, cycling must take place because fibers can shorten when allowed to do so.

Biochemical studies (10) have established that the MgATP complex, as the substrate, binds with the S-1 moiety of myosin while the latter is in a rigor-like linkage with actin. This binding promotes the dissociation of actin from myosin. When Huxley's (14) two-state (attached:detached) model is reconsidered in the context of this biochemical scheme, the apparent dissociation rate constant g must be proportional to substrate concentration, because the rate of the reaction is proportional to both myosin and substrate concentrations and the subsequent dissociation is nearly instantaneous (10). In a two-state model the overall cycling rate is also a function of the attachment rate constant f, and it is proportional to $f \cdot g/(f + g)$ (24, 25).

The change in the stiffness ratio with substrate can be interpreted in this model as follows. In low substrate concentration (pS > 5) the cycling rate is limited by g. In this condition the cycling rate is so slow that length oscillation at 5 Hz or 100 Hz does not interfere with it. Both oscillations just stretch and release elastic elements in the cross-bridges (8, 26) or in series with them. At physiological substrate concentration (pS ~3) we can assume that the cycling rate is comparable to the 5-Hz oscillation, because progressively less stiffness is detected at 5 Hz than at 100 Hz as substrate is increased (Fig. 3). At 5 Hz the cross-bridges can readjust to external length change by cycling, or perhaps by a conformational change (8).

When the 100-Hz length oscillation is used, cross-bridges adjust little to external length changes in high substrate con-

centration because we can assume that critical transitions cannot take place during this fast length change. This results in a simple stretch and return cycle in the elastic elements, just as in the low substrate or rigor case. Thus it is expected that 100-Hz stiffness detects a greater proportion of the attached cross-bridges than the 5-Hz stiffness, and that the systematic change in stiffness ratio with change in substrate concentration indicates the presence of increasingly faster transitions.

Transitions in the frequency range 5–100 Hz have been observed in intact crayfish (5) and frog (5, 8) muscles, and in glycerinated insect (27, 28) or rabbit (29) muscles. They were not systematically studied as a function of MgATP concentration. In crayfish, transitions are slower in skinned than in intact fibers (30).

The decrease in 100-Hz stiffness with an increase in substrate concentration above 0.1 mM (pS < 4), but with no parallel change in tension, is not consistent with a two-state model in which g only is affected by substrate concentration. In this model the increase in g with substrate would necessarily lead to a parallel loss in tension and stiffness. One simple explanation is that the attachment rate constant f can also be assumed to increase in higher substrate concentrations (we note, however, that there is no clear biochemical evidence for an effect of MgATP on f). With such an assumption, the attached cross-bridge population may remain constant and the high frequency stiffness will remain proportional to tension. Therefore, a plot of stiffness against substrate concentration, such as that of Fig. 3, should be increasingly flatter at higher frequencies of length oscillation.

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