brief communication

Are weakly binding bridges present in resting intact muscle fibers?

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ABSTRACT Several experimental results (Schoenberg, M. 1988. *Biophys. J.* 54:135–148) have shown that the force response of relaxed skinned muscle fibers to fast stretches arises from the presence of cross-bridges rapidly cycling between attached and detached states. These bridges were identified with the M.ATP ⇌ AM.ATP and M.ADP.Pi ⇌ AM.ADP.Pi states seen in solution and are commonly referred to as weakly binding bridges. In this paper we have investigated the possibility that weakly binding bridges are also present in resting intact muscle fibers. The force response to fast stretches can be accounted for by assuming the presence in the fiber of a viscous and a viscoelastic passive component arranged in parallel. None of these components has the properties previously attributed to weakly binding bridges. This shows that in intact resting fibers there is no mechanical evidence of attached cross-bridges, suggesting that, under physiological conditions, either the M.ATP or M.ADP.Pi states have a negligibly small affinity for actin or the AM.ATP and AM.ADP.Pi cross-bridge states are unable to bear tension and contribute to fiber stiffness.

INTRODUCTION

The development of force in a skeletal muscle is due to the attachment of the myosin projections, the cross-bridges, to specific sites on the actin filament upon increase of myoplasmic Ca^{2+}. However, it has been shown that in skinned muscle fibers at low ionic strength a significant portion of cross-bridges can be attached to actin also in the absence of Ca^{2+} (1–4). These bridges have many of the properties of the M.ATP ⇌ AM.ATP and M.ADP.Pi ⇌ AM.ADP.Pi states seen in solution and are referred to as weakly binding states or rapid equilibrium states. Since evidence of reduced numbers of these bridges was also found at normal ionic strength (5, 6), it was suggested that: (1) weakly binding states constitute an essential step of the force-generating process and (2) the major role of Ca^{2+} in the regulation process is to induce the transition of cross-bridges from the non–force-generating weakly bound states to more strongly bound and force-generating states (5, 6). In view of the noteworthy implications of this hypothesis, it is important to establish whether these weakly binding bridges can also be detected in intact muscle cells. We have therefore examined the mechanical properties of resting intact single frog muscle fibers to determine whether weakly binding bridges were present.

As shown by Schoenberg (7), the force response of a rapid equilibrium cross-bridge model to stretches of constant velocity, having only one attached state and only one detachment rate constant, is equivalent to that of a simple viscoelastic system having a relaxation time equal to the reciprocal detachment rate constant. On this basis, by analogy with skinned fiber results, if weakly binding bridges spend a significant fraction of time in the attached state in the absence of Ca^{2+} we would expect to find in resting intact fibers a viscoelasticity with an appropriate relaxation time.

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This prediction was tested by studying the force response of resting intact frog fibers to ramp stretches at different velocity. We found that the force response can be accounted for by assuming the presence in the fiber of a viscous element arranged in parallel with a viscoelastic one. None of these elements has the properties of weakly bound bridges as previously described. This result indicates that at variance with skinned fibers results, in intact resting fibers there is no mechanical evidence of attached bridges.

MATERIALS AND METHODS

Single intact fibers, 2.0–2.5 mm long, isolated from the lumbricalis digiti IV muscle of the frog (*Rana esculenta*), were mounted by means of aluminum foil clips between the lever arms of a force transducer (35–60 kHz resonance frequency) and a fast displacement generator (minimum ramp time 40 μs) in a chamber fitted with a glass floor for ordinary and laser light illumination. The initial sarcomere length (l_0) was usually adjusted to ~2.15 μm. Ramp stretches at velocity between 5 and 250 sarcomere lengths per second (l_0/s) and amplitude up to 6% l_0 were applied to one end of an unstimulated fiber, and force responses were measured at the other. Sarcomere length changes were measured by means of a laser diffractometer (8) in a fiber region (~200 μm long) located ~500 μm from the force transducer. This procedure virtually eliminated measurement artifacts due to the delay line behavior of the fiber. A low coherence solid state laser (power 3 mW, wavelength 0.787 μm, model GALA 0-78-04-44; D.O. Industries, Rochester, NY) was used as a light source for the diffractometer. The incident angle of the laser beam on the fiber was adjusted to obtain the maximum intensity of the first-order diffraction line used to monitor the sarcomere length. The rise time of the system was <3 μs and the peak-to-peak noise corresponded to ~0.2 nm. The output from the diffractometer was electronically differentiated (rise time of the differentiator, 3 μs) to obtain the instantaneous velocity of sarcomere length change.

To check fiber viability and measure active tension, fibers were stimulated at regular intervals with brief (0.6–0.8 s duration) tetanic volleys. Experiments were made at 5 and 15°C.

Force, fiber length (l_f), sarcomere length, and sarcomere-lengthening velocity were measured on a digital oscilloscope (model 4094; Nicolet Instrument Corp., Madison, WI) and stored in floppy disks for further analysis. To improve the signal-to-noise ratio, especially for the sarcomere-lengthening velocity signal, up to 10 responses were often averaged.

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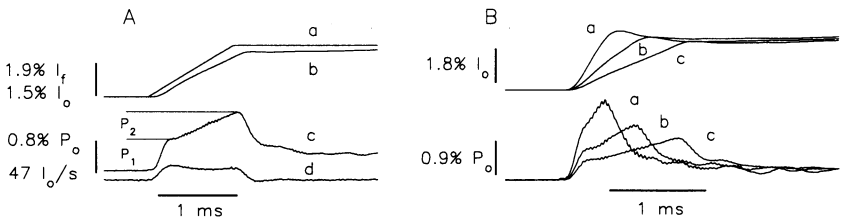

FIGURE 1 (*A*) Records from a typical experiment on a resting fiber subjected to a ramp lengthening at a velocity of 22 l_0/s. (*a*) Fiber length; (*b*) sarcomere length; (*c*) tension; and (*d*) rate of sarcomere length changes. The initial force response is clearly composed of two phases, indicated as P_1 and P_2. The sharpness of the P_1–P_2 transition depends on the shape of the sarcomere length change and this in turn depends on the mechanical properties of the fiber and on the compliance of the fiber connections to the transducers. (*B*) Effects of stretching velocity on the force responses: (*a*) 89 l_0/s; (*b*) 57 l_0/s, and (*c*) 24.5 l_0/s. Stretching amplitude: 2.77% l_0.

RESULTS AND DISCUSSION

Fig. 1 *A* shows a typical force response of a resting fiber subjected to a constant-velocity stretch. As shown previously (9), the rising part of the force transient is composed of two phases: (1) a fast initial tension increase at the beginning of the stretch and (2) a much slower tension rise ending at the end of the stretch. Comparison of force and sarcomere-stretching velocity traces shows that phase 1 (P_1) corresponds to the acceleration period during which the lengthening velocity rises, almost linearly, to the steady-state value, and that phase 2 (P_2) coincides with the period of stretching at constant velocity. As can be seen from the velocity record, the acceleration period at sarcomere level is relatively long ($\sim$200 μs) while the fiber length acceleration period lasts only $\sim$20 μs. This difference is due to the propagation of the mechanical perturbation along the fiber that delays and distorts the sarcomere length changes, as compared with the fiber length changes.

Force responses at three different stretching velocities are shown in Fig. 1 *B*. It can be seen that P_1 amplitude increases with the velocity while the amplitude of P_2 remains almost constant. Note that the breaking point between P_1 and P_2, which corresponds to the end of the acceleration period, is reached at the same time in all the records. This means that the sarcomere elongation occurring during phase 1 increases with the velocity. At the velocities shown in Fig. 1 *B*, the elongations were 3, 4.8, and 8.5 nm/half sarcomere. It should be pointed out that the sarcomere elongations occurring during phase 1 correspond to those applied to skinned fibers for studying the properties of the weakly binding bridges.

Fig. 2 *A* shows that P_1 amplitude increases linearly with the velocity in all the velocity range used. In contrast, P_2 amplitude reaches a plateau at relatively low velocities. These results suggest that P_2 is a viscoelastic response, while P_1 is consistent with either a viscous system or with a viscoelastic system having a relaxation time (τ) much shorter than 230 μs, the minimum stretch duration used in the experiment. Since P_1 probably corresponds to the force responses studied in skinned fibers, it was essential to clarify this point. This may be done by comparing the time courses of stretching velocity and force output during the period (t) of stretching at constant acceleration (Fig. 2 *B*). It can be shown that in this condition a simple viscoelastic system would give a force output (F_o) equal to:

$$F_o = a\eta(t - \tau) + a\eta\tau \exp(-t/\tau), \qquad (1)$$

where a = acceleration and η = viscous coefficient. Therefore, if P_1 were a viscoelastic response, the force should be distorted and delayed with respect to the stretching velocity. If, as suggested by Fig. 2 *A*, $t > \tau$, Eq. 1 reduces to:

$$F_o = a\eta(t - \tau)$$

and the delay between stretching velocity and force output corresponds to τ. In different preparations, we found that the delay varied between 0 and 5 μs (e.g., Fig. 2), strongly suggesting that P_1 is a viscous response. Since in terms of cross-bridge kinetics a viscous response implies a detachment rate constant of infinite value, it is very unlikely that the P_1 response arises from attached bridges. This conclusion is still valid even if we assume that P_1 is a viscoelastic response with a relaxation time of 5 μs. The corresponding detachment rate constant would be $2 \times 10^5\ s^{-1}$, a value $\sim$20 times higher than that found in skinned fibers. Finally, it may be of interest to see what the force output predicted by Eq. 1 would be for a relaxation time of $\sim$100 μs as that found in skinned fibers. In this case, $\tau > t$ and Eq. 1 reduces to:

$$F_o \approx a\eta t^2/2\tau.$$

Hence the system would act as an integrator, and a quadratic force response would be obtained for a linearly increasing velocity input. This behavior is clearly not in agreement with our results, as the relation between force and velocity during the acceleration period is highly linear (Fig. 3 *A*).

The conclusion that P_1 is a viscous response shows that the procedure used in skinned fibers to evaluate the number of attached cross-bridges from the fiber stiffness measured as the initial slope of the force-displacement

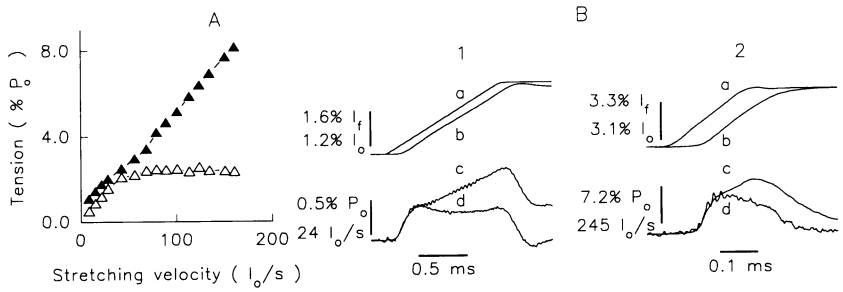

FIGURE 2 (*A*) P_1 (▲) and P_2 (△) amplitudes versus stretching velocity. P_1 increases linearly with the velocity, like a viscous response, while P_2 reaches a plateau at relatively low stretching velocity, resembling a viscoelastic response. The mean viscous coefficient of P_1 response, calculated by dividing the force per unit of cross-sectional area by the stretching velocity, is $\sim 0.7 \times 10^8$ Nsm^{-3}. P_2 values are corrected for the resting tension measured 50 ms after the end of the stretch. Stretch amplitudes: 3.7% l_0. (*B*) Fast time base records of passive force response to ramp stretches in two fibers. Stretching velocities were 24.4 l_0/s (*1*) and 245 l_0/s (*2*). Upper pair traces: (*a*) fiber length and (*b*) sarcomere length. Lower pair traces: (*c*) tension and (*d*) rate of sarcomere length change. The delay between velocity and force during the acceleration period, if present, is no longer than a few microseconds.

relationship or as chord stiffness is not applicable to intact resting fibers.

P_2 is a viscoelastic response and is therefore consistent in principle with the presence of attached weakly binding bridges. The relaxation time associated with this phase is ~ 1 ms, which would correspond, in terms of cross-bridge kinetics, to a detachment rate constant of 10^3 s^{-1}. As shown in Fig. 2 *A*, P_2 amplitude is independent of the stretching velocity for velocities > 80 l_0/s. In terms of weakly binding bridges this means that at these velocities all the attached bridges are sampled by the stretch and no detachment occurs during the stretch itself. Therefore, the whole length change applied would be taken up by the cross-bridges. As the cross-bridges have an elastic range of 12–15 nm/half sarcomere, a breaking point or "give" (10) should appear on the P_2 response when the stretch applied exceeds this limit. However, we found no sign of "give" on P_2 force records, even when the stretch was as high as 60 nm/half sarcomere. This observation makes the possibility that the P_2 response arises from attached bridges unlikely.

The nature of the force responses was further investigated by studying the effects of the initial sarcomere length. If P_1 and P_2 are due to cross-bridges, their amplitudes should decrease with the degree of overlap between thick and thin filaments. However, the relationships reported in Fig. 3 *B* show exactly the opposite: increasing the sarcomere length from 2.15 to 2.65 μm increases both P_1 and P_2 amplitudes. These findings confirm that neither P_1 nor P_2 arises from attached weakly binding bridges.

One interesting question is whether or not P_1 force can be attributed to the viscous resistance of the thick and thin filaments to the relative sliding motion. A rough calculation, made according to Huxley (1980), but using the geometry of actin and myosin filaments resulting from recent studies (11–14), shows that the viscous force predicted is of the same order of magnitude as the P_1 response. It should also be pointed out that, owing to the constant myofilament lattice volume behavior (15, 16), the rapid elongation of the resting fiber produces a rapid movement of the myofilaments towards the center

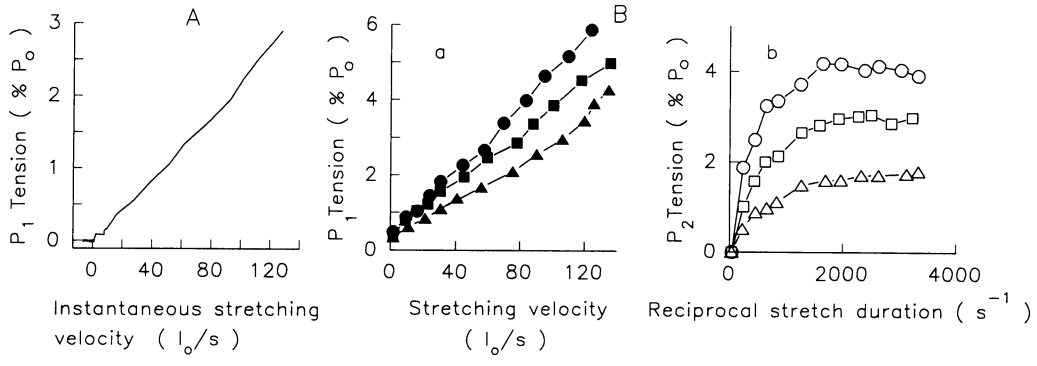

FIGURE 3 (*A*) Instantaneous relation between P_1 tension and stretching velocity as measured during the acceleration period (140 μs), corresponding, in this fiber, to the initial 12 nm/half sarcomere of elongation. (*B*) P_1 amplitude versus stretching velocity (*a*) and P_2 amplitude versus reciprocal stretch duration (*b*) at 2.15 μm (*triangles*), 2.50 μm (*squares*), and 2.65 μm (*circles*) sarcomere length. P_1 and P_2 amplitudes increase with sarcomere length in spite of the decreasing of the myofilament overlap. P_2 values corrected as in Fig. 2 *A*. As shown by Schoenberg (3), the relative force response of a viscoelastic system depends on the stretch duration rather than on stretching velocity.

of the fiber, and this could contribute to the viscous resistance during elongation. A possible explanation for P_2 could be related to the presence of the titin elastic filaments that join thick filaments to Z discs (17). These filaments, which account for most of the resting tension (17), could have a viscoelastic behavior.

To summarize, the analysis of the force response of resting intact fibers to fast stretches indicates the presence of a viscous and a viscoelastic passive component in the fiber and shows no mechanical evidence of attached cross-bridges. This result suggests that under physiological conditions at resting Ca^{2+} concentration either the M.ATP and M.ADP.Pi states have a negligibly small affinity for actin, or the AM.ATP and AM.ADP.Pi states are completely unable to bear tension and contribute to fiber stiffness.

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