ELSEVIER

Biophysical Chemistry 68 (1997) 1–8

Biophysical Chemistry

Crossbridge viscosity in activated frog muscle fibres

G. Cecchi *, M.A. Bagni, E. Cecchini, B. Colombini, F. Colomo

Dipartimento di Scienze Fisiologiche, Università di Firenze, Viale G.B. Morgagni, 63, I-50134 Firenze, Italy

Received 30 October 1996; accepted 5 December 1996

Abstract

Force responses to fast ramp stretches at various velocities were recorded in single muscle fibres isolated from tibialis anterior muscle of the frog (*Rana esculenta*) at a sarcomere length between 2.15 and 3.25 μm at 15°C. Stretches were applied at the tetanus plateau and during tetanus rise. Length changes were recorded at the sarcomere level using either a laser diffractometer or a striation follower apparatus. The immediate force response to the stretch is not simply elastic, as is usually assumed, but is composed of the sum of at least two components: (i) elastic (force proportional to the amount of stretch); and (ii) viscous (force proportional to the rate of stretch). The viscous response is associated with a short (about 10 μs) relaxation time. The amplitude of the viscous component increases progressively with tension during the tetanus rise and scales down with sarcomere length approximately in the same way as the tetanic tension. These results suggest that the viscosity of activated fibres may arise from crossbridge kinetics. © 1997 Elsevier Science B.V.

Keywords: Crossbridges; Muscle fibre; Myofilaments; Skeletal muscle; Stretches; Viscosity

1. Introduction

Several reports in the literature have shown that resting muscle fibres, either cardiac or skeletal, intact or skinned, when subject to fast length change exhibit a significant internal viscosity, the nature of which has not been identified [1–3]. Experiments on frog skeletal muscle fibres showed that a viscosity similar to that at rest was present at the tetanus plateau at a sarcomere length greater than 2.8 μm. However, at optimum overlap (at a sarcomere length of around 2.2 μm), very small or no viscosity resulted from the analysis of the force transients produced by step length changes [1,4]. The resting viscosity therefore should have disappeared or been greatly reduced as a consequence of fibre activation. On this basis, it has been generally assumed that activated fibres have negligible viscosity. Thus, the instantaneous force changes caused by step length changes (T_1 curve) are considered to be exclusively elastic and are attributed to a change in the length of the elastic components of the sarcomere located principally in the crossbridges and in the myofilaments [1,4–6]. On the other hand, preliminary results with ramp stretches of frog muscle fibres seemed to show the presence of a significant internal viscosity also during twitch force generation [7]. The presence of viscosity similar or higher than that at rest was also

* Corresponding author. Tel.: + +39-55-4237-340/311; fax.: +39-55-4379506; e-mail: cecchi@fisio.unifi.it

PII S0301-4622(96)02263-6

postulated to explain the reduction of the unloaded shortening velocity found at low tension in activated rat trabeculae [3]. The presence of an internal viscosity (or viscoelasticity with a short relaxation time) similar to that found in resting fibres would modify appreciably the instantaneous force response to a fast length change. For example, Ford et al. [1], using a step length change complete in 200 μs, have shown that the resting viscosity, if present at the tetanus plateau, would reduce the value of y_0 (the instantaneous length change necessary to bring to zero the tetanic tension) by about 15%. But most important is the possibility that viscous (or viscoelastic) behaviour of the active fibre could arise from actomyosin interaction since, in this case, the analysis of the viscoelastic properties could provide information about crossbridge kinetics. As shown by Schoenberg [8], for example, a simple model of crossbridges in rapid equilibrium between the attached and the detached state (weakly binding bridges) gives a force response to fast length changes equivalent to that of a viscoelastic system (elasticity in series with viscosity) having a relaxation time equal to the reciprocal of the detachment rate constant of the crossbridges. If the duration of the length change is much longer than the reciprocal of the detachment rate constant, the weakly binding bridge response will increase linearly with stretching velocity appearing as purely viscous.

In view of all these important implications, it is of interest to further investigate the possibility that some internal viscosity could be present in fully activated frog muscle fibres. This was done by analysing the force response of frog single muscle fibres to ramp stretches applied at various force levels during a tetanic contraction. The main results show that (1) the viscosity in activated fibres is greater than at rest; and (2) the increase of viscosity upon activation is associated with crossbridge formation.

2. Methods

Frogs (*Rana esculenta*) were killed by decapitation followed by disruption of the spinal cord. Single fibres, dissected from tibialis anterior muscle (4.5–6.5 mm long) were mounted by means of aluminium foil clips [1] between the lever arms of a fast stretcher (minimum rise time, 40 μs) and a fast capacitance force transducer (resonance frequency, 40–70 kHz) in a chamber fitted with a glass floor for ordinary and laser light illumination. Experiments were carried out at a sarcomere length from 2.15 to 3.25 μm. The initial sarcomere length, defined as l_0, was usually set at about 2.15 μm. Stimuli of alternate polarity of 0.5 ms duration and 1.5 times threshold strength were applied to the fibre by means of a pair of platinum plate electrodes at the appropriate frequency (40–55 Hz) to produce fused tetanic contractions of 150–350 ms duration at the experimental temperature of 15°C. Ramp stretches (0.5–1% l_0 amplitude; 5–50 l_0 s^{-1} velocity) were applied to one end of the fibre, and force responses were measured at the other. The sarcomere length was measured either by means of a diffractometer [9] or by means of a striation follower device [10] in a fibre segment selected for striation uniformity. The segment (1–1.5 mm long when using the striation follower or about 300 μm when using the diffractometer) was located as close as possible (usually within 1.5 mm) to the force transducer. The instantaneous stretching velocity was obtained by differentiating electronically the sarcomere length signal (rise time of the differentiator, 3 μs). To reduce the artefacts due to the thickness of the fibre on the sarcomere length measurements by diffraction [11], a low-coherence infrared light emitting diode (Hamamatsu L3302) was used as the light source for the diffractometer. Length ramps were applied at rest, at the tetanus plateau and throughout the tetanic tension rise. The force, sarcomere length, fibre length and instantaneous stretching velocity were recorded on a digital oscilloscope (model 4094; Nicolet Instrument Corporation, Madison, WI, USA) at double sampling intervals (externally controlled) of 1 ms and 2 or 5 μs, and were stored on floppy disks for subsequent analysis. In some cases, tension responses were evaluated after digital notch filtering to eliminate the spurious oscillations (produced by the stretcher) at about 20 kHz present on most of the records. The filtering was carried out utilising a program of the Nicolet oscilloscope and it did not change the shape of the force response.

The composition of the normal Ringer solution was 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 3 mM phosphate buffer at pH 7.1.

3. Results

Fig. 1A shows a typical force response to a ramp stretch applied at the tetanus plateau in a fibre from tibialis anterior muscle. It can be seen that, similarly to the response recorded in relaxed fibres [1,2], an inflection separates the rising part of the force transient into two distinct phases: a fast initial one (phase 1) lasting for about 50 μs or less, and a slower one (phase 2) lasting up to the end of the stretch. The presence of two phases on the force response suggests that the immediate force change of the activated fibre to a fast length change cannot be considered to be purely elastic. A comparison of force and stretching velocity records, reported in Fig. 1A and in Fig. 3 (taking into account the reasons for the delay between the two records presented in the caption of Fig. 3) shows that phase 1 corresponds to the period of acceleration during which the sarcomeres are stretched at increasing velocity while the slower phase 2 corresponds to the period of sarcomere elongation at constant velocity. The results of Fig. 1 can be explained by assuming that the force response is composed of the sum of (at least) two components: a viscous one and an elastic one. The amplitude of the viscous component, which is proportional to the instantaneous stretching velocity, would rise during the period of acceleration to reach, at the inflection point, a constant value which is maintained during the remainder of the stretch. In contrast, the elastic component would be proportional to the sarcomere elongation (assuming linear behaviour of the sarcomere elasticity) increasing during the whole stretch. The increase of force during the fast phase is therefore produced by the simultaneous rise in both the viscous and the elastic forces. At the end of the acceleration period (at the inflection point) the viscous force attains a constant value and the slope of the force response decreases since the force increase is now brought about only by the elastic component. While the viscous component was not detected previously, the elastic component represents the well known elastic response (associated with a fast recovery) due to the elongation of cross-bridge and myofilament elasticity. The roughly linear increase of tension during phase 2 is very likely justified by the small extent of the fast recovery and by the non-linearity of the force response during stretches [1,4].

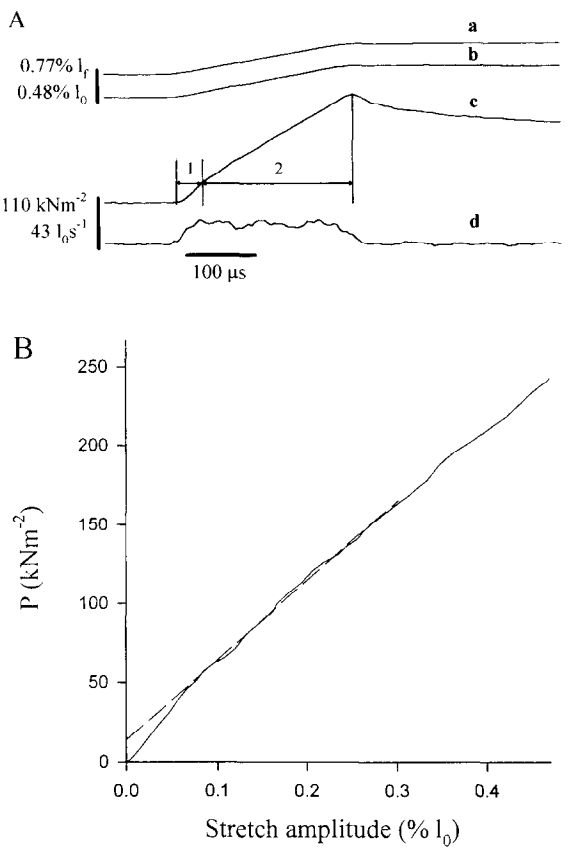

Fig. 1. (A) Typical force response to a ramp stretch at a velocity of 19 l_0 s^{-1}, applied at the tetanus plateau in a fibre from tibialis anterior muscle. The response is composed of a fast and a slow phase indicated on the figure as 1 and 2, respectively. From top to bottom: curve a, fibre length (l_f); curve b, sarcomere length (l_0); curve c, force; curve d, instantaneous stretching velocity. (Fibre length, 5010 μm; sarcomere length, 2.23 μm; segment length, 1170 μm.) (B) Same data as in (A) plotted as instantaneous force against instantaneous sarcomere length. The amplitude of phase 1 (P_1) was measured from the intercept obtained by extrapolating back to the ordinate the straight line fitted on the initial linear part (from 0.1–0.3% l_0) of the slower phase of the force response.

The separated contributions of the viscous and the elastic components to the force response can be determined from the instantaneous sarcomere length–tension plot as reported in Fig. 1B. The amplitude of the viscous component (P_1) is found by subtracting from the tension at the inflection point the elastic component (assumed to be linear). This can be done graphically by extrapolating back to the

ordinate the straight line fitted on the initial linear part of the elastic response during phase 2. It should be noted that if the force response were purely elastic, with no viscous force, the extrapolated line would have passed through the origin. The peak amplitude of the elastic component (P_2) is then calculated simply by subtracting from the peak of the tension transient the viscous component P_1. An important aspect of this analysis is that the value of P_1 is neither produced nor influenced by fibre inertia. This is because the force response during phase 2 on which the extrapolation is made is occurring at zero acceleration.

To elucidate further the nature of the fast phase, we examined the effects of the stretching velocity on the force response. An example of the records obtained at three different stretching velocities is reported in Fig. 2A, while in Fig. 2B is shown the relationship between P_1 and the stretching velocity. It can be seen that P_1 increases linearly with the velocity in the whole range tested (5–24 l_0 s^{-1}) in agreement with the idea that, at least in this velocity range, P_1 shows viscous behaviour.

Considering P_1 as a viscous response, a viscous coefficient (η), estimated as the ratio between P_1 (expressed in kilonewtons per square metre) and lengthening velocity (expressed in l_0 per second) can be calculated from the slope of the regression line fitted to the data of Fig. 2B. The resulting value of η was 0.83 kN m^{-2} s (in other experiments, $n = 9$, the value of η ranged from about 0.6 to 1.2 kN m^{-2} s).

It is not immediately clear that P_1 is a pure viscous response since a viscoelastic element (viscosity in series with elasticity) with a relaxation time much shorter than 200 μs (the shortest stretch duration used) would be characterised by the same linear relationship shown in Fig. 2B behaving like a viscous element. Since we were not able to decrease the stretch duration below 200 μs while maintaining a well ramp shaped stretch at sarcomere level, a different approach, based on the comparison between force response and instantaneous stretching velocity, was used to clarify this point (see also Ref. [2]). It can be shown in fact that the force response of a simple viscoelastic element (elasticity in series with viscosity), stretched at constant acceleration for a period longer than its relaxation time, is approximately delayed by a relaxation time in respect to the velocity record. In contrast, in a pure viscous response, stretching velocity and tension are expected to be synchronous. A comparison between force and velocity records on a fast time basis is reported in

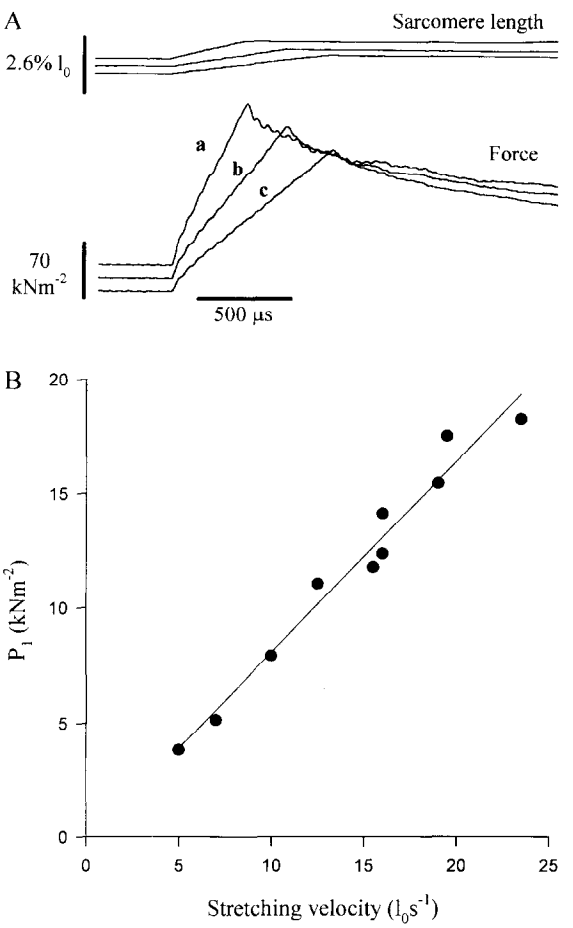

Fig. 2. (A) Sample records of force response at various stretching velocities at the tetanus plateau. Stretching velocities: curve a, 18 l_0 s^{-1}; curve b, 12 l_0 s^{-1}; curve c, 9.2 l_0 s^{-1}. The spurious oscillations at about 20 kHz superimposed on the force signal in these and in other records are produced by the lever of the stretcher when sudden length changes are generated, and are transmitted to the force transducer through the fibre. Their frequency is always the same, being independent of fibre length and of the tension developed. (Fibre length, 4890 μm; sarcomere length, 2.15 μm; segment length, 1460 μm.) (B) Amplitude of the fast phase of the force response (P_1) as a function of the stretching velocity. The viscous coefficient (η) calculated from the fitting of the data was 0.83 kN m^{-2} s. Same fibre as in (A).

Fig. 3. It can be seen that the force is delayed by about 20 μs with respect to the velocity. Since the transmission time of the mechanical wave is about 10 μs, these data would indicate, with the precision reached in these experiments, that P_1 is effectively a viscoelastic response with a relaxation time of about 10 μs. Values between 8 and 15 μs were obtained in other experiments.

In principle, the presence of an inflection on the force record could also be due to forced crossbridge detachment (give) or the yielding of some other elastic structures in the fibre. In this case, however, the inflection point, which would represent the yield point, is expected to correspond to a given amount of sarcomere elongation or a given increase in force. This is in contrast with the data reported in Fig. 2A from which it can be seen that both force and sarcomere elongation at the time of the inflection largely change with stretching velocity. In addition, it should be considered that the amount of length change at the time of inflection (0.05% l_0 at the lowest velocity and 0.12% l_0 at the highest) is much smaller than the 1.2–1.5% l_0 expected from crossbridge yielding. Finally, the viscous response is present also during fast ramp releases where crossbridge breaking is not expected (data not reported).

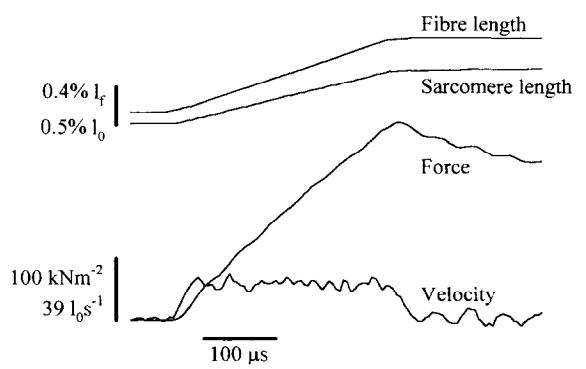

Fig. 3. Comparison between force response and instantaneous stretching velocity at a fast time base. The initial fast phase of the force response lags behind the velocity by about 18 μs. Since the transmission time is about 10 μs, the real delay between the two records is reduced to about 8 μs. The presence of this delay suggests that P_1 is not a purely viscous force but a viscoelastic one. (Fibre length, 5795 μm; sarcomere length, 2.19 μm; segment length, 1250 μm.)

3.1. Elastic response

Although this paper is dedicated to the study of the viscous component of the force response, it is necessary to make some observations concerning the elastic component. The behaviour of the elastic response is particularly clear during phase 2, which occurs at constant speed. Since during this period the viscous force is constant, the elastic response is simply equal to the total force response reduced by the constant viscous force. Graphically, the elastic force response during phase 2 can be found by shifting downward the total force response by an amount equal to P_1. The force response during phase 2 is actually not elastic but viscoelastic (because of the fast recovery mechanism [1]), but for the purposes of this paper it has been considered simply elastic and Hookean, which is equivalent to assuming that the force increase during phase 2 is linear. This is clearly an approximation, however, as it can be seen from most of the records, the deviation from linearity, especially during the initial part of phase 2, is not important. The elastic response has been estimated (see Fig. 5B) by measuring the sarcomere stiffness expressed as the ratio of the changes in force over the length changes during the initial 50 or 100 μs of phase 2. It should be pointed out that this stiffness measurement is not affected by the viscosity of the fibre as the viscous force during this phase is constant.

3.2. Force response during tetanus rise

Fig. 4A shows some typical experimental records obtained at the different tension levels of 0.34, 0.62 P_0 during the tetanus increase, and at the tetanus plateau. It can be seen that both P_1 and P_2 increase with the tension developed at the time of the stretch. Fig. 4B shows the relationship between relative viscosity and relative tension during the tetanus rise from the same data. The viscous coefficient η calculated from these data increased from 0.65 kN m^{-2} s at 0.34 P_0, to a maximum of 0.99 kN m^{-2} s at the tetanus plateau.

3.3. Force responses at longer sarcomere length

The observation that fibre viscosity increases with tension during the tetanus rise suggests the interest-

ing possibility that fibre viscosity could arise from attached crossbridges. To test this hypothesis, we have examined the effects of the sarcomere length on the force response. If an active fibre viscosity is due to crossbridges, a direct proportionality is expected between myofilament overlap and P_1. Sample records at 2.18, 2.75 and 3.25 μm sarcomere length are shown in Fig. 5A, while in Fig. 5B is reported the relationship between sarcomere length, viscosity and stiffness. It can be seen that fibre viscosity decreases with sarcomere length in approximately the same way as tension and stiffness, in agreement with the idea that the viscosity is proportional to the number of attached crossbridges. It is important to point out

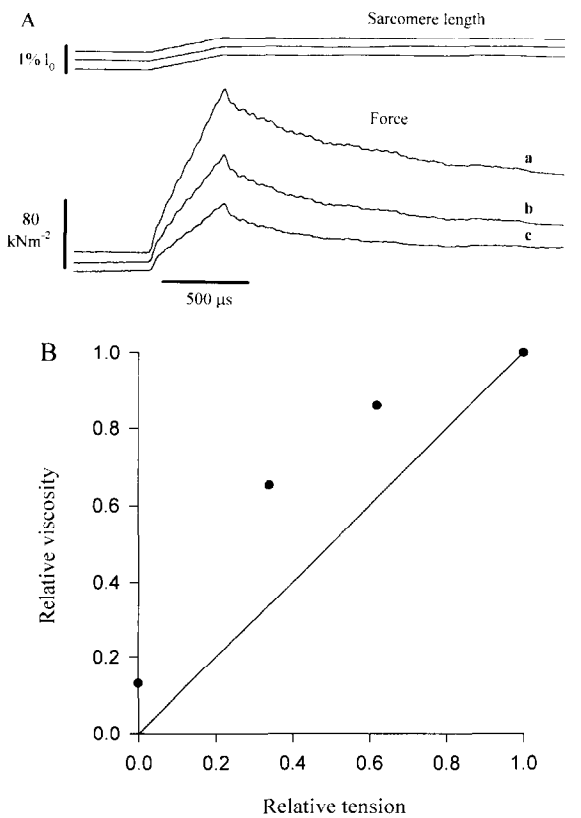

Fig. 4. (A) Sample records of force response to fast stretches (at about 15 l_0 s^{-1}) applied at various tension levels during the tetanus rise: curve a, plateau; curve b, 0.62 P_0; curve c, 0.34 P_0. (Fibre length, 4810 μm; sarcomere length, 2.25 μm; segment length, 1519 μm.) (B) Relationship between the viscosity and the tension developed during the tetanus rise calculated from the records in (A). The point on the ordinate represents the viscosity of the passive fibre.

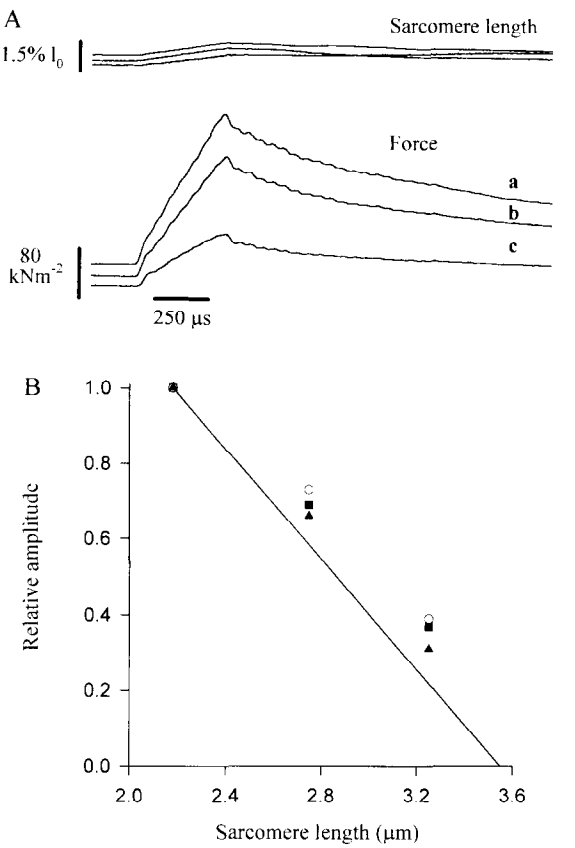

Fig. 5. (A) Sample records of force response at various sarcomere lengths: curve a, 2.18 μm; curve b, 2.75 μm; curve c, 3.25 μm. (Fibre length, 5180 μm; segment length, about 300 μm.) (B) Effects of the sarcomere length on (■) tension, (▲) stiffness, and (○) viscosity, expressed relatively to their maximum values. Note that the viscosity decreases roughly with tension and stiffness when the overlap between myofilaments is decreased. The continuous line represents the change in myofilament overlap with sarcomere length. Same fibre as in (A).

that this behaviour is just the opposite of that found in resting fibres in which the viscous response increased more than proportionally with sarcomere length [2].

4. Discussion

Our results show that the force response of activated fibres during ramp stretching is composed of an initial fast phase followed by a slower one. This observation cannot be accounted for by assuming

that the immediate response of the active fibre to a fast length change is simply elastic, but suggests a more complex behaviour. As assumed in calculating P_1, a simple model appropriate for describing the force response may comprise an elastic element (representing sarcomere elasticity) in parallel with a viscoelastic element (viscosity in series with elasticity) characterised by a very short relaxation time. In the range of length change duration of around 100–200 μs (values commonly used for quick release or stretch experiments) P_1 behaves as a purely viscous force increasing linearly in proportion to the elongation velocity, as shown in Fig. 2B. However, its viscoelastic nature results from the observation that during phase 1, the tension lags behind the instantaneous stretching velocity by some microseconds. As has been already pointed out, assuming that P_1 is equivalent to the response of a simple elastic element in series with a viscous one, this delay is equal to the relaxation time.

Fig. 5B shows that fibre viscosity scales down with the amount of overlap approximately in the same manner as tetanic force and stiffness. The previously reported finding that the viscosity in a relaxed fibre either intact or skinned depends on the sarcomere length in the opposite way [2] indicates that active and passive viscosities have a different mechanism. A relationship between tension and P_1 is present also during the increase of tension in a tetanus. Although the precise time course of P_1 development has not yet been determined, it is quite clear that P_1 increases with the tension developed.

In principle, fibre viscosity may be due to the resistance to the relative sliding of the myofilaments in the intracellular fluid. Upon activation, crossbridges swing away from the backbone of the myosin filaments to reach out to the actin filament, thus offering a larger surface to the intracellular fluid. This would increase the viscous resistance to the sliding in comparison with the rest. However, as shown previously [12], it seems unlikely that this mechanism can account for a viscosity as great as that found here. Another aspect to be considered is the following observation: the viscous resistance to the sliding should decrease with increase of sarcomere length because of the reduction in overlap; however, the reduction of lateral filament spacing at longer sarcomere length should produce an increase of viscosity. It is unlikely that the combination of these two opposite effects could produce the good correlation between viscosity and tension and stiffness found experimentally at various filament overlaps.

We believe that it is more likely that the viscosity of the active fibre is due to the crossbridges mechanism. In this case, our viscoelastic response could be justified by the presence of weakly binding crossbridges detaching with a rate constant of around 10^5 s^{-1}. This crossbridge state could correspond to the unspecific weak binding of myosin to actin that precedes force generation [13]. According to this idea, the viscoelastic response would not imply the presence of real mechanical elements, but it is implicit in the rapid equilibrium crossbridge mechanism. It should be pointed out, however, that since our data suggest that fibre viscosity is located in the overlap region, a real elasticity in series with viscosity exists in the sarcomere and is represented by the myofilament compliance. Although at present our data do not allow an estimate of this effect, the presence of the myofilament compliance will reduce the relaxation time effectively attributable to the crossbridge mechanism. Consequently, the detachment rate constant of rapid equilibrium bridges would be higher than 10^5 s^{-1}.

Previous experiments by Ford et al. [1,4] have shown that, at optimum length, the active fibre viscosity is smaller than the resting viscosity, which therefore should have decreased as a consequence of stimulation. This result disagrees with our finding that the fibre viscosity increases upon activation. We have no precise explanation for this discrepancy; there are, however, some experimental differences that could be considered.

1. We used ramp length changes instead of step length changes. During the steps, the stretching velocity and therefore the viscous force never attains a steady value, making difficult its quantification.
2. In calculating the effect of viscosity, Ford et al. [1] considered the velocity of shortening or lengthening of the whole fibre while we measured the instantaneous velocity directly at sarcomere level, therefore taking into account the distortion associated with the propagation of the mechanical wave.

3. The duration of phase 1 in our experiments was 50 μs or less. The limited frequency response of the force transducer used by Ford et al. [1] should have somewhat smoothed the phase 1 in their record, making its detection more difficult.

More recently, De Tombe and Ter Keurs [3] showed that the reduction of the maximum shortening velocity of cardiac trabeculae found at a low level of activation could be accounted for by assuming the presence of a velocity-independent internal load due to a viscosity similar to that present in resting trabeculae. They measured a viscous coefficient of 0.47–0.77 kN m^{-2} s, a value similar to ours at full activation. Our fibre viscosity, however, would differ from that postulated by De Tombe and Ter Keurs, in two important aspects: (1) it is not constant but proportional to the tension developed; and (2) it very likely arises from crossbridge interaction and not from passive structures of the fibre. This excludes the possibility of the active fibre viscosity acting as an internal load during shortening.

Finally, it should be pointed out that an internal viscosity similar to that found here was necessary in order to fit the mechanical resonance curves of activated frog fibres [14].

As was already reported in Section 1, the presence of significant internal viscosity in the activated fibres would affect the instantaneous T_1 curve (and the value of y_0) and should therefore be considered especially in experiments in which fast length changes are used.

In conclusion, the data reported in this paper show that activated frog fibres have an internal viscosity greater than at rest. This viscosity is associated with a very short relaxation time and it is roughly proportional to the isometric tension developed. It is suggested that this viscosity arises from crossbridges weakly bound in a non-force-generating configuration.

Acknowledgements

The present research was financed by Universita' degli Studi di Firenze, European Economic Community (contract no. ERBCHRXT 940606, HCM Programme), Telethon-Italy and C.N.R.-Italy.

References

[1] L.E. Ford, A.F. Huxley and R.M. Simmons, J. Physiol., 269 (1977) 441–515.
[2] M.A. Bagni, G. Cecchi, F. Colomo and P. Garzella, J. Physiol., 482 (1995) 391–400.
[3] P.P. de Tombe and H.E.D.J. ter Keurs, J. Physiol., 454 (1994) 619–642.
[4] L.E. Ford, A.F. Huxley and R.M. Simmons, J. Physiol., 311 (1981) 219–249.
[5] H.E. Huxley, A. Stewart, H. Sosa and C.T. Irving, Biophys. J., 67 (1994) 2411–2421.
[6] K. Wakabayashi, Y. Sugimoto, H. Tanaka, Y. Hueno, Y. Takezama and Y. Amemiya, Biophys. J., 67 (1994) 2422–2435.
[7] M.A. Bagni, G. Cecchi, F. Colomo and P. Garzella, Adv. Exp. Med. Biol., 332 (1993) 703–712.
[8] M. Schoenberg, Biophys. J., 48 (1985) 467–475.
[9] M.A. Bagni, G. Cecchi and F. Colomo, J. Muscle Res. Cell Motil., 6 (1985) 102a.
[10] A.F. Huxley, V. Lombardi and L.D. Peachey, J. Physiol., 317 (1981) 12P–13P.
[11] K. Burton and A.F. Huxley, Biophys. J., 68 (1995) 2429–2443.
[12] A.F. Huxley, Reflections on Muscle, Liverpool University Press, Liverpool, UK, 1980.
[13] I. Rayment, H.M. Holden, M. Whittaker, C.B. Yohn, M. Lorenz, K.C. Holmes and R.A. Milligan, Science, 261 (1993) 58–65.
[14] G. Cecchi, P.J. Griffiths and S. Taylor, Biophys. J., 49 (1986) 437–451.