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Effects of intracellular acidification and varied temperature on force, stiffness, and speed of shortening in frog muscle fibers

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Radzyukevich, T., and K. A. P. Edman. Effects of intracellular acidification and varied temperature on force, stiffness, and speed of shortening in frog muscle fibers. Am J Physiol Cell Physiol 287: C106–C113, 2004. First published March 3, 2004; 10.1152/ajpcell.00472.2003.—This study aimed to establish whether the temperature-dependent effect of acidification on maximum force observed in mammalian muscles also applies to frog muscle. Measurements of force, stiffness, and unloaded velocity of shortening in intact single muscle fibers from the anterior tibialis muscle of Rana temporaria were performed between 0 and 22°C during fused tetani in H2CO3-CO2-buffered Ringer solution with pH adjusted to 7.0 and 6.3, respectively. The force-to-stiffness ratio increased as a rectilinear function of temperature between 0 and 20°C at pH 7.0. Lowering the pH to 6.3 reduced the tetanic force by 13.5 ± 1.2 and 11.5 ± 1.4% at 2.8 and 20.5°C, respectively, with only a minor reduction in fiber stiffness. The maximum speed of shortening was decreased by lowered pH by 12.9 ± 1.5 and 7.8 ± 1.1% at low and high temperature, respectively. Acidification increased the time to reach 70% of maximum force by 18.0% at ~2°C, the same pH change performed at ~20°C in the same fibers reduced the rise time by 24.1%. The same increase in the rate of rise of force at high temperature was also found at normal pH after the fibers were fatigued by frequent stimulation. It is concluded that, in frog muscle, the force-depressant effect of acidification does not vary significantly with temperature. By contrast, acidification affects the onset of activation in a manner that is critically dependent on temperature.

METHODS

Preparation and mounting. The experiments were performed on single fibers dissected from the anterior tibialis muscles of Rana temporaria. The experimental procedures used were approved by the

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Animal Ethics Committee of the University of Lund. Frogs were stored at 4 °C for at least 1 wk before use. The animals were killed by decapitation followed by destruction of the spinal cord.

The fiber was mounted horizontally in a Perspex chamber between a force transducer and an electromagnetic puller. The fiber tendons were held by aluminium clips as described previously (20, 23). The muscle chamber was covered to provide a constant environmental temperature around the fiber. The experiments were carried out at a resting sarcomere length of 2.1 μm. Fiber length, cross-sectional area, and sarcomere length were measured as described by Edman and Reggiani (23).

**Solutions.** The dissection of the muscle fibers was carried out in a standard Ringer solution containing (in mM) 115.5 NaCl, 2.0 KCl, 1.8 CaCl₂, 2.0 Na₂HPO₄ + NaH₂PO₄, pH 7.0, at 2.0 °C. For the actual experiments, a bicarbonate Ringer solution of the following composition was used (in mM): 108.0 NaCl, 2.0 KCl, 1.8 CaCl₂, 10.0 NaHCO₃. The solution was bubbled with mixtures of CO₂ and O₂ to give a pH of 7.0 or 6.2–6.3 at the temperature used in the muscle chamber (14). The bathing solution, which was thermostatically controlled to within 0.2 °C, was continuously perfused through the muscle chamber (volume ca. 2.5 ml) at a speed of 2 ml/min. The experiments were performed at a given temperature within the range of 2–22 °C as stated in RESULTS. Both temperature and pH of bathing solution in the chamber were measured periodically during the experiment.

**Stimulation.** Fibers were stimulated by passing rectangular current pulses between two platinum-plate electrodes placed symmetrically on either side of the fiber ~2 mm from it. The pulse duration was 0.2 ms, and the frequency used for tetanic stimulation (16–22 Hz at low temperature and 65–80 Hz at high temperature) was adjusted to give a fused tetanus of 600–1,000-ms duration. The interval between tetani was 3 min, except in some experiments where the interval was reduced from 180 to 15 or 30 s to induce fatigue of the fiber (fatiguing stimulation; Ref. 22). The strength of the electrical stimulus was set 15% above the twitch threshold and was adjusted, if required, during the experiment as the stimulation threshold changes with both temperature and pH.

**Force transducer.** The principal unit of the transducer was a semiconductor strain-gauge element (AE 801, Aksjeselskapet Mikroelektronikk, Horten, Norway). To make the transducer suitable for stiffness measurements, a distal portion (~2 mm in length) of the silicon bar was removed. The force transducer so modified had a resonant frequency of 19 kHz when submerged in the bathing fluid. There was no detectable “creep” of the transducer signal after a sudden unloading of the transducer (see Ref. 20).

**Electromagnetic puller.** Two different electromagnetic pullers were used in this study. Motor number 1 allowed relatively large semifast movements and was employed in experiments for force-velocity measurements and slack tests (for description, see Ref. 23). Motor number 2 had the capacity to produce fast, low-amplitude length changes and was used in experiments that involved stiffness measurements. A description of motor number 2 has been given by Edman and Lou (20).

**Measurement of fiber stiffness.** Stiffness was measured by recording the changes in force that occurred in response to an imposed sinusoidal length oscillation as fully described previously (20). The frequency of the length oscillation was 2 kHz, and the peak-to-peak amplitude was 10–11 μm, corresponding to 1.6–1.8 nm per half sarcomere. This means that the fiber underwent alternating stretches and releases of <1 nm/half sarcomere in amplitude, about the unperturbed length of the fiber. The stiffness measurement was thus performed within the straight portion of the stress-strain relationship of the sarcomere elasticity located above and below the isometric force (see Refs. 25, 37). The stretch and release movements produced during the stiffness measurement were completed in 0.250 ms. The cyclic length changes may thus be considered rapid enough to provide a useful index of the instantaneous stiffness of active muscle (25, 31).

The oscillation was initiated just before the onset of stimulation and usually continued until the shoulder of the relaxation phase had been passed. A stiffness signal was formed by passing the signal from the force transducer through a narrow band-pass filter (Q value of 5.5), the optimum frequency of which was set to the actual frequency of the length oscillation used. By rectifying the filtered signal, a direct read-out of the fiber stiffness could be obtained during the course of the tetanus (see Fig. 1 in Ref. 20). The bandwidth of the rectified signal was DC-1.3 kHz. The force signal without the superimposed force oscillation was recorded simultaneously by using a notch filter, which produced maximum attenuation at the frequency (2 kHz) used for the length oscillation. The signal from the position transducer of the puller producing the fast length oscillation was treated in the same way as described for the force transducer to provide a measurement of the peak-to-peak amplitude of the length oscillation.

The immediate force response to the rapid length change, used for measuring instantaneous stiffness, might be attenuated due to an early component of tension recovery. This would lead to some underestimation of the cross-bridge stiffness and, furthermore, make a comparison of the measured stiffness at different temperatures uncertain.
RESULTS

Differential effects of temperature on tetanic force and stiffness at pH 7.0. The influence of temperature on tetanic force and instantaneous stiffness was studied within the range of 2.4–21.7°C in frog single muscle fibers immersed in bicarbonate Ringer solution at pH close to 7.0. The fibers were stimulated to produce a 0.8-s isometric tetanus at 3-min intervals at each selected temperature, and the stimulus frequency was adjusted to provide complete mechanical fusion in each case. The stimulations were continued until the maximum tetanic force had settled at a constant level, with six to eight tetani usually being required to attain constant responses. After a completed series of recordings at a given temperature, the fiber was rested for 60 min, during which time the bath temperature was readjusted.

Example records of tetanic force and instantaneous stiffness derived at 2.4 and 19.4°C are illustrated in Fig. 1. Figure 1A shows two superimposed records of the tetanic force recorded at low and high temperature, and Fig. 1B illustrates the corresponding stiffness myograms. It can be seen, in accordance with earlier observations (see Ref. 56), that raising the temperature led to a steeper rise of force, a greater amplitude of force, and a faster relaxation phase. In 30 fibers investigated, the maximal tetanic force increased by 38.4 ± 2.4% as the temperature changed from 2.9 ± 0.1 to 20.5 ± 0.2°C. Stiffness, on the other hand, was found to decrease by 10.6 ± 2.9% as the temperature was raised over the same interval (see below for further details).

In accordance with previous observations at low temperature in frog muscle (3, 8, 20, 26, 29), Fig. 1. C and D, demonstrates that stiffness was in the lead of tension during the rising phase of the tetanus, and this applies to both low and high temperatures. The stiffness remained constant during the plateau phase of the tetanus and lagged behind tension during the relaxation phase.

In three experiments, simultaneous measurements of tetanic force and tetanic stiffness were performed at four to five different temperatures within the range 2.4–20.1°C. The results show (Fig. 2A) that force and stiffness do not undergo proportional changes as the temperature is altered. Thus, by raising the temperature from a range of 2.4–3.5°C to 8.2, 8.4, and 9.0°C in the three different experiments, the stiffness increased by merely 1.2, 2.4, and 6.5%, respectively. At the same time, tetanic force increased by 17.7, 45.9, and 24.1% of the value recorded at 2.4–3.5°C. By stepwise increasing the temperature from −9 to 20°C, there was a slight further increase in tetanic force (Fig. 2A) while, at the same time, the stiffness decreased to a value below that derived at 2.4–3.5°C.
Figure 3 illustrates typical records of force and stiffness during isometric tetani at normal pH and during acidification. At both high and low temperatures, the maximum tetanic force was moderately reduced by lowering the pH. In 10 fibers investigated, the reduction of force by acidification was 13.5 ± 1.2% at low temperature, whereas the corresponding decrease recorded at the high temperature was 11.5 ± 1.4% (Table 1). The difference between these two values was not statistically significant (P > 0.1, n = 10). As illustrated in Fig. 3B, stiffness was reduced less by acidosis than force was, and this applies to measurements at both high and low temperature. In five fibers, the decrease in stiffness during acidification was merely 5.6 ± 2.8% (P < 0.08) and 4.0 ± 2.6% (P < 0.16) at low and high temperature, respectively; these changes were not statistically significant.

pH affected the rising phase of force and stiffness differentially at high and low temperatures despite the fact that maximum force and maximum stiffness were both reduced at the two temperatures as just described. This is illustrated in Fig. 4, which shows the rising phases of the records in Fig. 3 on a faster time base. It can be seen that, whereas acidification reduced the rate of rise of both force and stiffness at low temperature, the opposite was true at high temperature, i.e., there was a steeper rise of force and stiffness in response to lowered pH in the latter situation (Table 1). In 10 experiments, the time to reach 70% of maximum force increased by 18% (from 79 ± 6 to 93 ± 8 ms, P < 0.05) in response to acidification at low (~2°C) temperature. By contrast, at high (~20°C) temperature, the same pH change reduced the time to reach 70% of maximum force by 24% (from 31 ± 3 to 23 ± 2 ms, P < 0.001). These disparate effects by acidification on the onset of activation at high and low temperatures have not, to our knowledge, been reported previously.

Effects of intracellular acidification on tetanic force and tetanic stiffness: influence of temperature. The fibers, immersed in bicarbonate-CO₂-buffered bathing solution at 2–3°C, were stimulated to produce a fused tetanus at regular 3-min intervals with recording of force and, in some experiments, with simultaneous measurements of fiber stiffness. After constant responses had been attained at pH 7.0, the pH of the bathing fluid was reduced to 6.3 by raising the CO₂ concentration of the medium, and the fiber was tetanized at 3-min intervals as before. By reducing the pH, the tetanic force declined, reaching a constant level within ~30 min. The above sequence of events was thereafter carried out at a temperature close to 20°C. In other experiments, the order of temperature changes was the opposite, i.e., measurements at normal and reduced pH were first carried out at the high temperature level (~20°C) and were followed by similar measurements near 2°C.

Recent evidence suggests that the thick and thin filaments have a finite stiffness comparable to that residing in the myosin cross bridges during maximal activity (30, 32, 36, 50). However, there is still a lack of information as to whether the filament elasticity is Hookean and whether it is uniformly distributed along the filaments or merely involves the free portions of the filaments outside the overlap region. The existence of filament compliance makes a quantitative evaluation of the cross-bridge stiffness somewhat uncertain. Assuming that the filament compliance has the characteristics of a Hookean spring acting in series with the cross bridges, a change in cross-bridge stiffness will be underestimated by using the total sarcomere stiffness as an index. For example, if the filament compliance were to account for as much as 50% of the sarcomere compliance, a 10% increase in sarcomere stiffness would, in reality, correspond to a 22% increase in cross-bridge stiffness. On the same assumption, a 10% decrease in sarcomere stiffness would correspond to an 18% decline in cross-bridge stiffness.

The dotted curves in Fig. 2B illustrate the estimated force-stiffness relationship of the cross bridges at different temperatures when account is made of the presence of thin-filament compliance amounting to 50% of the total sarcomere compliance. It is reasonable to assume that the elasticity of the free portion of thin filament has a low temperature dependence (see Ref. 38). The closed squares in Fig. 2B show the calculated force-to-stiffness ratio when the series compliance is assumed to be independent of temperature, whereas the closed triangles show the situation when the series compliance varies with temperature with a Q₁₀ of 1.20. It can be seen that, even in the latter case, there is a substantial increase (~40%) of the force-to-stiffness ratio as the temperature is raised from 3 to 20°C.

Intracellular acidification and temperature

Figure 2A, also see Fig. 1B). The unequal temperature dependence of force and stiffness depicted in Fig. 2A results in a rectilinear increase of the force-to-stiffness ratio (Q₁₀ = 1.41) as the temperature is raised from ~3 to 20°C (Fig. 2B). A similar observation was reported by Galler and Hilber (27) in studies on skinned rat skeletal muscle fibers. Assuming that the measured stiffness reflects the number of attached cross bridges (31), the results provide evidence that the average force produced by a cross bridge during a working cycle steadily increases as the temperature is raised within the temperature range considered.

Effects of intracellular acidification on tetanic force and tetanic stiffness: influence of temperature. The fibers, immersed in bicarbonate-CO₂-buffered bathing solution at 2–3°C, were stimulated to produce a fused tetanus at regular 3-min intervals with recording of force and, in some experiments, with simultaneous measurements of fiber stiffness. After constant responses had been attained at pH 7.0, the pH of the bathing fluid was reduced to 6.3 by raising the CO₂ concentration of the medium, and the fiber was tetanized at 3-min intervals as before. By reducing the pH, the tetanic force declined, reaching a constant level within ~30 min. The above sequence of events was thereafter carried out at a temperature close to 20°C. In other experiments, the order of temperature changes was the opposite, i.e., measurements at normal and reduced pH were first carried out at the high temperature level (~20°C) and were followed by similar measurements near 2°C.

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The time from the last stimulus to the occurrence of the “shoulder” of the force myogram was used as a measure of the duration of the linear phase of relaxation during tetanus, i.e., the part of the relaxation where no gross sarcomere length changes occur (11, 12, 14, 19). The reciprocal of this measurement thus provides a useful index of the rate of isometric relaxation of the muscle fiber. Decreasing the pH markedly reduced the rate of relaxation (Fig. 3 and Table 1). In 10 experiments, the duration of the linear phase of relaxation increased by 56.8 ± 6.0% during acidification at low (2.8 ± 0.3°C) temperature and by 47.6 ± 3.8% at high (20.5 ± 0.3°C) temperature. The difference between the measured effects of acidification at the two temperatures was not statistically significant ($P > 0.2$).

It was of interest to learn whether the increase in speed of force development induced by acidification at high temperature also occurred in response to fatiguing stimulation. Figure 5 shows an experiment to test this point, and Table 1 summarizes the results from a whole series of similar experiments at 20°C. The fiber, initially equilibrated at pH 7.0 (control 1, Fig. 5), was subjected to acidosis at pH 6.27. This caused a substantial increase in the rate of rise of force (acidification in Fig. 5), in accordance with the results presented in Fig. 4. After return to the control medium of pH 7.0, which restored the rate of force development to its original value (control 2, Fig. 5), the fiber was subjected to a period of fatiguing stimulation at pH 7.0 by reducing the intervals between tetani from 3 min to 15 s. This led to a steepening of the rising phase of the tetanus (fatigue in Fig. 5), which agreed well with the effects induced by acidification at the high temperature. The mean values of the effects produced by acidification and fatiguing stimulation are presented for comparison in Table 1. The results show that, as maximum tetanic force was depressed by ~12% by acidification and fatiguing stimulation, the time to 70% of maximum force was reduced by 20–25% by the two interventions. These findings provide yet another example of the striking similarities between the contractile effects of fatiguing stimulation and acidification that have previously been demonstrated in frog muscle fibers (22).

**Effects of intracellular acidification on maximum speed of shortening at low and high temperature.** The velocity of unloaded shortening was measured with the slack-test method (17) as outlined in METHODS and illustrated in Fig. 6. The slack tests were carried out during the plateau phase of nine consecutive tetani using three different amplitudes of release, each one repeated three times in separate runs. The regression of slack time on amplitude of release provided a measure of $V_0$. As illustrated by a representative experiment in Fig. 6, decreasing the pH of the extracellular medium from 7.02 to 6.26 caused a moderate decrease of $V_0$ at both low (2.5°C) and high (20.1°C) temperature. The depression of $V_0$ was, however, less pronounced at the higher temperature. In 10 experiments, in which measurements were made at the two temperature levels...
in the same fibers (Table 1), acidification depressed $V_0$ by 12.9 ± 1.5% at low temperature and by 7.8 ± 1.1% at high temperature, the difference between these two values being statistically significant ($P < 0.01$, paired observations).

**DISCUSSION**

The present experiments provide new information on the temperature dependence of the contractile effects that are induced by intracellular acidification in frog muscle fibers. The results also contain information of relevance to evaluate the variation in force output of attached cross bridges as the temperature is changed. These two aspects of the study are discussed separately below.

**Temperature dependence of the contractile effects of intracellular acidification.** Previous studies have demonstrated that frog muscle fibers that are brought to fatigue by frequent activation at low (2–3°C) temperature lose part of their capacity to produce force at the same time as their maximum speed of shortening is substantially reduced (15, 22). These changes were found to accord remarkably well with the effects induced by intracellular acidification (20, 22), supporting the view that lowered intracellular pH is an important factor in the development of fatigue induced by frequent stimulation of amphibian muscle. Similar to the observation in frog muscle, lowered intracellular pH does have a depressant effect on both force and shortening velocity also in mammalian muscle at relatively low temperature (20°C). The results obtained so far would thus seem to make clear that intracellular acidification puts the muscle in a state that is virtually indistinguishable from moderate fatigue, i.e., the milder form of fatigue (18, 21), in which the tetanic force is depressed by not more than ~30% of the rested-state value. This by no means excludes that accumulation of other products of the ATP hydrolysis, such as Mg-ADP and Pi, also affects the cross-bridge function during moderate fatigue. The actions of ADP and Pi on force and shortening velocity have been reported in several studies on skinned muscle fibers (1, 10, 13, 28, 34, 48, 49), and a summary of these effects has been presented earlier (15, 22). It is clear, however, that the combined actions of the two hydrolysis products do not eliminate the close relationship that exists between the contractile changes recorded during intracellular acidification and moderate fatigue in frog muscle.

It is well known from previous studies of both amphibian and mammalian muscles that the decrease in tetanic force during fatigue and intracellular acidification is associated with a lower rate of force development. This is regularly observed in frog muscle preparations at the low temperatures generally used (e.g., Refs. 20, 22, 44). The reduced speed of force development is at least partly attributable to the change of the force-velocity relationship that occurs under these conditions leading to a lower speed of shortening at low and intermediate loads (15, 22). The present results demonstrate that fatigue and acidification of frog muscle fibers at high temperature (20°C) affect the rising phase of the tetanus in a way that is opposite to that observed at low temperature. That is, whereas the maximum tetanic force is depressed to the same extent at low and high temperature (Table 1), the rate of rise of force is increased by fatigue and acidification at the high temperature. These patterns of effects were fully reversible when the temperature was changed intermittently in each individual fiber tested.

The mechanism underlying the increased rate of rise of force during fatigue and acidification at high temperature is unclear and requires further investigation. As pointed out above, the basic effect of the two interventions can be presumed to be a slowing of the tension rise during tetanic stimulation at both low and high temperature in view of the fact that the speed of
shortening is depressed in both cases. It is, therefore, logical to presume that the two interventions, fatigue and acidification, in addition to their depressant effect on cross-bridge function (20, 21), also increase the rate of activation of the contractile system, an effect that may become predominant at high temperatures. The possibility that the faster rise of force at high temperature might reflect an increased rate of attachment of the myosin cross bridges during fatigue and acidification can probably be excluded, because this would enhance the maximum tetanic force as well above the control level.

Variation in force output of attached cross bridges with altered temperature. According to the cross-bridge model of muscle contraction, the measured isometric force is a function of the number of attached cross bridges and the average force generated per cross bridge. A change in either of these two variables may be evaluated by simultaneously recording the force output of the muscle fiber and the instantaneous stiffness of the fiber, the latter measurement being used as an index of the number of attached cross bridges. Results from several previous studies on both frog and mammalian muscle preparations have shown that an increase in temperature results in a smaller rise in stiffness than in force, suggesting that the force output per bridge is enhanced with increasing temperature (7, 25, 27). The present study extends earlier work by providing a detailed account of the simultaneous changes in force and stiffness that occur as the temperature is altered in discrete steps between 2 and 22°C. By using the ratio between force and stiffness, it has been possible to derive an approximate index of the average force output per cross bridge and its variation with temperature. For the following discussion, it is essential to point out that the frog muscle fibers used are likely to be maximally, or supramaximally, activated over the entire range of temperatures investigated. This follows from the fact that the tetanic force is not enhanced by addition of caffeine or other twitch-potentiating agents at either low (1–3°C; Refs. 20, 35) or high (15–30°C; Refs. 9, 33, 45; see also Ref. 6) temperatures.

Our results show that the average force generated per cross bridge, as indicated by the force-to-stiffness ratio, increases steadily as the temperature is raised between 3 and 20°C. This conclusion remains after account has been made of the possibility that 50% of the fiber compliance resides in the free portions of the thin filaments and, furthermore, that the filament compliance increases moderately with temperature (see Fig. 2B). In contrast to the force-to-stiffness ratio, the tetanic stiffness by itself changes in a more complex manner with temperature. Thus, as the temperature was raised from ~3 to 20°C, the fiber stiffness increased to a maximum near 9°C. However, the tetanic stiffness decreased to a level below that recorded at 3°C by further increasing the temperature to 20°C. These findings suggest that the relatively steep rise in maximum tetanic force that occurs as the temperature is raised from 3 to 10°C is due to an increased number of active cross bridges in combination with increased force output per bridge. By raising the temperature beyond this level, however, the steady increase in force production of the active cross bridges will be counterbalanced by a decrease in the number of attached bridges, resulting in a flat maximum of the tetanic force between 15 and 20°C, as observed in the present study.

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DISCLOSURES

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