

## DEPRESSION OF $\text{Ca}^{2+}$ INSENSITIVE TENSION DUE TO REDUCED pH IN PARTIALLY TROPONIN-EXTRACTED SKINNED SKELETAL MUSCLE FIBERS

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**ABSTRACT** Previous studies on skinned muscle fibers have demonstrated a direct effect of elevated levels of  $\text{H}^+$  ion to depress force production; however, the molecular basis for this effect is presently unknown. Here, whole troponin complexes were removed from skinned single fiber preparations of rat slow-twitch and fast-twitch muscles, and the effect of  $\text{H}^+$  ions on the resultant  $\text{Ca}^{2+}$ -insensitive force was examined. The effect of  $\text{H}^+$  ions to depress force was found to be virtually identical in untreated control fibers activated in the presence of  $\text{Ca}^{2+}$  and in fibers activated in the absence of  $\text{Ca}^{2+}$  by troponin removal. Thus, the effect of  $\text{H}^+$  ions to depress force occurs at a step in activation beyond the disinhibition of the thin filament by  $\text{Ca}^{2+}$ , probably involving reductions in the number of attached cross-bridges or in the force per attachment.

### INTRODUCTION

Muscle fatigue can be defined as the inability of a maximally stimulated fiber to develop maximum force (Fitts et al., 1982). Depending upon the intensity of activity, muscle fatigue will develop in a characteristic pattern of mechanical and biochemical alterations. Indeed, numerous reports have shown a strong correlation between muscle fatigue and intracellular pH during intense contractile activity and subsequent recovery (Hermansen and Osnes, 1972; Dawson et al., 1978; Metzger and Fitts, 1987a). Active tension has been found to decrease even when intracellular pH is reduced by means other than intense contractile activity (Edman and Mattiazzi, 1981; Sahlin et al., 1983; Mainwood and Renaud, 1985). In attempts to determine whether changes in pH could account for muscle fatigue, several studies have been conducted using the skinned muscle fiber preparation, in which the sarcolemma is removed by chemical or mechanical techniques (Fabiato and Fabiato, 1978; Donaldson and Hermansen, 1978; Robertson and Kerrick, 1979; Donaldson, 1984; Metzger and Moss, 1987). After removal of the sarcolemma, the ionic composition of the fluid bathing the myofilaments can be directly controlled while leaving the myofilament lattice intact. Thus, intracellular pH can be varied precisely and independently of other factors, such as the concentrations of  $\text{Ca}^{2+}$ , ATP, inorganic phosphate, or

creatine phosphate, thought to have roles in fatigue. This experimental approach allows for a direct test of the effect of pH on contractile function.

Such studies have provided evidence that an increased concentration of  $\text{H}^+$  ions could be responsible for at least part of the observed reduction in maximum force during fatigue. As the pH of the solution bathing the contractile apparatus was lowered from a resting value of 7.00 to 6.20, which is the value observed after intense contractile activity (Hermansen and Osnes, 1972; Metzger and Fitts, 1987a), both maximum force and the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus were significantly reduced (Fabiato and Fabiato, 1978; Metzger and Moss, 1987). Recently, we showed that  $\text{H}^+$  ion-mediated reductions in force and velocity are greater in muscles containing fast isoforms of contractile and regulatory proteins as compared with muscles with slow isoforms (Metzger and Moss, 1987). While these studies clearly indicate that variations in the concentration of  $\text{H}^+$  ion modulate contractile performance, the mechanism by which this occurs remains unknown.

In an attempt to explain the  $\text{H}^+$  ion-induced reduction in the  $\text{Ca}^{2+}$  sensitivity of tension development, various hypotheses have been proposed, including a  $\text{H}^+$  ion-induced decrease in the affinity of troponin C for  $\text{Ca}^{2+}$  (Blanchard et al., 1984) and a decrease in the electrostatic attraction of  $\text{Ca}^{2+}$  to the negatively-charged thin filament as pH is reduced (Godt, 1981). While these two possibilities separately or perhaps together could account for the reduced  $\text{Ca}^{2+}$ -sensitivity, neither appears to be appropriate to explain the observed  $\text{H}^+$  ion-mediated decline in maxi-

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imum force. As discussed elsewhere (Fabiato and Fabiato, 1978; Metzger and Moss, 1987), if  $H^+$  ions depress maximum force through a mechanism involving incomplete activation of the thin filament by  $Ca^{2+}$  the effect should be overcome by increasing the  $[Ca^{2+}]$ . However, at low pH the sigmoidal relationship between force and pCa reaches a plateau at high  $[Ca^{2+}]$  and typically declines somewhat when the concentration of  $Ca^{2+}$  is increased to supersaturating levels (Fabiato and Fabiato, 1978; Metzger and Moss, 1987). This result suggests that the molecular basis for the effect of  $H^+$  ions to reduce maximum force involves a step in activation beyond disinhibition of the thin filament by  $Ca^{2+}$ .

Here, this possibility was tested directly by examining the effect of pH upon force production in fibers that were treated to remove some of the whole troponin complexes from the thin filament (Moss et al., 1986). In a partially troponin deficient fiber, portions of the thin filament are permanently (though reversibly) disinhibited allowing for cross-bridge cycling and force production in the absence of  $Ca^{2+}$ .

## MATERIALS AND METHODS

Female Sprague-Dawley rats (average weight 270 g) were injected with Nembutal (50 mg/kg body weight, intraperitoneally) and the slow-twitch soleus (sol) and fast-twitch superficial portion of the vastus lateralis (svl; 100% type IIb, Baldwin et al., 1972) muscles were removed and placed in cold relaxing solution (described below). Bundles of ~50 fibers were dissected from each muscle and tied with surgical silk to glass capillary tubes. Bundles were stored for up to 3 wk at  $-22^\circ C$  in relaxing solution which contained 50% (vol/vol) glycerol. Before an experiment bundles were placed for 30 min in cold relaxing solution containing 0.5% (wt/vol) Brij-58 in order to disrupt the sarcoplasmic reticulum (Moss, 1979), after which individual fibers were pulled free from one end of the bundle. While in relaxing solution, the fiber was mounted between a force transducer (model 403, Cambridge Technology Inc., Cambridge MA; sensitivity 20 mV/mg) and a DC torque motor (model 300s, Cambridge Technology Inc.). Complete details of the connectors, mounting procedures, and experimental apparatus have been reported previously (Moss, 1979). The connectors consisted of troughs fashioned from 29 gauge stainless steel tubing attached to wires from the force transducer and motor. The ends of the fiber were secured in the troughs by overlaying short lengths of 5-0 monofilament suture, which in turn was tied in place with 10-0 suture. This connector minimized the amount of end compliance so that upon exposing the fiber to  $Ca^{2+}$ -containing activating solution, sarcomere length varied <2% of the value obtained in relaxing solution.

The mounted fiber was viewed through an inverted microscope (model WL, Carl Zeiss Inc., Thornwood, NY) and end-to-end length was set to achieve a resting sarcomere length of  $\sim 2.50 \mu m$  by adjusting the three-way positioner to which the motor was attached. Mean data for soleus ( $n = 6$ ) and svl ( $n = 7$ ) fibers were as follows: end-to-end length, 2.74 and 2.39 mm; sarcomere length in relaxing solution, 2.51 and 2.58  $\mu m$ ; sarcomere length during maximum  $Ca^{2+}$  activation, 2.48 and 2.52  $\mu m$ , respectively.

Relaxing and activating solutions contained (in millimolar): EGTA, 7; free  $Mg^{2+}$ , 1; imidazole, 20; ATP, 4.42; creatine phosphate, 14.5; and sufficient KCl to yield an ionic strength of 180 mM. Solution pH was adjusted to 7.00 or 6.20 with KOH; however, for relaxing solution at pH 6.20, HCl was added to adjust pH. Relaxing solution had a pCa (i.e.,  $-\log [free Ca^{2+}]$ ) of 9.0 while pCa for maximum activation was 4.5. The computer program of Fabiato and Fabiato (1979) was used to calculate the final concentrations of each metal, ligand and metal-ligand complex, based upon the stability constants reported by Godt and Lindley (1982).

The apparent stability constant for  $Ca^{2+}$ -EGTA was corrected for temperature ( $15^\circ C$ ), pH (7.00 or 6.20) and ionic strength (Fabiato and Fabiato, 1979). All force measurements were determined at  $15^\circ C$ .

To determine maximum  $Ca^{2+}$ -activated force at pH 7.00 or 6.20, fibers were exposed to activating solution and steady isometric tension was allowed to develop. Subsequently, overall muscle length was rapidly (<1 ms) reduced by 400  $\mu m$  so that force fell to zero. The fiber was then transferred to relaxing solution and muscle length was re-extended. The difference between steady developed tension and the tension baseline was measured as total tension. Active tension was determined by subtracting resting tension (tension at pCa 9.0) from total tension. In fibers in which troponin was removed, active tension was determined as the difference between the resting tension value before troponin removal and the total tension. Initial control measurements of maximum isometric force at pCa 4.5 were made on each fiber first at pH 7.00, then at pH 6.20, and again at pH 7.00.

Subsequently, whole troponin complexes were removed from the fibers using a modification of previously developed methods (Moss et al., 1986), and steady  $Ca^{2+}$  insensitive tension at pCa 9.0 was measured using the same sequence of pH values. The solution for removal of troponin consisted of (in millimolar): EDTA, 10; phosphate, 5; imidazole, 5; KCl, 50; pH 7.00; and 0.9 mg/ml light chain-2S from bovine masseter muscle which contains a protease activity that selectively cleaves whole troponin complexes from the thin filament (Moss et al., 1986). Since significant amounts of rigor tension developed upon transferring the fiber into this solution, end-to-end fiber length was reduced so that the tension borne was similar to that at pCa 9.0 before removal of troponin. The amount of troponin removed was dependent upon solution temperature ( $15$ – $35^\circ C$ ) and the duration of treatment (1–5 h) with higher temperatures and longer durations resulting in greater amounts of troponin removed and thus greater amounts of  $Ca^{2+}$  insensitive tension. Solutions in which masseter  $LC_{IS}$  was substituted for  $LC_{2s}$  removed substantially less Tn, while solutions without masseter light chains resulted in virtually no  $Ca^{2+}$  insensitive force. After the removal of whole troponin and the return of temperature to  $15^\circ C$ , the fiber was transferred to relaxing solution and end-to-end muscle length was readjusted to the initial value. In a number of fibers, temperature stabilization and length adjustments were carried out after the transfer of the fiber to a relaxing solution containing ATP $\gamma$ S in order to inhibit force production during these procedures.

To determine the contractile and regulatory protein content before and after an experiment, fiber segments were placed in 0.5 ml microfuge tubes containing sodium dodecyl sulfate (SDS) buffer (10  $\mu l/mm$  segment length), and stored at  $-22^\circ C$  for subsequent analysis of contractile and regulatory protein content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (Giulian et al., 1983; Moss et al., 1986). We have previously published representative gels from control sol and svl fibers (Metzger and Moss, 1987) as well as densitometric scans of gels of fibers after the removal of whole troponin complexes (Moss et al., 1986).

Values are reported as means  $\pm$  standard error of the mean. Differences between two means were determined by a two-tailed t-test for unpaired data. A level of  $P < 0.05$  was selected as indicating significance.

## RESULTS

As shown in Fig. 1, substantial amounts of  $Ca^{2+}$  insensitive force can be achieved in a skinned fiber from which whole troponin has been partially removed; however, developed force in highly troponin deficient fibers fell progressively with time in relaxing solution. This is not unexpected since the thin filament is fixed in a state of nearly complete activation allowing for maintained interaction of cross-bridges such that the fiber progressively deteriorates. For this reason, the experiments investigating the effects of pH

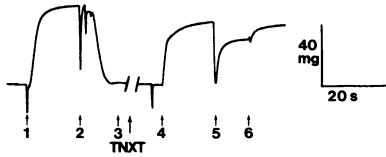


FIGURE 1 Slow time-base recording of force during maximum  $\text{Ca}^{2+}$  activation of a slow-twitch soleus fiber before partial removal of whole troponin complexes (*left*) and in the absence of  $\text{Ca}^{2+}$  after removal of some whole troponin complexes (*right*). At point 1, the fiber was transferred from a relaxing solution to a maximally activating solution (i.e., pCa 4.5) and steady isometric force was allowed to develop. All measurements of force were done at 15°C. At point 2, slack (400  $\mu\text{m}$ ) was rapidly (<1 ms) introduced into the fiber so that overall force fell to zero (not observed on slow time-base scale), after which the fiber was returned to relaxing solution and its length was re-extended (at point 3). The fiber was then exposed for 5 h (35°C) to the solution to remove whole troponin complexes (TNXT). Temperature was subsequently reduced to 15°C and the fiber was placed in relaxing solution (pCa 9.0) at point 4. In some fibers, temperature was reduced while the fiber was in a relaxing solution containing a nonhydrolyzable form of ATP ( $\text{ATP}\gamma\text{S}$ ) instead of ATP in order to inhibit force development during temperature stabilization. At point 5, slack was introduced to the fiber after the development of steady isometric force in relaxing solution to determine the amount of  $\text{Ca}^{2+}$  insensitive force, which in this fiber was 0.78  $P_0$ . Finally, at point 6, fiber length was re-extended. Overall fiber length was 2.28 mm. Average sarcomere length was determined optically (Moss, 1979); a value of 2.49  $\mu\text{m}$  was measured both at pCa 9.0 and during steady tension generation at pCa 4.5.  $P_0$  in this fiber was 57 mg or 1,264  $\text{g}/\text{cm}^{-2}$ .

involved the removal of less troponin and hence lower (~50% of maximum control force at pCa 4.5, pH 7.00; i.e., 0.50  $P_0$ ) amounts of  $\text{Ca}^{2+}$  insensitive force. In this way measurements could be made at pH 7.00 and 6.20 without a significant decline in the force developed upon returning

to pH 7.00. The effects on tension development due to troponin removal were reversed by recombination of purified whole troponin into the treated fibers (see also Moss et al., 1986).

In Fig. 2, original records of force are shown for soleus (*top*) and svl (*bottom*) skinned single fibers at pH 7.00 and 6.20 before and after partial removal of whole troponin complexes. In agreement with earlier findings (Metzger and Moss, 1987), maximum force in the untreated soleus fiber was reduced to 0.74  $P_0$  when pH was decreased from 7.00 to 6.20. After partial removal of troponin complexes,  $\text{Ca}^{2+}$  insensitive force at pH 7.00 was 0.365  $P_0$ . Upon lowering pH to 6.20, this force decreased to 0.263  $P_0$  and the ratio of  $\text{Ca}^{2+}$  insensitive forces (i.e., force at pH 6.20/force at pH 7.00) was 0.72. Returning the fiber to relaxing solution at pH 7.00 completely restored  $\text{Ca}^{2+}$  insensitive force to 0.365  $P_0$ . In the presence of maximally activating  $\text{Ca}^{2+}$  (pCa 4.5), total force production at pH 7.00 was 0.93  $P_0$ , indicating that the maximum force generating capability of the fiber was virtually unchanged after the procedure to remove troponin. In this case, also, developed force at pH 6.20 was 0.72 of to the value at pH 7.00. Qualitatively similar results were obtained in the svl fibers (Fig. 2, lower). However, as shown previously (Metzger and Moss, 1987), the effect of  $\text{H}^+$  ions to depress force was significantly greater in the svl compared with soleus fibers. Force at pH 6.2 relative to that at pH 7.00 was ~0.64 in both the control fibers during maximum activation with  $\text{Ca}^{2+}$  and also when activated in the presence or absence of  $\text{Ca}^{2+}$  by partial removal of troponin.

In Table I, mean values of the ratios of active forces

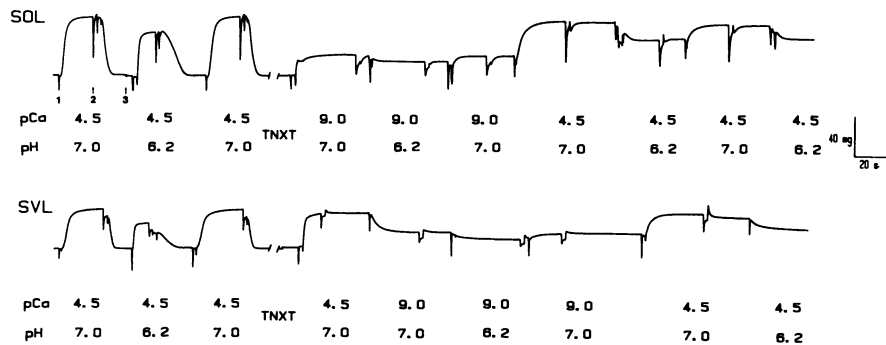


FIGURE 2 Slow time-base recordings of isometric force for soleus (*upper*) and svl (*lower*) fibers at pH 7.00 and 6.20, before and after partial removal of whole troponin, and in the absence (pCa 9.0) and presence (pCa 4.5) of maximally activating  $\text{Ca}^{2+}$ . The events marked 1, 2, and 3 are as described in the legend to Fig. 1. In the soleus fiber before removal of whole troponin, peak force at pH 6.20 relative to that at pH 7.00 ( $P_0$ ) was 0.74. After removal of some whole troponin (TNXT, 2.5 h at 35°C),  $\text{Ca}^{2+}$  insensitive force at pH 7.00 was 0.365  $P_0$ , while in the presence of  $\text{Ca}^{2+}$  force was 0.93  $P_0$ . Force at pH 6.20 was 0.72 of the force at 7.00, both at pCa 9.0 and pCa 4.5.  $P_0$  in this fiber was 65 mg; 1,362  $\text{g}/\text{cm}^{-2}$ . In the svl fiber (*lower trace*) before partial removal of whole troponin, peak force at pH 6.20 was 0.66  $P_0$ . After partial removal of whole troponin (2.5 h at 35°C),  $\text{Ca}^{2+}$  insensitive force at pH 7.00 was 0.45  $P_0$ , while in the presence of calcium (pCa 4.5) force was 0.97  $P_0$ . Forces at pH 6.20 relative to those at pH 7.00 were 0.61 and 0.64 respectively, in the absence and presence of maximally activating  $\text{Ca}^{2+}$ .  $P_0$  in this fiber was 43 mg; 841  $\text{g}/\text{cm}^{-2}$ . It was apparent during the course of this investigation that troponin C was, to some extent, extracted from otherwise intact functional groups. Therefore, in many fibers, as was done in the two fibers included here, purified troponin C (0.5 mg/ml in a relaxing solution containing  $\text{ATP}\gamma\text{S}$ ) was recombined into the fiber in order to restore  $\text{Ca}^{2+}$  sensitivity to these functional groups (Moss et al., 1985).

TABLE I  
RELATIVE FORCE\*

pCa	4.5		9.0	4.5
SOL	75.1 ± 0.8 <sup>†</sup>	Troponin Removal	72.3 ± 1.6 <sup>†</sup>	73.6 ± 0.9 <sup>†</sup>
SVL	67.0 ± 0.9		62.7 ± 1.8	66.7 ± 1.2

The sequence of events proceeded from left to right in this table, i.e., tension was first measured at pCa 4.5; troponin was removed; and tension was subsequently measured at first pCa 9.0 and then pCa 4.5. Values are means ± SEM of 6 SOL and 7 SVL fibers. Control results obtained at pCa 4.5 and 15°C are in good agreement with earlier findings on skinned fibers at higher temperatures (22–23°C; Fabiato and Fabiato, 1978; Donaldson and Hermansen, 1978).

\*Force at pH 6.20/force at pH 7.00.

<sup>†</sup>Significant differences between the SVL and SOL data,  $P < 0.05$ .

obtained at pH 6.20 compared with pH 7.00 are shown for soleus and svl fibers both before and after partial removal of whole troponin. Two main conclusions can be drawn from these results: (a) within fibers from a particular type of muscle (i.e., fast or slow) the effect of H<sup>+</sup> ions to depress force is similar in control fibers during maximum Ca<sup>2+</sup> activation and in partially troponin depleted fibers in the presence and absence of Ca<sup>2+</sup>; and (b) in both the control and troponin-deficient fibers, there is a consistent and statistically significant difference in the H<sup>+</sup> ion-induced diminution of force between the fast and slow muscle fibers.

## DISCUSSION

From the findings of the present study it is clear that the mechanism by which H<sup>+</sup> ions depress maximum force involves a step in contraction subsequent to Ca<sup>2+</sup> activation of the thin filament. This is apparent because Ca<sup>2+</sup> sensitive tension in control fibers and Ca<sup>2+</sup> insensitive tension in troponin extracted fibers were similarly affected by H<sup>+</sup> ions. It is likely that the effect of H<sup>+</sup> ions to depress maximum force involves a reduction in the number of cross-bridge attachments to the thin filament and/or a reduction in force per cross-bridge attachment. Fabiato and Fabiato (1978) proposed a similar mechanism upon observing a 30% decline in rigor tension (no added Ca<sup>2+</sup>, pMgATP 5.5) in skinned preparations of skeletal and cardiac muscles upon lowering pH from 7.00 to 6.20. Unlike their study in which cross-bridges were bound and not cycling, we have tested whether, in the absence of Ca<sup>2+</sup>, the properties of normally cycling cross-bridges were affected by pH. In this context, we have previously provided evidence that cross-bridge interaction in troponin extracted fibers is similar to controls at comparable levels of thin filament activation in terms of tension development and shortening velocity (Moss, 1986; Moss et al., 1986; Allen and Moss, 1987).

Our results may also provide a mechanism for the well documented effect of H<sup>+</sup> ions to reduce the Ca<sup>2+</sup> sensitivity of tension development (see Introduction). If, in fact, H<sup>+</sup>

ions depress maximum force by decreasing the number of cross-bridge attachments, this could also account for the reduced Ca<sup>2+</sup> sensitivity of tension development at low pH. There is evidence that the presence of both rigor (Bremel and Weber, 1972) and cycling (Gordon and Ridgway, 1987; Guth et al., 1987) cross-bridges increases the affinity of thin filament binding sites for Ca<sup>2+</sup>, which contributes to the apparent cooperativity of tension development (Moss et al., 1985; Moss et al., 1986). Thus, the decreased Ca<sup>2+</sup> sensitivity of tension development at low pH could be a result of a decrease in the cooperative activation of the thin filament due to fewer attached cross-bridges.

While it is evident that there is no single cause of muscle fatigue (see Mainwood and Renaud, 1985; Metzger and Fitts, 1987b; Metzger and Moss, 1987), our results indicate that under conditions in which intracellular pH falls to low levels, such as occur in living fibers during intense contractile activity, H<sup>+</sup> ions may account for as much as 40% of the decrease in contractile force. Also, the effect of H<sup>+</sup> ions to depress maximum force is greater in fibers containing fast isoforms of contractile and regulatory proteins compared with those with slow isoforms. Since the isoform-dependent variation in the effect of H<sup>+</sup> ions to depress tension was similar in controls and in troponin-extracted fibers, it is probable that the mechanism for this difference between fiber types is related to the isoform of myosin present rather than the regulatory proteins.

The authors gratefully acknowledge the contributions of Dr. James Graham, Mr. Gary Giulian and Dr. Marion Greaser to this study, and thank Ms. Susan Krey for preparation of the manuscript.

This work was supported by a National Institutes of Health grant (HL25861) to R.L. Moss and a National Institutes of Health post-doctoral fellowship (AR07811) to J.M. Metzger.

Received for publication 15 April 1988 and in final form 22 August 1988.

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