

Increased Excitability of Acidified Skeletal Muscle: Role of Chloride Conductance

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ABSTRACT Generation of the action potentials (AP) necessary to activate skeletal muscle fibers requires that inward membrane currents exceed outward currents and thereby depolarize the fibers to the voltage threshold for AP generation. Excitability therefore depends on both excitatory Na^+ currents and inhibitory K^+ and Cl^- currents. During intensive exercise, active muscle loses K^+ and extracellular K^+ ($[\text{K}^+]_o$) increases. Since high $[\text{K}^+]_o$ leads to depolarization and ensuing inactivation of voltage-gated Na^+ channels and loss of excitability in isolated muscles, exercise-induced loss of K^+ is likely to reduce muscle excitability and thereby contribute to muscle fatigue in vivo. Intensive exercise, however, also leads to muscle acidification, which recently was shown to recover excitability in isolated K^+ -depressed muscles of the rat. Here we show that in rat soleus muscles at 11 mM K^+ , the almost complete recovery of compound action potentials and force with muscle acidification (CO_2 changed from 5 to 24%) was associated with reduced chloride conductance (1731 ± 151 to $938 \pm 64 \mu\text{S}/\text{cm}^2$, $P < 0.01$) but not with changes in potassium conductance (405 ± 20 to $455 \pm 30 \mu\text{S}/\text{cm}^2$, $P < 0.16$). Furthermore, acidification reduced the rheobase current by 26% at 4 mM K^+ and increased the number of excitable fibers at elevated $[\text{K}^+]_o$. At 11 mM K^+ and normal pH, a recovery of excitability and force similar to the observations with muscle acidification could be induced by reducing extracellular Cl^- or by blocking the major muscle Cl^- channel, ClC-1 , with 30 μM 9-AC. It is concluded that recovery of excitability in K^+ -depressed muscles induced by muscle acidification is related to reduction in the inhibitory Cl^- currents, possibly through inhibition of ClC-1 channels, and acidosis thereby reduces the Na^+ current needed to generate and propagate an AP. Thus short term regulation of Cl^- channels is important for maintenance of excitability in working muscle.

KEY WORDS: lactic acid • muscle fatigue • action potentials • Na^+ channels • Cl^- channels

INTRODUCTION

The activation of a mammalian skeletal muscle fiber requires that action potentials can be generated at the neuromuscular junction and subsequently propagate along the surface membrane and deep into the muscle fiber along the t-tubular system (t-system). To initiate an action potential, the inward membrane current must be sufficient to bring the membrane potential (V_m) above the voltage threshold for action potential generation. Because the change in membrane potential in response to excitatory currents depends on the passive membrane properties and since the chloride conductance (G_{Cl^-}) accounts for around 80% of the total membrane conductance at rest, action potential generation and propagation strongly depend on the balance between the excitatory Na^+ currents and the inhibitory or shunting Cl^- currents. The importance of this balance for muscle excitability is perhaps best illustrated by the dramatic hyperexcitability observed in myotonia congenita, a disease that is caused by a mutation in the gene coding for the major muscle Cl^- channel ClC-1 (Koch et al., 1992). Moreover, myotonia congenita is often treated with Na^+ channel blockers (Lehmann-

Horn and Jurkat-Rott, 1999), again indicating that the initiation of action potentials in skeletal muscles depend on the interplay between excitatory Na^+ currents and shunting Cl^- currents.

During exercise, active muscle lose K^+ and extracellular K^+ ($[\text{K}^+]_o$) has been reported to increase from around 4 to 10 mM in plasma, and even higher levels of $[\text{K}^+]_o$ have been reported in the interstitium (Sreter, 1962; Hnik et al., 1976; Hermansen et al., 1984; Hallen et al., 1994; Green et al., 2000; Juel et al., 2000; Sejersted and Sjøgaard, 2000). Exposure of isolated rat muscles to elevated $[\text{K}^+]_o$ corresponding to the levels measured in vivo starts a chain of events that primarily affects the excitatory Na^+ currents. At elevated $[\text{K}^+]_o$, fibers become depolarized, Na^+ channels become slow inactivated (Ruff, 1996), the amplitude of single action potentials becomes reduced (Rich and Pinter, 2001), and the amplitude and integrated area of compound action potentials (M-waves) decrease. Based on such findings, elevated $[\text{K}^+]_o$ leading to reduced muscle excitability through loss of excitatory Na^+ currents is believed to be a component in muscle fatigue in working muscle (Sejersted and Sjøgaard, 2000).

Abbreviations used in this paper: 9-AC, 9-anthracene-carboxylic acid; t-system, t-tubular system.

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Intensive exercise, however, is usually associated with an intracellular accumulation of lactic acid and consequently a drop in muscle pH. Interestingly, Lehmann-Horn et al. (1987) have shown that the contractile force of isolated fiber bundles from intercostal muscles of a patient suffering from hyperkalaemic periodic paralysis show partial recovery from depression caused by increased $[K^+]_o$ when exposed to lowered pH. Likewise, in studies on isolated rat muscles depressed by increased $[K^+]_o$, it was found that within the physiological range, reductions in muscle pH produce a substantial recovery of excitability and force (Nielsen et al., 2001). Later, a study on mechanically skinned fibers of the rat showed a similar enhancement of t-system excitability in depolarized fibers with intracellular acidosis (Pedersen et al., 2004). Importantly, this latter study showed that the effect of acidosis was caused by a down-regulation of t-tubular Cl^- permeability. These findings suggest that the effect of pH on excitability of skeletal muscle is related to a change in G_{Cl^-} . This notion tallies with previous studies on both amphibian and mammalian skeletal muscle tissue, which indicate that reduced muscle pH can lower G_{Cl^-} (Hutter and Warner, 1967; Palade and Barchi, 1977). Based on these observations and the importance of G_{Cl^-} in action potential generation and propagation, we hypothesize that the increase in excitability in K^+ -depressed muscles induced by muscle acidification is caused by a reduction in G_{Cl^-} . To evaluate this hypothesis, the aim of the present study was to examine the effects of muscle pH on G_{Cl^-} and, further, to examine whether the observed changes in G_{Cl^-} can explain the recovery of excitability and force observed when pH is lowered in muscles depressed by elevated $[K^+]_o$ (Nielsen et al., 2001). Part of the results has been presented in a preliminary form (Pedersen, 2004).

MATERIALS AND METHODS

Animal Handling and Muscle Preparation

All experiments were performed using soleus muscles from Wistar rats. If not otherwise indicated, tetanic force production and compound action potentials (M-waves) were measured in muscles from 4-wk-old male or female animals (60–70 g). In addition, a few measurements of contractile force were done on muscles from adult female animals (3–5 mo, 230–300 g). Measurements of cable parameters and action potentials were done on muscles from adult female animals. Animals were fed ad libitum and were living under 12 h light/dark conditions at a thermostated temperature of 21°C. Rats were killed by cervical dislocation followed by decapitation, and intact soleus muscles were dissected out. In experiments where muscles were stimulated via the nerve, muscles were dissected out with ~10 mm of intact nerve attached. All handling of animals was in accordance with Danish Animal welfare regulations.

Muscles were incubated in standard Krebs-Ringer bicarbonate buffer containing (in mM) 122 NaCl, 25 $NaHCO_3$, 2.8 KCl, 1.2 KH_2PO_4 , 1.2 $MgSO_4$, 1.3 $CaCl_2$, and 5.0 D-glucose. Total buffer $[Cl^-]$ was 127.4 mM. In Cl^- -free buffer, methanesulphonate salts

replaced NaCl and KCl, and $Ca(NO_3)_2$ replaced $CaCl_2$. In buffers with reduced $[Cl^-]$, NaCl was partly replaced by sodium methanesulphonate. In buffers with high K^+ , KCl replaced part of the NaCl whereby $[Cl^-]$, osmolarity, and ionic strength were kept constant. The pH of all standard buffers equilibrated with 5% CO_2 in O_2 was 7.4 (referred to as normal pH). To acidify muscles, buffers were equilibrated with 24% CO_2 in O_2 (low pH). In a previous study, we showed that when CO_2 was increased to 23%, the pH of the standard Krebs-Ringer buffer decreased by 0.56 units and produced a maintained reduction in the intracellular pH of the muscles of 0.39 units (Nielsen et al., 2001). All experiments were carried at 30°C.

Isometric Force and Compound Action Potentials (M-waves)

Muscles were stimulated to contract either by field stimulation or by stimulating the nerve using a suction electrode. In experiments using field stimulation, muscles were mounted on force transducers at optimal length and stimulated by applying an electrical field across the central part of the muscle through platinum electrodes. Tetani were elicited every 10 min using 2-s trains of 0.2-ms pulses at 30 Hz. An electrical field of 24 V/cm was used, which was supramaximal stimulation for muscles incubated in standard Krebs-Ringer buffer at both pH 7.4 and at 6.8. In some experiments with 4-wk-old animals, force and M-waves were recorded simultaneously. In these experiments, tetanic contractions were evoked via the nerve using 5 mA constant current pulses with 0.15-ms duration delivered at 30 Hz. M-waves were recorded from a circular silver electrode with a recording area of 0.79 mm² (Overgaard and Nielsen, 2001).

Intact muscles were used in experiments with 4-wk-old animals. To minimize problems with diffusion of oxygen and other compounds between the core of the muscles and the buffer, muscles from adult animals used for experiments involving contractions were split in two equal halves from tendon to tendon with only one of them being used for experiments. In pilot experiments, intact muscles from adult animals showed the same contractile properties and the same loss of force at elevated $[K^+]_o$ as did the split muscles but the time to reach a steady force at elevated $[K^+]_o$ was considerably longer for the whole muscles.

Recordings of Cable Parameters and Single Action Potentials

Recordings of cable parameters and action potentials were done using a two-electrode constant current technique. Both electrodes were connected to an Axoclamp-2a amplifier. One electrode was used for passing current into the cells (current electrode), the other for measuring the intracellular potential (voltage electrode). Resistances of microelectrodes were between 5 and 20 M Ω . The current electrode was filled with 2 M K-citrate and the voltage electrode was filled with 3 M KCl. The membrane potential recorded by the microelectrodes and the current pulses injected in the fiber were displayed on an oscilloscope and recorded by a computer. Pilot experiments showed that when two electrodes were placed in the same fiber in muscles from 4-wk-old animals, the fiber depolarized rapidly, and the use of large holding currents would be necessary for maintaining a constant resting membrane potential. To avoid this problem, all experiments in which fibers were penetrated with two electrodes were done on muscles from adult animals.

The cable parameters were measured by injecting hyperpolarizing constant current pulses of 75 ms duration. The steady displacement of the membrane potential (ΔV_m) during a constant current pulse (I) was recorded at three to five locations in each fiber. For each fiber investigated, the three to five $\Delta V_m/I$ ratios were plotted on a log scale against the inter-electrode distance (x) on a linear scale and fitted to a two parameter exponentially

decaying function ($y(x) = y_0 e^{-bx}$), giving a straight line (see Fig. 2). From fibers that showed an accurate fit ($r^2 \geq 0.99$), the ordinate intercept (y_0) was taken as the input resistance (R_{in}), and the length constant (λ) was calculated from the slope of the fitted line ($b = 1/\lambda$). Assuming an internal resistivity (R_i) of 180 Ωcm (Albuquerque and Thesleff, 1968), R_{in} and λ were used to calculate the membrane conductance in accordance with the methods of Boyd and Martin (1959) based on the theory derived by Hodgkin and Rushton (1946). The values of the longitudinal resistance of the fiber (r_i) and the membrane resistance (r_m) were calculated as $r_i = 2R_{in}/\lambda$ and $r_m = 2R_{in}\lambda$. Fiber diameter (d) was calculated as $d = (4R_i/(\pi r_i))^{1/2}$ and specific membrane resistance (R_m) as $R_m = \pi d r_m$. Membrane conductance (G_m) was then calculated as $G_m = 1/R_m$. The component conductance of K^+ (G_{K^+}) was taken as the membrane conductance in Cl^- -free buffer, disregarding the contribution of other ion conductances. G_{Cl^-} was calculated by subtracting G_{K^+} from G_m in Cl^- -containing buffer. All fibers in which the resting potential depolarized more than 7 mV, while the two electrodes were inserted in the fiber, were disregarded. Likewise, fibers with a resting membrane potential more depolarized than -60 mV at a $[K^+]_o$ of 4 mM or more than -50 mV at a $[K^+]_o$ of 11 mM were disregarded.

For the measurement of action potentials, the current and the recording electrode were placed 0.3 mm apart in the same fiber, and depolarizing constant current pulses were injected through the current electrode while the response of the membrane was recorded by the voltage electrode. Pilot experiments showed that in muscles at 4 mM K^+ , action potentials always occurred within the first 25 ms of the current pulses, so to obtain the highest possible sampling frequency for data acquisition (81 KHz) the pulse duration was set to 25 ms. To determine the rheobase current in muscles at 4 mM K^+ , single depolarizing current pulses with increasing strength (steps of 5 nA starting at 10 nA) were injected until an action potential was elicited. If fibers did not produce an action potential in response to a 100 nA current, they were considered to be unexcitable. In the excitable fibers, the rate of rise of the action potential was calculated from the slope of the action potential upstroke between 10 and 90% of the action potential peak.

Intracellular Water Content

For determination of intracellular water content, muscles from adult rats at 11 mM K^+ were equilibrated for 100 min in buffer containing ^{14}C -sucrose (0.1 $\mu\text{Ci}/\text{ml}$ and 1 mM nonlabeled sucrose). After incubation, the tendons were cut off and the muscles were blotted, weighed for determination of wet weight, and subsequently dried overnight at 60°C. After determination of dry weight, the muscles were soaked for 20 h in 0.12 M trichloroacetic acid (TCA), and the content of ^{14}C -sucrose in the supernatant was determined by scintillation counting. Intracellular water content, expressed per gram dry weight, was calculated from the total water content by subtracting the distribution volume of ^{14}C -sucrose.

Chemicals and Isotope

All chemicals were of analytical grade. 9-Anthracene-carboxylic acid (9-AC) and methanesulphonate salts were from Sigma-Aldrich and ^{14}C -sucrose was from Amersham Biosciences.

Statistics

All data are expressed as means \pm SEM. The statistical significance of any difference between groups was ascertained using Student's two-tailed t test for nonpaired observations between two groups and one-way Anova between more than two groups.

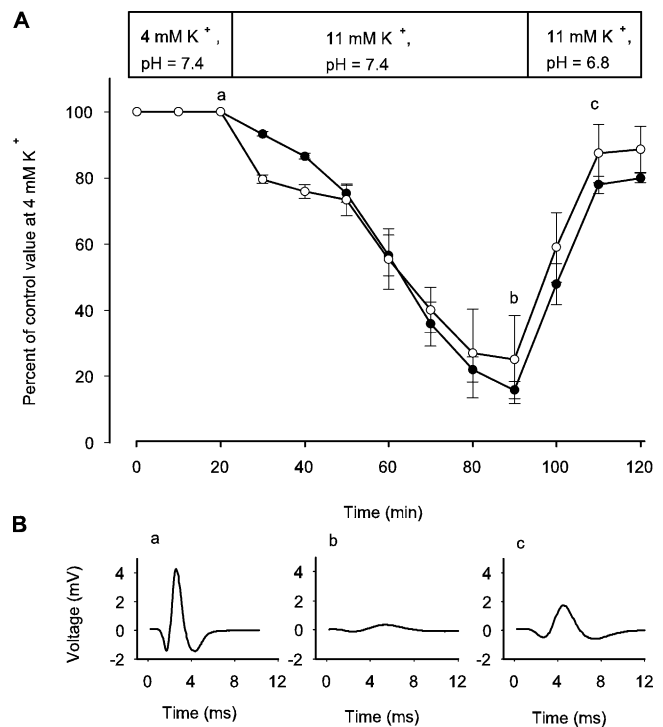


FIGURE 1. Effect of high $[K^+]_o$ and muscle acidification on M-wave area and tetanic force in muscles from 4-wk-old animals. Muscles were incubated in Krebs-Ringer buffer equilibrated with 5% CO_2 in O_2 . After determination of control values at 4 mM K^+ , $[K^+]_o$ was increased to 11 mM, and after 70 min the muscles were acidified by increasing CO_2 to 24%, as indicated in A. Muscle contractions were elicited every 10 min via stimulation of the motor nerve with a 1.5-s train of 30-Hz pulses. (A) Time course of M-wave area (\circ) and tetanic force (\bullet). Data show means \pm SEM from three muscles with 100% force corresponding to 0.32 ± 0.01 N. (B) Representative M-wave recordings from a single muscle obtained at the time points indicated by a, b, and c in A.

RESULTS

Acidification Recovers M-waves and Force Production in Muscles at Elevated $[K^+]_o$

Fig. 1 shows the effect of elevated extracellular K^+ concentration and acidification on M-waves and force in isolated muscles from 4-wk-old animals. At normal pH (5% CO_2), an increase in $[K^+]_o$ from 4 to 11 mM caused a 85% reduction in tetanic force, which was closely associated with a comparable reduction in M-wave area, indicating that the loss of force was caused by a loss of excitability. Fig. 1 A also shows that when muscle acidosis was induced by increasing CO_2 to 24%, an almost complete recovery of both M-wave area and tetanic force took place. Fig. 1 B shows representative M-wave recordings from one of the muscles included in Fig. 1, and illustrates the effect of elevated $[K^+]_o$ and the subsequent reduction in muscle pH on the shape of the M-wave trace. In the experiments depicted in Fig. 1, muscle excitation took place via the nerve, but

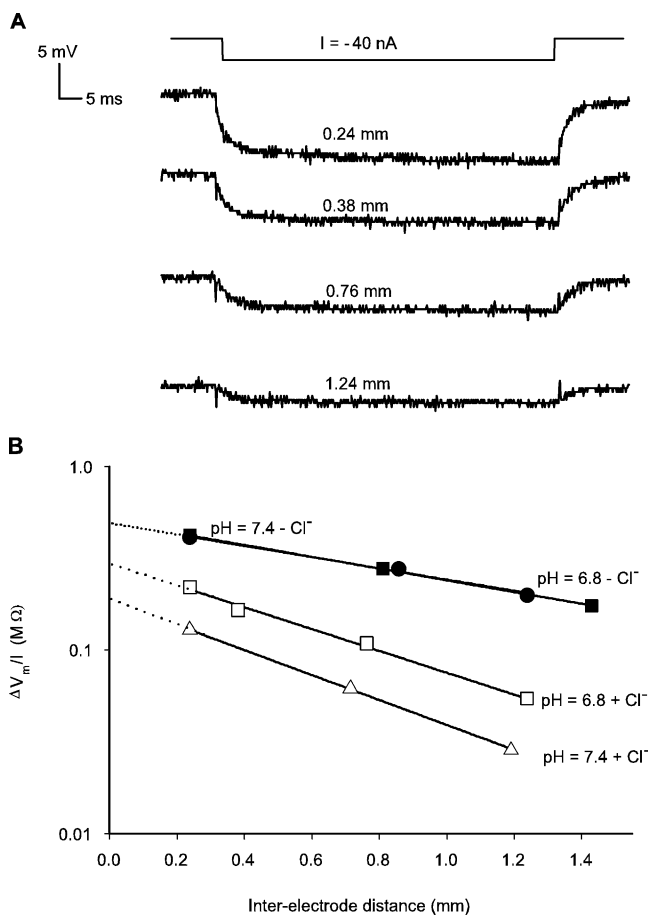


FIGURE 2. Effect of acidosis on membrane properties determined at 11 mM K^+ in muscle fibers from adult rats using two intracellular microelectrodes inserted into the same fiber. One electrode was used to pass hyperpolarizing constant current pulses, and the other electrode (voltage electrode) was used to measure the membrane potential responses at three to five different locations along the fiber. (A) Response of the membrane potential to injection of hyperpolarizing constant current pulses (-40 nA) recorded by the voltage electrode at four different inter-electrode distances (0.24, 0.38, 0.76, 1.24 mm) in a fiber from a muscle placed in Cl^- -containing solution at pH 6.8. Vertical and horizontal calibration bars show membrane potential and time, respectively. (B) Representative plots of the ratios between the change in steady-state membrane potential and the amount of current injected as a function of the inter-electrode distance in a single fiber from each of the four groups of fibers at 11 mM K^+ and pH 7.4 + Cl^- (Δ), pH 6.8 + Cl^- (\square), pH 7.4 - Cl^- (\bullet), pH 6.8 - Cl^- (\blacksquare), respectively. For group average, see Table I.

similar effects of increased CO_2 were seen in tubocurarine-treated muscles stimulated directly by use of field stimulation (unpublished data). This demonstrates that the effect of acidosis was related to changes distal to the neuromuscular junction. Other experiments showed that in muscles maintained at 4 mM K^+ throughout the experiment, an increase in CO_2 to 24% only had a minor effect, giving rise to a temporary $4 \pm 1\%$ increase in tetanic force ($n = 4$).

Acidification of Muscles Reduces Resting Cl^- Conductance

To determine whether the effect of acidosis on muscle excitability at 11 mM K^+ (Fig. 1) could be related to a change in G_{Cl^-} , cable parameters from four groups of muscles at 11 mM K^+ (pH 7.4 or 6.8, and with or without Cl^- in the buffer) were measured. Penetration of fibers from 4-wk-old animals with two electrodes at the same time led, however, to large depolarizations, making the use of large holding currents necessary to maintain a constant resting potential. To avoid this problem, the examination of cable parameters was done in larger muscles from adult rats. Fig. 2 A shows representative membrane potential responses to a hyperpolarizing current of -40 nA recorded at four different locations in a fiber incubated at pH 6.8 in Cl^- -containing buffer. From these recordings, the $\Delta V_m/I$ ratios were calculated and related to the inter-electrode distance as illustrated in Fig. 2 B, which shows measurements from single fibers from each of the four groups of muscles reflecting each group average. In Cl^- -containing buffer, low muscle pH led to larger $\Delta V_m/I$ ratios than normal pH, indicating that the membrane conductance had been reduced by acidification. In marked contrast, fibers in Cl^- -free buffer appeared unaffected by acidification, indicating that the effect of pH on membrane conductance only was related to a change in G_{Cl^-} . To investigate the effect of acidosis on the component conductance of K^+ and Cl^- in more qualitative terms, λ , R_{in} , and G_m were determined in each of the four groups in accordance with the methods of Boyd and Martin (1959). Table I shows the calculated values of λ , R_{in} , and G_m for fibers at 4 mM K^+ (normal pH with Cl^-) and from the four groups of fibers at 11 mM K^+ . When pH was reduced from 7.4 to 6.8 in muscles incubated in Cl^- -containing buffer at 11 mM K^+ , λ increased significantly by 18% ($P < 0.01$) and R_{in} by 40% ($P < 0.01$). Consequently, muscles incubated in Cl^- -containing buffer showed a reduction in membrane conductance from $2136 \pm 150 \mu S/cm^2$ at pH 7.4 to $1393 \pm 56 \mu S/cm^2$ at pH 6.8 ($P < 0.01$). In muscles incubated in Cl^- -free buffer, there were no significant changes in λ or R_{in} with acidification, which was also reflected by a small and insignificant change in G_K^+ from $405 \pm 20 \mu S/cm^2$ at normal pH to $455 \pm 30 \mu S/cm^2$ at low pH ($P < 0.16$). Based on these results, acidification from 7.4 to 6.8 by increasing CO_2 from 5 to 24% caused a reduction of G_{Cl^-} from $1731 \pm 151 \mu S/cm^2$ at normal pH to $938 \pm 64 \mu S/cm^2$ at low pH corresponding to a 46% decrease ($P < 0.01$).

To test whether the measurements of G_{Cl^-} were influenced by an effect of acidosis on the diameter of the muscle fibers, the intracellular water content was measured in each of the four groups of muscles from adult animals at 11 mM K^+ and compared with the average fiber diameter calculated from measurements of cable parameters. Since the calculated diameter did not dif-

T A B L E I

Effect of pH on Electrical Properties of Muscle Fibers from Adult Rats at 4 and 11 mM K⁺

[K ⁺] _o	[Cl ⁻] _o	pH	<i>n</i>	V _m	λ	R _{in}	Diameter	G _m	G _{K⁺}	G _{Cl⁻}
<i>mM</i>	<i>mM</i>			<i>mV</i>	<i>mm</i>	<i>MΩ</i>	<i>μm</i>	<i>μS/cm²</i>	<i>μS/cm²</i>	<i>μS/cm²</i>
4	127.4	7.4	10/19	-69 ± 1	0.89 ± 0.03	0.35 ± 0.02	55 ± 2	1017 ± 58		
11	127.4	7.4	10/23	-52 ± 1	0.65 ± 0.02	0.20 ± 0.01	62 ± 2	2136 ± 150		1731 ± 151
11	0	7.4	9/28	-55 ± 1	1.45 ± 0.06	0.52 ± 0.02	57 ± 2	405 ± 20	405 ± 20	
11	127.4	6.8	7/29	-52 ± 1	0.77 ± 0.03	0.28 ± 0.01	57 ± 2	1393 ± 56		938 ± 64
11	0	6.8	8/26	-53 ± 1	1.37 ± 0.07	0.51 ± 0.02	56 ± 2	455 ± 30	455 ± 30	

Hyperpolarizing current pulses of 75 ms duration were injected through the current electrode, and the voltage electrode recorded the membrane responses at three to five locations in each fiber. $\Delta V_m/I$ ratios were plotted on a log scale against inter-electrode distance and fitted to a two-parameter exponentially decaying function, giving a straight line. The slope of the line was used to calculate the length constant, λ , and the ordinate intercept gave the input resistance, R_{in} (Fig. 2). Conductance was calculated from λ and R_{in} according to Boyd and Martin (1959) using an assumed internal resistivity (R_i) of 180 Ωcm . n gives muscles/fibers used in each group. Values are means \pm SEM.

fer significantly between the groups (Table I), the intracellular water content would be expected to be the same in all groups. In muscles incubated in Cl⁻-containing buffer at a pH of 7.4, the intracellular water content was 2.75 ± 0.05 g H₂O/g dry weight, which is consistent with previously published values for adult rats (Cieslar et al., 1998). Neither incubation in Cl⁻-containing buffer at pH 6.8 or in Cl⁻-free buffer at pH 7.4 or 6.8 caused any change in the intracellular water content of the muscles, the contents being 2.78 ± 0.02 , 2.72 ± 0.01 , and 2.79 ± 0.02 g H₂O/g dry weight, respectively ($P < 0.46$, $n = 3$ in all groups).

Reduction in G_{Cl⁻} Recovers Excitability and Force Production in Muscles at Elevated [K⁺]_o

To test if the improved excitability and force production that was induced by increasing CO₂ to 24% in muscles from 4-wk-old animals at elevated [K⁺]_o (Fig. 1) could be explained by a reduction in G_{Cl⁻}, as observed in muscles from adult animals (Table I), we examined the effect of reducing G_{Cl⁻} in muscles from young rats by lowering [Cl⁻]_o to 80 mM while keeping pH at 7.4. Measurements of membrane conductance showed that lowering [Cl⁻] in the buffer from the normal 127.4 mM to 80 mM reduced G_{Cl⁻} of muscles from adult rats at 11 mM K⁺ to 761 ± 102 $\mu\text{S}/\text{cm}^2$ ($n = 15$), which was similar to the reduction seen when these muscles were acidified with 24% CO₂ (Table I, $P < 0.1$). Fig. 3 A shows that in addition to the reduction in G_{Cl⁻}, the lowering of [Cl⁻]_o to 80 mM led to a recovery of M-wave area and force production of similar magnitude as the recoveries induced by increasing CO₂ to 24%. In accordance with the data shown in Table I, the reduction in [Cl⁻]_o had no effect on the membrane potential, the potentials being -55 ± 1 mV at both 127.4 mM Cl⁻ and 80 mM Cl⁻. Fig. 3 A further shows that recovery of excitability and force in muscles at 11 mM K⁺ also could be induced by addition of 100 μM of the specific muscle Cl⁻ channel (ClC-1) inhibitor 9-AC.

To ensure that these effects of reduced [Cl⁻]_o and 9-AC were not caused by effects on the motor nerve, a series of experiments was performed in which force was measured in muscles from 4-wk-old animals at 11 mM K⁺ that were stimulated directly with field stimulation. Fig. 3 B illustrates that also in these experiments, a substantial recovery of force could be induced by reducing buffer [Cl⁻] to 80 or even 0 mM, or by adding 100 μM 9-AC. Moreover, adding 30 μM 9-AC, which in rat diaphragm has been shown to reduce G_{Cl⁻} to a similar degree as when CO₂ was increased to 24% in the present study (Palade and Barchi, 1977), led to a recovery of force that was similar to the recovery of force induced by increasing CO₂ to 24%. Other experiments showed that similar results were obtained in muscles pretreated with 10⁻⁵ tubocurarine (unpublished data).

Acidosis Decreases Rheobase Current and Increases the Number of Excitable Fibers in Muscles at Elevated [K⁺]_o

The recovery of M-waves induced by acidification in muscles at elevated [K⁺]_o (Figs. 1 and 3) strongly suggests that acidification improves muscle excitability. To further investigate the effects of acidification on the excitability of muscles at normal and elevated [K⁺]_o, intracellular recordings of action potentials were done in muscles at normal pH (5% CO₂) and in acidified muscles (24% CO₂). In these experiments, action potentials were elicited in single fibers by injecting depolarizing constant current pulses of 25 ms duration and recording the membrane response by another intracellular electrode. Since this method required the penetration of fibers with two electrodes, the experiments were done on muscles from adult rats. Table II shows that in muscles at 4 mM K⁺, acidification produced a significant reduction in the rheobase current of 26%, corresponding to a 15 nA decrease. When muscles were examined at 11 mM K⁺ (as used in Table I and Figs. 1 and 3), the examination of rheobase current and action potentials was encumbered by a complete loss of excitabil-

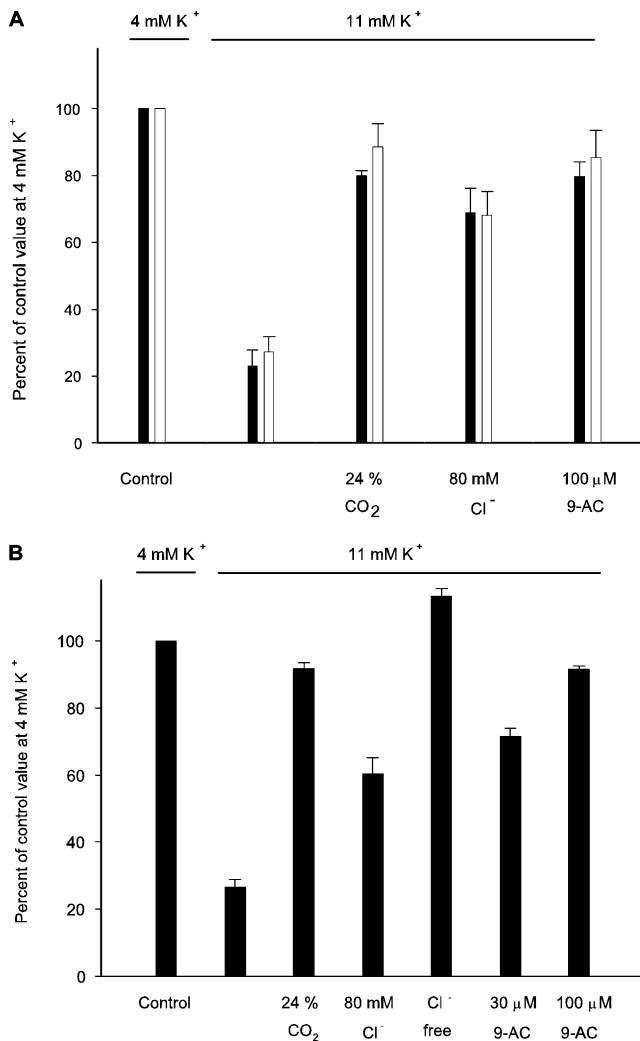


FIGURE 3. Effect of motor nerve stimulation and field stimulation on recovery of M-wave area and force at 11 mM K^+ in muscles from young rats by various ways of reducing G_{Cl^-} . The figures show steady-state values of force and M-wave area from experiments with time courses similar to the experiment in Fig. 1. (A) Force (solid bars) and M-wave area (open bars) under control conditions at 4 mM K^+ and normal pH, 11 mM K^+ and normal pH, and at 11 mM K^+ with reduced G_{Cl^-} . At 11 mM K^+ , G_{Cl^-} was reduced by muscle acidification, reduction in $[Cl^-]_o$ from 127 to 80 mM, or addition of 100 μ M 9-AC. Contractions were elicited via stimulation of the motor nerve. (B) Force responses obtained by field stimulation under control conditions at 4 mM K^+ and normal pH, 11 mM K^+ and normal pH, and at 11 mM K^+ with reduced G_{Cl^-} . At 11 mM K^+ , G_{Cl^-} was reduced by muscle acidification, Cl^- substitution, or addition of 9-AC. Data show means + SEM from 4–36 muscles.

ity in most of the fibers, as judged from their inability to respond to a 100 nA current injection with an action potential. For that reason, $[K^+]_o$ was only increased to 9 mM, and all fibers were excited by injection of a single 100-nA depolarizing constant current pulse. Fig. 4 A shows that when the muscles were incubated at normal pH, this procedure only elicited action potentials in 55% of the examined fibers ($n = 40$). In muscles at low

TABLE II

Effect of Muscle pH on Action Potential Parameters in Muscle Fibers from Adult Rats at 4 and 9 mM K^+

$[K^+]_o$	pH	n	Rheobase	Rate of rise	Peak potential
			nA	V/s	mV
4	7.4	4/38	57 ± 2	142 ± 9	6 ± 2
4	6.8	4/36	42 ± 2^b	178 ± 9^b	14 ± 1^b
9	7.4	4/22	–	39 ± 5	-12 ± 2
9	6.8	4/38	–	56 ± 5^a	-5 ± 1^b

To measure action potentials, the current and the voltage electrode were placed 0.3 mm apart in the same fiber, and by injecting depolarizing constant current pulses through the current electrode, action potentials were elicited and recorded by the voltage electrode. pH was lowered from 7.4 to 6.8 by increasing CO_2 from 5 to 24%. To determine the rheobase current in muscles at 4 mM K^+ , 25-ms pulses with increasing strength (steps of 5 nA starting at 10 nA) were injected until an action potential was elicited. In muscles at 9 mM K^+ , only a single depolarizing pulse of 100 nA was injected. In the excitable fibers (see text), the rate of rise of the action potential was calculated from the slope of the action potential upstroke between 10 and 90% of the action potential amplitude. n gives muscles/fibers used in each group. Values are means \pm SEM.

^a $P < 0.05$ (significantly different from corresponding value at the same $[K^+]_o$ and normal pH).

^b $P < 0.01$ (significantly different from corresponding value at the same $[K^+]_o$ and normal pH).

pH, however, the same stimulation elicited an action potential in 95% of the tested fibers ($n = 40$). This indicates that upon acidification, an additional 40% of the fibers responded with an action potential to the 100-nA stimulus. Examining the action potentials of those fibers that were excitable under the different conditions showed that acidification also had effects on action potential shape. Fig. 4 shows representative action potentials from fibers at 4 mM K^+ and normal pH (Fig. 4 B), at 9 mM K^+ and normal pH (Fig. 4 C), and at 9 mM K^+ and low pH (Fig. 4 D). Clearly, elevated $[K^+]_o$ caused a depression of the action potential, which was partly recovered by acidification. Table II shows that the potentiation of the action potentials by low pH was present both in muscles at 4 and 9 mM K^+ , producing a 25 and 43% increase in the rate of rise of the action potential and a 8 and 7 mV increase in the action potential peak in fibers at 4 and 9 mM K^+ , respectively.

Muscles from Adult Rats Have Less Tolerance to Elevated $[K^+]_o$ than Muscles from 4-wk-old Rats

Figs. 1 and 3 show that when muscles from 4-wk-old rats were exposed to 11 mM K^+ , force production was reduced to between 20 and 30% of control force. It was surprising, therefore, that the exposure of muscles from adult rats to 11 mM K^+ led to almost complete loss of the ability of the muscles fibers to respond to a 100-nA current injection with an action potential, indicating an almost complete loss of excitability. Based on this, we examined the tolerance of muscles from adult

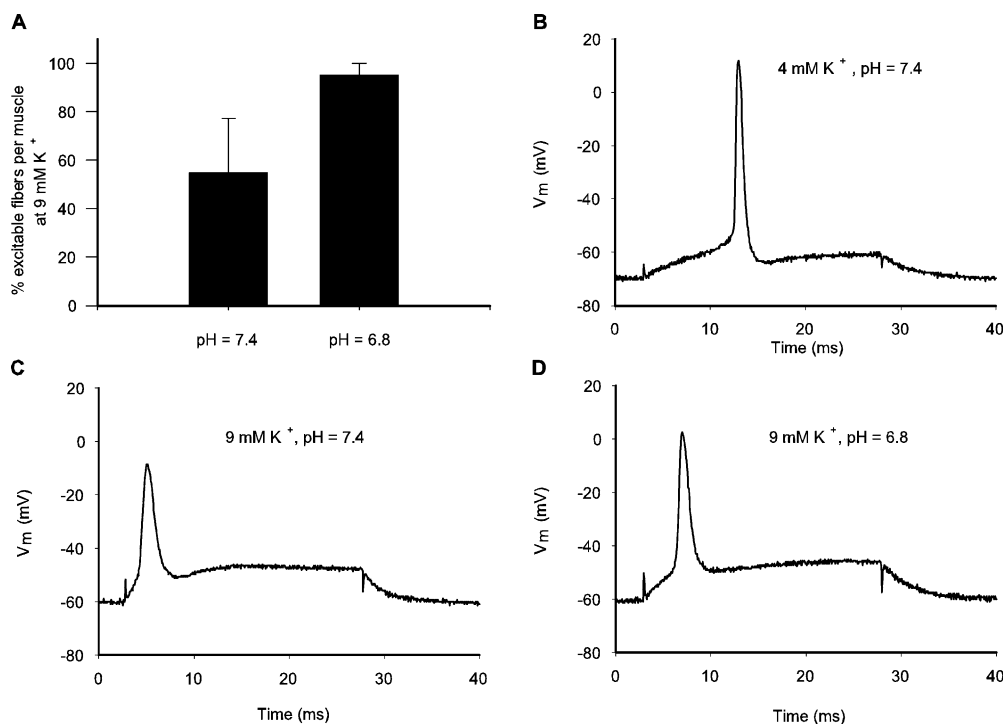


FIGURE 4. Effects of elevated $[K^+]_o$ and low pH on action potentials in muscles from adult rats. Data from the experiments are shown in Table II. (A) Effect of reduced pH on the number of fibers at 9 mM K^+ that responded with an action potential (excitable fibers) when injected with a 100-nA depolarizing constant current pulse of 25 ms duration. (B–D) Representative traces of membrane depolarization and action potentials in response to a depolarizing constant current pulse in fibers at 4 mM K^+ and normal pH (B), at 9 mM K^+ and normal pH (C), and at 9 mM K^+ and low pH (D). For other details, see legend to Table II.

rats to elevated $[K^+]_o$ by making three series of experiments essentially as illustrated in Fig. 1 but with muscles from adult rats stimulated to contract via field stimulation. In the three series of experiments, the muscles were exposed to 7, 9, or 11 mM K^+ at normal pH for 100 min to obtain a steady force response at the elevated $[K^+]_o$. Then CO_2 was increased to 24%, and the new steady force level was recorded. The summarized results are given in Fig. 5, which shows that when muscles at normal pH were exposed to a $[K^+]_o$ of 9 mM or more, it produced a large reduction in force. Comparing these results with the results illustrated in Fig. 1 and with previous results from 4-wk-old animals (Nielsen et al., 2001) clearly shows that muscles from adult animals have less tolerance to elevated $[K^+]_o$ than muscles from 4-wk-old animals. Thus, at normal pH, the force production of muscles from 4-wk-old animals incubated at 11 mM K^+ roughly corresponded to the force production of muscles from adult rats incubated at 9 mM K^+ .

Importantly, as for muscles from 4-wk-old rats, acidification caused a pronounced recovery of force in muscles from adult rats incubated at elevated $[K^+]_o$ (Fig. 5). This effect was largest at 9 mM K^+ , whereas only a minor effect was seen at 11 mM K^+ .

DISCUSSION

Effect of Acidosis on Muscle Excitability and Excitation-induced Force Production

Since exercise leads to accumulation of extracellular K^+ in active muscles, the decline in force in the isolated

muscles from 4-wk-old animals at high $[K^+]_o$ shown in Fig. 1 supports the traditional view that exercise-induced elevation of $[K^+]_o$ can contribute to muscle fatigue in vivo. The observation that muscles from adult animals show less tolerance to elevated $[K^+]_o$ (Fig. 5) than muscles from 4-wk-old animals further supports this view by indicating that the depression of muscle function takes place at concentrations of extracellular

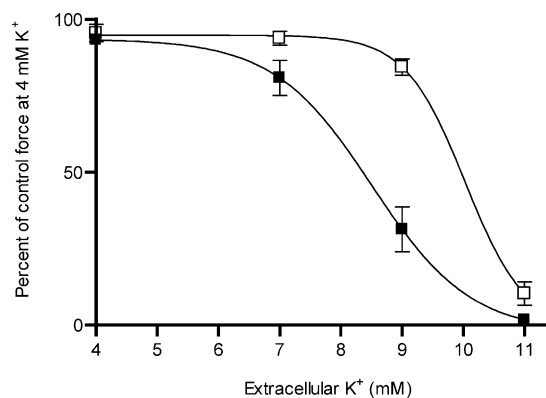


FIGURE 5. Effects of elevated extracellular K^+ and muscle acidification on steady tetanic force in split muscles from adult rats. The figure shows summarized results from experiments performed essentially like the experiments illustrated in Fig. 1 except that muscles were stimulated by field stimulation. When force at elevated $[K^+]_o$ had reached a steady level at normal pH (●), muscles were acidified by increasing the percentage of CO_2 in the gas from 5 to 24% (○). Data were fitted to a Boltzmann sigmoidal curve. Data show means \pm SEM from four muscles with 100% corresponding to 0.60 ± 0.03 N.

K^+ that is appreciably lower than what has been reported for $[K^+]_o$ during exercise in humans (for review see Sejersted and Sjøgaard, 2000). The force-depressing effect of elevated $[K^+]_o$ is most likely related to the depolarization of the muscle fibers, which has been shown to cause a loss of Na^+ current due to slow inactivation of Na^+ channels (Ruff, 1996), leading to a loss of the ability of the fibers to initiate and propagate action potentials (Cairns et al., 1995, 1997; Overgaard et al., 1999; Sejersted and Sjøgaard, 2000; Rich and Pinter, 2003). This reduction in excitability can be seen in Fig. 1, which in agreement with other studies (Overgaard et al., 1999; Overgaard and Nielsen, 2001) shows that the reduction in force at 11 mM K^+ was well correlated with a concomitant reduction in M-wave area. Interestingly, at 11 mM K^+ , the membrane potential of muscles from adult animals was the same as in muscles from 4-wk-old animals. Thus, the reduced tolerance to elevated $[K^+]_o$ in muscles from adult rats could not be related to a larger effect of increased $[K^+]_o$ on the membrane potential but must result from a reduced tolerance to depolarization per se.

During intensive exercise, the accumulation of extracellular K^+ occurs simultaneously with an accumulation of lactic acid and ensuing reduction in muscle pH (Fitts, 1994). Thus, to determine the effect of high $[K^+]_o$ on muscle function under circumstances that approach the conditions during exercise, muscles were acidified by elevated CO_2 pressure, which acidifies both the intra- and extracellular compartment of the muscles (Nielsen et al., 2001). Similar to previously reported effects of lactic acid (Nielsen et al., 2001), lowering pH by increasing the CO_2 tension clearly increased the tolerance of the muscles to elevated $[K^+]_o$. This effect was present in muscles from both 4-wk-old (Fig. 1) and adult animals (Fig. 5).

Judged from the increase in M-wave area (Fig. 1), the recovery of force induced by low pH was related to an increase in the number of fibers producing an action potential in response to electrical stimulation of the preparation. Since similar increase in the tolerance to elevated $[K^+]_o$ was found in curarized muscles activated by field stimulation, the mechanism for the protective effect of acidosis had to reside distally to the neuromuscular junction. Together this indicates that the increase in force production in muscle at elevated $[K^+]_o$ upon acidification is caused by an increase in the ability of the muscle fibers to initiate and propagate action potentials. This conclusion is supported by the finding that in muscles from adult rats that were incubated at 9 mM K^+ , the relative force (Fig. 5) and the proportion of fibers that produced an action potential in response to injection of a 100-nA current (Fig. 4) were closely related before and after acidification. Interestingly, in muscles from adult animals incubated at

4 mM K^+ , acidification reduced the rheobase current and caused a greater rate of rise and higher peak of the action potentials (Table II), indicating that acidification also causes an increase in the excitability of muscles incubated at normal $[K^+]_o$.

Effect of Acidosis on Membrane Conductance

Table I shows that within the physiological range, a reduction in pH, obtained by raising CO_2 from 5 to 24%, in muscles at 11 mM K^+ led to a large reduction in G_m . Since G_{Cl^-} is known to account for the largest fraction of the total membrane conductance in resting muscle fibers (Bretag, 1987), the reduction in G_m at low pH was most likely related to a reduced G_{Cl^-} . Indeed, low pH was without effect on G_m in muscles incubated in Cl^- -free buffer, showing that the effect of low pH on G_m was the result of a 46% reduction in G_{Cl^-} . This effect of pH is in accordance with other reports on the influence of acidosis on G_{Cl^-} in native tissue (Hutter and Warner, 1967; Palade and Barchi, 1977). The finding that low pH was without effect on G_m in muscles incubated in Cl^- -free buffer also indicates that reduced pH did not change G_{K^+} significantly. This conclusion is further supported by the finding that the reduction in pH had no effect on V_m . In muscles at 11 mM K^+ and normal pH, G_{Cl^-} accounted for around 81% of the total membrane conductance, which agrees convincingly with previous investigations conducted at 5 mM K^+ under otherwise similar conditions (Albuquerque and Thesleff, 1968; McArdle and Albuquerque, 1973). The large increase in total membrane conductance at 11 mM K^+ compared with 4 mM K^+ roughly agrees with the constant field theory (Hodgkin and Katz, 1949) and with the results of Kwiecinski et al. (1984).

The Role of Reduced G_{Cl^-} and $ClC-1$ Channels in the Protective Effect of Acidosis

The finding that acidification of muscles at 11 mM K^+ caused a reduction in G_{Cl^-} (Table I) suggests that the concomitant improvement in the ability of the muscle fibers to respond to electrical stimulation can be explained by a reduction in Cl^- currents, which changes the balance between excitatory Na^+ currents and the shunting Cl^- currents in favor of the excitatory currents. This role for G_{Cl^-} is supported by the observation that in K^+ -depressed muscles at normal pH, substantial recovery of excitability and force could be induced by reducing buffer Cl^- or by adding 9-AC. Importantly, in these experiments, large recoveries were seen even when 80 mM Cl^- or 30 μ M 9-AC were used to obtain graded reductions in G_{Cl^-} , thereby mimicking the reduction in G_{Cl^-} measured in acidified muscles. These results indicate that a reduction in G_{Cl^-} plays a major role in the recovery of muscle function induced by acidification at elevated $[K^+]_o$. At variance with this,

Cairns et al. (2004) recently reported a synergistic effect of increasing $[K^+]_o$ and decreasing $[Cl^-]_o$ on loss of force in soleus and extensor digitorum longus muscles from mice. This loss of force was probably due to the depolarization they observed when $[Cl^-]_o$ was lowered at elevated $[K^+]_o$, perhaps indicating some active transport mechanism of Cl^- at elevated $[K^+]_o$ not seen in our results or in human skeletal muscle where it has been shown that Cl^- is passively distributed over a range of $[K^+]_o$ from 1 to 7 mM (Kwiecinski et al., 1984). In accordance with our results, van Emst et al. (2004) recently reported a recovery of twitch force in K^+ -depressed rat soleus muscles upon reduction of extracellular Cl^- .

It should be noted that despite the tendency for G_{Cl^-} to be lower in muscles at 80 mM Cl^- and normal pH compared with acidified muscles (761 ± 102 vs. $938 \pm 64 \mu S/cm^2$), the recovery of excitability and force induced by 80 mM Cl^- was actually lower than the recovery induced by acidification (Fig. 3). One possible reason for this discrepancy could be that the cable parameters were measured in muscles from adult rats, whereas measurements of force and M-waves were done in muscles from young rats. Another possible explanation for the discrepancy could be that acidification, in contrast to reduced extracellular Cl^- , leads to an increased screening of fixed negative charges on the outer membrane surface, effectively making the surface potential of the membrane more positive and consequently elevating the electric field within the membrane. According to Woodhull (1973), such a screening effect of extracellular H^+ ions could shift the gating of inactivation of voltage-gated Na^+ channels in the depolarizing direction and thereby increase the sodium currents during an action potential in depolarized fibers. If so, such an effect of low extracellular pH would contribute to the recovery of excitability seen in K^+ -depressed muscles upon acidification. At variance with this, a reduction of extracellular pH has also been found to block voltage-gated Na^+ channels, which would counteract restoration of excitability upon muscle acidification (Hille, 1968; Woodhull, 1973; Kuzmenkin et al., 2002). In our results, however, this direct blocking effect of low extracellular pH must be minor since muscle acidification caused a pronounced increase in rate of rise of the action potentials and a reduced rheobase current in muscles at 4 mM K^+ , where a screening effect of low extracellular pH on membrane excitability would be of less significance compared with K^+ -depressed muscles. In contrast, intracellular acidification seems to have only minor effects on the steady-state activation and inactivation of voltage-gated Na^+ channels in skeletal muscles, (Kuzmenkin et al., 2002).

Since the weakly voltage-dependent ClC-1 channel is believed to account for the largest part of G_{Cl^-} in skele-

tal muscle (Jentsch et al., 2002), the above findings suggest the involvement of ClC-1 channels in the protective effect of acidosis. This is further indicated by the protective effect of 9-AC on excitability and force in K^+ -depressed muscles (Fig. 3). The activation of muscles requires a maintained excitability of both the sarcolemma and the t-tubular system. The measurements of M-waves and action potentials in the present study mainly reflect the function of the sarcolemma. Studies on skinned fibers (Coonan and Lamb, 1998; Pedersen et al., 2004), however, demonstrate that Cl^- channels play a similar role for the function of the t-tubular system, which is further supported by the close correlation between force and sarcolemma excitability in the present study. When ClC-1 channels are expressed in heterologous expression systems, however, the observed effects of acidosis on ClC-1 channels are unable to explain the reduced G_{Cl^-} at low pH observed in native tissue (Rychkov et al., 1996). The apparent discrepancy between these observations from native tissue and expression systems is not clear. Irrespective of this, the present study demonstrates that muscle excitability strongly depends on the balance between the number of Na^+ channels that can be activated and the prevailing G_{Cl^-} . Furthermore, it demonstrates that the mechanism underlying the protective effect of muscle acidification in K^+ -depressed muscles (Nielsen et al., 2001) is largely attributable to a down-regulation of G_{Cl^-} , possibly conveyed by an inhibition of ClC-1 channels. These results fully agree with the recent study of Pedersen et al. (2004) on mechanically skinned muscle fibers showing enhanced t-system excitability in depolarized fibers through a reduction in t-system Cl^- permeability. Together, these two studies underline the importance of Cl^- channel regulation for maintenance of excitability and contractile function in working muscle.

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