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RESEARCH ARTICLE

Recovery from acidosis is a robust trigger for loss of force in murine hypokalemic periodic paralysis

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Periodic paralysis is an ion channelopathy of skeletal muscle in which recurrent episodes of weakness or paralysis are caused by sustained depolarization of the resting potential and thus reduction of fiber excitability. Episodes are often triggered by environmental stresses, such as changes in extracellular K^+, cooling, or exercise. Rest after vigorous exercise is the most common trigger for weakness in periodic paralysis, but the mechanism is unknown. Here, we use knock-in mutant mouse models of hypokalemic periodic paralysis (HypoKPP; $Na_V1.4$-R669H or $Ca_V1.1$-R528H) and hyperkalemic periodic paralysis (HyperKPP; $Na_V1.4$-M1592V) to investigate whether the coupling between pH and susceptibility to loss of muscle force is a possible contributor to exercise-induced weakness. In both mouse models, acidosis (pH 6.7 in 25% CO_2) is mildly protective, but a return to pH 7.4 (5% CO_2) unexpectedly elicits a robust loss of force in HypoKPP but not HyperKPP muscle. Prolonged exposure to low pH (tens of minutes) is required to cause susceptibility to post-acidosis loss of force, and the force decrement can be prevented by maneuvers that impede Cl^- entry. Based on these data, we propose a mechanism for post-acidosis loss of force wherein the reduced Cl^- conductance in acidosis leads to a slow accumulation of myoplasmic Cl^-. A rapid recovery of both pH and Cl^- conductance, in the context of increased $[Cl]_{in}/[Cl]_{out}$, favors the anomalously depolarized state of the bistable resting potential in HypoKPP muscle, which reduces fiber excitability. This mechanism is consistent with the delayed onset of exercise-induced weakness that occurs with rest after vigorous activity.

Introduction

The familial periodic paralyses are rare inherited disorders of skeletal muscle in which mutations of ion channels ($Na_V1.4$, $Ca_V1.1$, or Kir2.1) result in susceptibility to recurrent episodes of weakness or even paralysis (Cannon, 2015). In all forms of periodic paralysis, the transient attacks of weakness are caused by sustained depolarization of the resting potential (V_{rest}) and the accompanying loss of fiber excitability (Lehmann-Horn et al., 1983; Rüdel et al., 1984). While spontaneous episodes do occur, many attacks are induced by environmental triggers, such as diet (carbohydrate or salt content), exercise, muscle cooling, or stress. Exercise is one of the most consistent provocative factors (Miller et al., 2004). Curiously, the weakness does not begin during exercise. In fact, an impending attack of weakness can be attenuated by performing light exercise. Susceptibility to exercise-induced weakness occurs a few minutes after stopping to rest, especially after a prolonged period (tens of minutes or longer) of vigorous exercise.

Despite the fact that exercise is a common trigger for all forms of familial periodic paralysis, the mechanism is unknown. Previous suggestions have included the efflux of K^+ out of working muscle, adrenaline release, or a decrease in local pH (Ricker et al., 1989). Two lines of evidence support the notion that acidosis may be protective and thereby explain why attacks do not occur during exercise. First, carbonic anhydrase inhibitors, such as acetazolamide, reduce attack frequency and severity for ∼50% of patients with hypokalemic periodic paralysis (HypoKPP; Matthews et al., 2011) or hyperkalemic periodic paralysis (HyperKPP; Sansone et al., 2016). At therapeutic dosages, carbonic anhydrase inhibitors cause systemic metabolic acidosis, and this effect has been proposed to be an essential feature for the therapeutic benefit (Griggs et al., 1970). Clinical studies support this hypothesis, as shown by the fact that metabolic acidosis produced by oral administration of NH_4Cl for 3 d also provided protection from provoking an attack of HypoKPP (Jarrell et al., 1976). Maren and colleagues have proposed the benefit of acidosis for HypoKPP comes from an effect on K^+ flux (Vroom et al., 1975; Jarrell et al., 1976). Acidosis decreases the influx of K^+ into many tissues, including skeletal muscle. During an attack of HypoKPP, serum K^+ is low because K^+ shifts into muscle fibers (Zierler and Andres, 1957), which in a

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positive-feedback loop aggravates the paradoxical depolarization and decreases contractility. Attenuation of the K^+ influx by acidosis may therefore be beneficial. Additional support for this hypothesis comes from our HypoKPP mouse models (Wu et al., 2013a) because acetazolamide is effective in vivo but much less so for the ex vivo tissue bath assay (where $[K^+]_o$ is fixed and acetazolamide does not produce acidosis). A second line of evidence shows benefit from acidosis in HyperKPP. In vitro studies on human intercostal fibers from a patient with HyperKPP showed that a reduction of pH, using CO_2 or HCl, attenuated or prevented the loss of force triggered by elevated $[K^+]_o$ (Lehmann-Horn et al., 1987). The improvement in force generation occurred even though the excessive depolarization of V_{rest} persisted at low pH. The authors proposed that a left shift in the apparent voltage dependence of sodium channel inactivation from higher extracellular $[H^+]$ could explain the improvement of fiber excitability.

In this study, we initially set out to define the mechanism by which acidosis may be protective against an attack of weakness induced by known triggers, such as reduced K^+. The approach was to perform in vitro contraction studies with our knock-in mutant mouse models of periodic paralysis (Hayward et al., 2008; Wu et al., 2011, 2012), where environmental variables such as $[K^+]$, pH, and temperature could be controlled in an organ bath. While acidosis (bicarbonate bath gassed with 25% CO_2) increased the force in HyperKPP muscle ($Na_V1.4$-M1592V) and did provide modest protection from a low K^+ (2 mM)–induced loss of force in the soleus muscle from HypoKPP mice ($Na_V1.4$-R669H), the more robust finding was a transient loss of force elicited by the return to normal pH (5% CO_2). This post-acidosis loss of force occurred in both models of HypoKPP ($Na_V1.4$-R669H and $Ca_V1.1$-R528H), but not for HyperKPP ($Na_V1.4$-M1592V). A prolonged exposure to low pH was required to induce susceptibility to loss of force, and the force decrement could be prevented with ionic conditions or drugs that impede the entry of Cl^- into muscle.

We propose a mechanism for the post-acidosis loss of force, based on accumulation of myoplasmic Cl^- during acidosis. Acidosis is known to decrease the chloride conductance of muscle, G_{Cl}, which is the dominant membrane conductance in resting fibers (Palade and Barchi, 1977; Pedersen et al., 2005). A lower G_{Cl} impedes the major efflux pathway and promotes Cl^- accumulation (Aickin et al., 1989) resulting from the basal influx via the Na^+-K^+-$2Cl^-$ (NKCC1) cotransporter. Myoplasmic $[Cl^-]$ is normally low, ∼4 mM, and so after several minutes the relative change in concentration is sufficient to substantially depolarize the equilibrium potential, E_{Cl}. The resting potential, V_{rest}, does not change much during acidosis (low G_{Cl}), but upon rapid recovery from acidosis, G_{Cl} rapidly increases and the established shift of E_{Cl} causes depolarization of V_{rest}. HypoKPP muscle has a bistable V_{rest}, with the anomalous depolarized state caused by a gating pore current from mutant channels (Cannon, 2018). Consequently, HypoKPP fibers, but not WT, become stably depolarized, refractory, and unable to contract because of Na_V channel inactivation.

Materials and methods

Mouse models of periodic paralysis

The generation of knock-in mutant mouse lines, molecular confirmation of mutant allele expression, and functional characterization of the skeletal muscle phenotype with periodic paralysis have been previously described (Hayward et al., 2008; Wu et al., 2011, 2012). Knock-in mutant mice were backcrossed with 129/Sv mice for more than 15 generations to produce congenic lines. We adopted a nomenclature for our mouse lines based on the missense mutation in the human channel so that cross-referencing to the patient literature on periodic paralysis would be facilitated. Our mouse model for HyperKPP plus myotonia contained a point mutation in *SCN4A* that encoded M1592V in the human $Na_V1.4$ sodium channel (M1586V in mouse; Hayward et al., 2008). We created two mouse models of HypoKPP, one with a missense mutation of *SCN4A* encoding the equivalent of human R669H in $Na_V1.4$ (R663H in the mouse; Wu et al., 2011) and a second with a missense mutation in *CACNA1S* encoding the R528H mutations in $Ca_V1.1$ (Wu et al., 2012).

In vitro contraction studies

Mice were euthanized with isoflurane inhalation followed by cervical dislocation. The soleus muscle was quickly removed and suspended vertically between a fixed post and a force transducer (Fort 25, World Precision Instruments or 305C Dual Mode Lever-Arm System, Aurora Scientific) in a tissue bath maintained at 37°C. The standard bath solution was a bicarbonate-buffered Krebs solution containing (in mM) 118 NaCl, 4.75 KCl, 1.18 $MgSO_4$, 2.5 $CaCl_2$, 1.18 NaH_2PO_4, 24.8 $NaHCO_3$, and 10 glucose. The Krebs solution was supplemented with 0.5 µM D-tubocurarine, except for the compound muscle action potential (CMAP) experiments in Fig. 4 (see below), to provide consistency of direct field stimulation to muscle fibers rather than have an uncontrolled contribution from motor nerve endings (Wu et al., 2011). For some of the fibers included in Figs. 1 and 2 A, the Krebs bath was also supplemented with 20 U/liter (158 nM) insulin to prolong viability of the ex vivo preparation. Subsequently, we found there was no improvement, and so insulin was omitted for the majority of the data in this paper. Moreover, there was no difference in the loss of force elicited by recovery from acidosis with or without insulin. The tissue bath was continuously bubbled with a mixture of N_2, O_2, and CO_2 that was set by a computer-controlled gas mixer (Gas Blender Series 100, MCQ Instruments). The standard mixture was 95% O_2/5% CO_2, and periods of acidosis were imposed by rapid exchange with the same Krebs solution that had been preequilibrated with 7.5–40% CO_2 by using a commensurate reduction in the percentage of O_2. The possibility that changes in muscle force were from hypoxia, rather than acidosis, was excluded by showing the same changes were produced when the O_2 was kept constant but CO_2 and N_2 were varied: 75% O_2/20% N_2/5% CO_2 exchanged with 75% O_2/25% CO_2. Tetanic contraction was induced by constant-current stimulation (80 mA; model A385 World Precision Instruments or model 301C Aurora Scientific) with 0.5-ms pulses (40 × 100 Hz) applied to a pair of platinum pins oriented

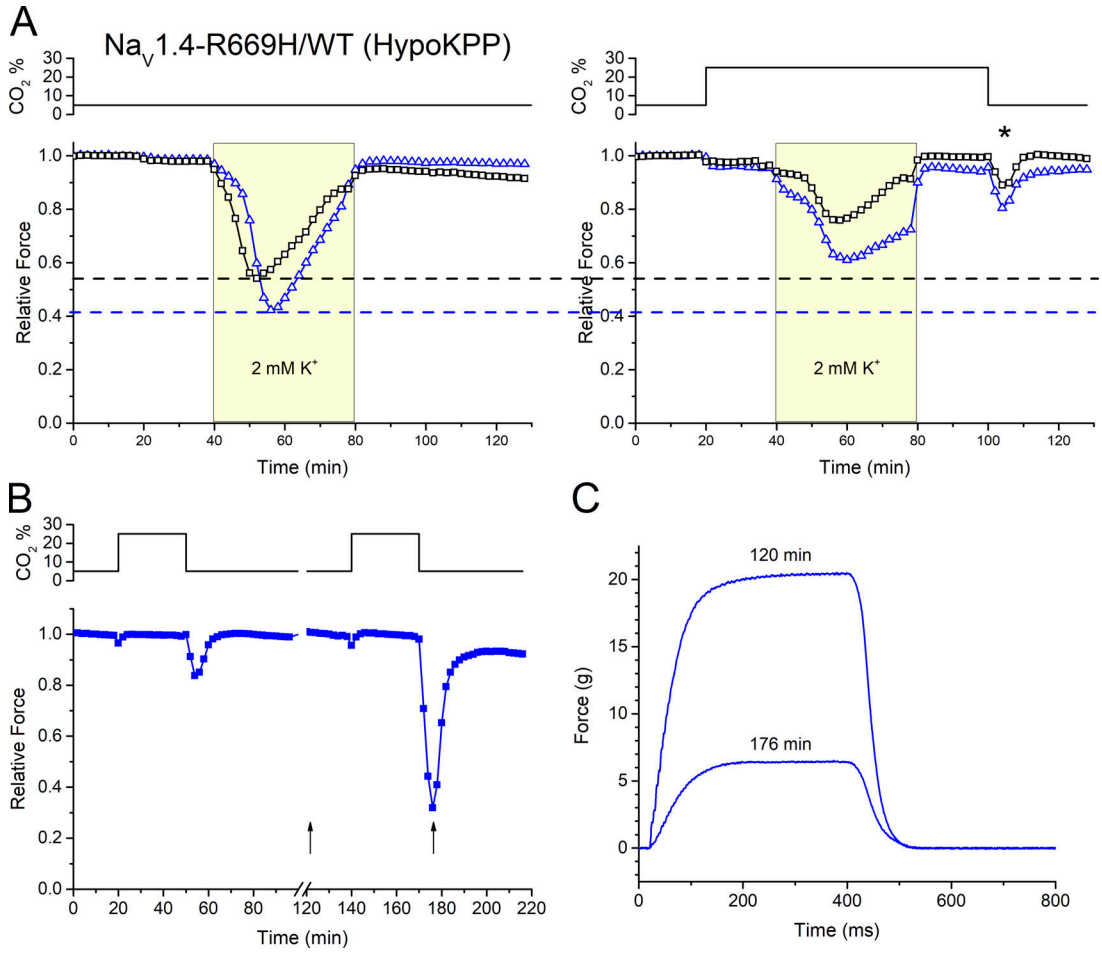

Figure 1. **Acidosis attenuates the loss of force elicited by a low-K⁺ challenge.** Isometric contractions were recorded once every 2 min in response to tetanic stimulation (0.5-ms pulses at 100 Hz × 40) of the isolated soleus muscle ex vivo. **(A)** Reduction of $[K^+]_o$ from 4.7 to 2 mM revealed the HypoKPP phenotype as a large decrease in force (~50%, left), as shown by exemplary responses from two different heterozygous mice ($Na_V1.4$-R669H/WT). When the paired muscles from the opposite limb of these same two mice were preequilibrated in acidified Kreb's solutions (25% CO_2/75% O_2, pH 6.71), the loss of force for a 2-mM K^+ challenge was attenuated to 30% (right). The return to normal Kreb's (5% CO_2/95% O_2, pH 7.43; asterisk) triggered a second transient loss of force. **(B)** Recovery from acidosis alone, without a hypokalemic challenge, also elicited a transient decrease in force. Data show consecutive acidosis challenges applied to the same soleus muscle from a heterozygous $Na_V1.4$-R669H/WT mouse. **(C)** Force transients recorded during the control interval (120-min trace) and at the nadir of the transient decrease in force (176-min trace).

perpendicular to the muscle long axis. Figs. 2, 3, 5, and 6 show relative force ([peak force for test]/[peak force for control]), and the error bars indicate standard error of the mean.

In vitro CMAP

The soleus muscle with a stump of the intact sciatic nerve was dissected from the mouse and mounted to a force transducer in a horizontal tissue chamber (Kent Scientific). The nerve was drawn into a suction electrode and stimulated (8 mA) at 100 Hz × 60 pulses of 0.5-ms duration (A385 Stimulator; World Precision Instruments). Muscle excitability was assessed by measuring the CMAP as a differential voltage between two electromyography needles inserted into the soleus, with both on the same side relative to the neuromuscular junction. The differential signal was amplified (3-Hz to 3-KHz bandwidth; Grass P511) and sampled at 10 KHz. The tissue bath contained the standard Krebs solution at 37°C as above, except the D-tubocurarine was omitted.

Intracellular pH (pH_i)

Myoplasmic pH was measured with the ratiometric fluorescent probe BCECF (2′,7′-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein) (Westerblad and Allen, 1992). Fibers from the flexor digitorum brevis were loaded with AM-BCECF (10 µM, ThermoFisher Scientific) in HEPES buffered saline (in mM): 135 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.4 NaH_2PO_4, 5.5 glucose, 20 HEPES, pH 7.4 with NaOH for 30 min at 37°C. Fibers were then imaged using an Andor Neo sCMOS spinning-disc confocal microscope (dual excitation at 445 nm and 488 nm and emission filter 535 nm) in the UT Southwestern Live Cell Imaging Core Facility. Images were acquired every 2 min over a 1-h session during which a 25% CO_2 challenge was applied for 20 min. Calibration was then performed at the end of every experimental run by applying the H^+ ionophore nigericin (10 µM) and then superfusing the fibers with high K^+ solutions (in mM: 140 KCl, 0.5 $MgCl_2$, 1.2 $KHPO_4$, 20 pH buffer, pH with KOH) buffered with Mes (pH 4.5, 5.5), a 50/50 mixture of Mes and HEPES (pH

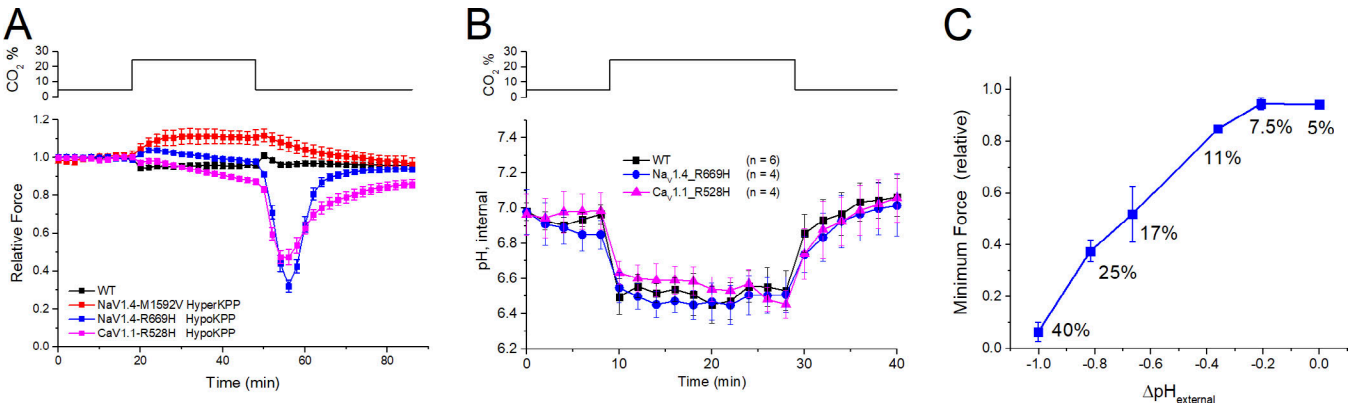

Figure 2. **Recovery from acidosis triggers a transient loss of force in HypoKPP muscle. (A)** Peak tetanic force was maintained for soleus muscle from all mice during a 30-min challenge with 25% CO_2. Upon return to 5% CO_2, a transient loss of force occurred for muscle from both HypoKPP mouse models (n = 6 for each), but not WT (n = 7) or HyperKPP (n = 6). **(B)** A comparable decrease of pH_i, as measured by BCECF fluorescence, occurred for HypoKPP and WT muscle during a 25% CO_2 challenge. **(C)** The dose–response relation measured over a range of 20-min CO_2 challenges (5% to 40%) for $Na_V1.4$-R669H HypoKPP soleus muscle shows a threshold for detecting a loss of force at 11% (–0.36 pH units) and near complete loss of force after a 40% challenge (–1.0 pH units).

6.5, 7.5) or Tris (pH 8.5, 9.36) in a high K solution (Westerblad and Allen, 1992). After subtraction of background fluorescence, the fluorescence ratio, R = F488/F445, was calculated using ImageJ and the calibration data were fit to the following equation:

$$pH_i = pKa + \log[(R - R_{min})/(R_{max} - R)].$$

Study approval

All procedures were in accordance with animal protocols approved by the Institutional Animal Care and Use Committees at the David Geffen School of Medicine, University of California, Los Angeles, CA and University of Texas Southwestern Medical Center, Dallas, TX.

Results

Acidosis attenuates the loss of force in HypoKPP muscle during low-K^+ exposure

Strenuous exercise is a well-known precipitant for attacks of periodic paralysis in susceptible individuals, with the weakness occurring after several minutes of rest but not during ongoing activity (Cannon, 2015). Nothing is known about the mechanism of this phenomenon, and our initial approach was to explore the possibility that muscle acidosis protects against the loss of force during exercise (Jarrell et al., 1976). We tested this hypothesis by using an in vitro contraction assay previously developed for our knock-in mutant mouse model of HypoKPP caused by the sodium channel $Na_V1.4$-R669H mutation (Wu et al., 2011). The force of an isometric tetanic contraction in the isolated soleus muscle was stable over 40 min (shown as relative force for two soleus muscles from different animals in Fig. 1 A, left) but transiently decreased when the bath [K^+] was reduced from 4.7 to 2 mM (40–80 min), which shows the HypoKPP phenotype. The soleus from the opposite hindlimb was tested in a separate organ bath for which acidosis was imposed by exchanging the bath with bicarbonate-buffered solution equilibrated with 25% CO_2/75% O_2 (pH 6.71) instead of the control 5% CO_2/95% O_2 (pH 7.43). Acidosis was well-tolerated as shown by the sustained level of contractile force (Fig. 1 A, right, 20–40 min), and the loss of force induced by a subsequent 2-mM [K^+] challenge was attenuated (40–80 min). In each of the four $Na_V1.4$-R669H mice tested, pretreatment with 25% CO_2 always attenuated the loss of force from a 2-mM K^+ challenge, similar to the exemplary responses shown in Fig. 1 A. On average, the relative force nadir was 0.53 ± 0.039 in 5% CO_2 and 0.71 ± 0.054 in 25% CO_2; $P < 0.05$.

Unexpectedly, a transient decrease in tetanic force consistently occurred upon recovery from acidosis when the bath was rapidly returned to Kreb's solution equilibrated with 5% CO_2/95% O_2 (Fig. 1 A, asterisk in the right panel). We next examined whether the low-K^+ challenge was necessary for this pH-dependent loss of force. A 20-min interval of acidosis alone (25% CO_2/75% O_2) was sufficient, and a second acidosis challenge applied to the same muscle 2 h later produced an even larger decline in force (Fig. 1 B). Example force transients selected at times 120 min (control) and 176 min (nadir for transient loss) are shown in Fig. 1 C. The rise time and decay time of the force transient during the nadir of peak force were indistinguishable from control conditions, and there was no sag in the peak force during a 400-ms tetanic contraction.

Recovery from acidosis triggers a large transient loss of force in HypoKPP muscle

The acidosis challenge was applied to soleus muscle from several mouse models of periodic paralysis and WT mice to determine which genotypes have susceptibility to the transient loss of force upon return to normal pH. Two established models of murine HypoKPP were examined: $Na_V1.4$-R669H (Wu et al., 2011) and $Ca_V1.1$-R528H (Wu et al., 2012). For both HypoKPP lines, soleus muscle from homozygous mutant animals was used herein and for the remainder of our studies to maximize the sensitivity of detecting an anomalous force response. The HyperKPP mouse model was studied in the heterozygous state ($Na_V1.4$-M1592V/WT)

because survival of homozygous mutant animals is very poor (Hayward et al., 2008). Maximal tetanic force of the soleus muscle was monitored every 2 min as the bath was exchanged with Kreb's solution equilibrated in 25% CO_2/75% O_2 and then 30 min later returned to Kreb's solution equilibrated with 5% CO_2/95% O_2. Force was maintained within ±15% during the acidosis interval for soleus muscle from all mouse genotypes, but only the HypoKPP soleus had a large transient decrease in maximal force upon return to 5% CO_2/95% O_2 (Fig. 2 A). We confirmed the transient loss of force was a consequence of the acidosis in 25% CO_2, and not the 20% reduction of O_2, by challenging $Na_V1.4$-R669H soleus with 5% CO_2/75% O_2/20% N_2, which did not cause a transient loss of force (data not shown). Onset for the loss of force began with a lag since the bath exchange (three times the chamber volume) was completed in ∼5 s, and 6–8 min was required for the force to reach a minimum. Recovery of force was consistently slower for the $Ca_V1.1$-R528H soleus than $Na_V1.4$-R669H, and the peak force for $Ca_V1.1$-R528H muscle had a slow decline during the acidosis interval. The response for HyperKPP soleus consistently had a small increase in force during the acidosis interval. One possible interpretation is that in 4.7 mM K^+ the baseline force was mildly compromised for HyperKPP muscle, but not HypoKPP, and acidosis improved performance analogous to the improvement in low K^+ for HypoKPP muscle (Fig. 1 A).

The pH_i change in response to the 25% CO_2 challenge was measured with a ratiometric fluorescent dye (BCECF) to determine whether the susceptibility to a transient loss of force in HypoKPP muscle was associated with a difference in pH_i homeostasis. A rapid exchange of the chamber bath to Kreb's buffer equilibrated with 25% CO_2 caused a 0.4-U decrease of pH_i (Fig. 2 B) that reached steady state within 2 min (the first measurement time point) and remained stable during the entire 20-min exposure. In all muscles tested, this hypercapnic load overwhelmed the compensatory capacity of a fiber to autoregulate pH_i. A rapid return to Kreb's buffer equilibrated with 5% CO_2 caused a gradual recovery of pH_i to baseline that occurred over a 10-min period. The responses were indistinguishable for muscle isolated from either HypoKPP mouse model or WT mice. In particular, the trajectory for the recovery of pH_i, when a transient loss of force would occur for HypoKPP muscle (30–40 min in Fig. 2 B), was the same for fibers from mutant and WT mice. These data show that the post-acidosis loss of force for $Na_V1.4$-R669H or $Ca_V1.1$-R528H HypoKPP muscle is not caused by a difference in pH_i regulation compared with WT muscle, which has no transient loss of force.

The sensitivity of HypoKPP muscle to pH-induced loss of force was determined by varying the percentage of CO_2 between 5% and 40% during the 20-min challenge for $Na_V1.4$-R669H soleus (Fig. 2 C). The threshold for eliciting a detectable loss of force was 11.2% CO_2 (ΔpH_e −0.4). The nadir in force decreased to 50%, with a 16.7% CO_2 challenge (ΔpH_e −0.6), and in 40% CO_2 (ΔpH_e −1.0) the force transiently decreased to nearly zero. By comparison, exhaustive exercise in humans reduces the pH_i of the quadriceps muscle by ∼0.5 (Sahlin et al., 1976), which implies that physiologically relevant changes in pH are sufficient to trigger a transient loss of force in HypoKPP muscle.

Prolonged acidosis, followed by rapid recovery, promotes a transient loss of force

The time course for the onset of susceptibility to loss of force was determined by varying the duration of the acidosis interval in Kreb's buffer preequilibrated with 25% CO_2. The loss of force upon return to 5% CO_2 became more severe as the acidosis exposure time was increased over many tens of minutes, as shown in Fig. 3 A. Each muscle preparation was tested with only a single interval of acidosis to avoid the complication of cumulative effects, as demonstrated by the repeated exposures separated by 2 h in Fig. 1 B. The peak tetanic force for soleus muscle from $Ca_V1.1$-R528H mice had a progressive, slow decline during the period of acidosis (Fig. 3 A, left panel), and so a baseline correction was made to define the relative loss of force upon return to 5% CO_2 (Fig. 3 A, right panel). The onset for susceptibility to loss of force was exponential with a time constant of ∼30 min. This slow time course is comparable to the phenomenon of postexercise weakness for individuals with HypoKPP, in which a period of vigorous exercise of 30 min or longer is typically required to trigger an episode of weakness during subsequent rest.

Light exercise may abort an impending attack of HypoKPP, and a gradual "warm down" after vigorous exercise may prevent an acute postexercise episode (Lehmann-Horn et al., 2004). We therefore wondered whether a gradual recovery of pH after 30 min of acidosis in 25% CO_2 might prevent the transient loss of force in our mouse model of HypoKPP. A slow recovery of pH for the muscle bath was achieved by adjusting the percentage of CO_2 in a mixture of O_2/N_2/CO_2 from a computer-controlled gas mixer that continuously bubbled the 25-ml chamber. A step change in the percentage of CO_2 from 25% back to 5% produced an exponential recovery of pH from 6.6 to 7.4 with a time constant of 50 s (Fig. 3 B, left). More gradual pH changes were applied by using a series of small incremental step changes in the percentage of CO_2 over several minutes. The pH of the muscle bath was monitored continuously with a reference electrode. A pair of soleus muscles from the same mouse was tested in parallel, but in separate chambers, with one having a rapid return of pH within 5 s by total exchange of the bath with preequilibrated Kreb's buffer and the other having a slow pH change controlled by the gas mixer. Slowing the pH recovery time from a few seconds to a few minutes did not prevent the transient loss of force (Fig. 3 B, left), and prolongation of the pH recovery time to 20 min was required attenuate the loss by ∼40% (Fig. 3 B, middle). Extending the pH recovery time to 50 min greatly reduced the loss of force and in some individual trials completely prevented the loss. Collectively, the data in Fig. 3 show that both the onset of acidosis-induced susceptibility to loss of force and the recovery from susceptibility are very slow, with a time course of many tens of minutes.

HypoKPP muscle fiber excitability is decreased during an acidosis-triggered loss of force

The episodic attacks of weakness associated with low extracellular [K^+] in patients with HypoKPP are caused by sustained depolarization of the resting potential (V_{rest}), which

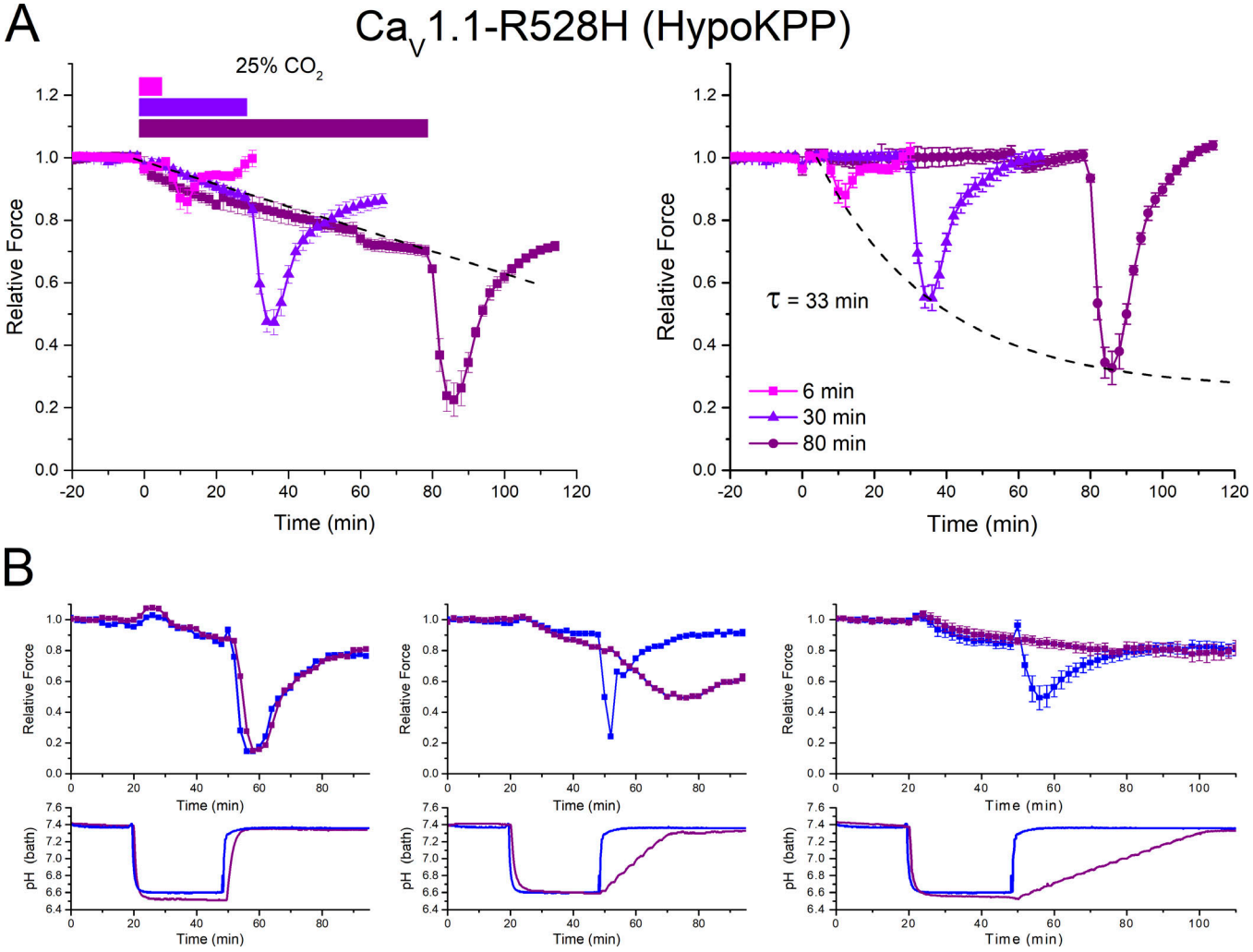

Figure 3. **Slow kinetics of acidosis-induced loss of force in HypoKPP muscle. (A)** The time course for the development of susceptibility to post-acidosis loss of force was determined by varying the duration of a 25% CO_2 challenge (bars, left panel). Plots show average responses from Ca$_V$1.1-R528H soleus (6 min: n = 9, 30 min: n = 6; reproduced from Fig. 2 A; 80 min: n = 4). **(B)** Data were corrected for the decline in force during the 25% CO_2 exposure (dashed line, panel A) to more clearly show the exponential kinetics for the onset of susceptibility with a time constant of 33 min. Plots in the top row show a pairwise comparison for the transient loss of force induced by a rapid transition of bath pH to 7.4 (blue) versus a slower pH recovery for the soleus from the other hind leg of the same Ca$_V$1.1-R528H mouse in a separate tissue bath (purple). The bottom row shows the bath pH monitored with a reference electrode. Exemplary comparisons for the two soleus muscles from a single mouse (left and middle panels) show an attenuation for the loss of force when the pH transition occurred slowly over 20 min. A slower recovery of bath pH over 50 min prevented the post-acidosis loss of force (right, n = 5).

inactivates Na^+ channels and reduces fiber excitability (Rüdel et al., 1984). We tested whether the acidosis-induced loss of force in mouse HypoKPP muscle was also associated with reduced fiber excitability. CMAP was measured as an index of fiber excitability in an ex vivo preparation for which the free ending of the sciatic nerve was stimulated with a suction electrode and force was monitored during an isometric contraction of the soleus muscle. A 30-min challenge with 25% CO_2 was well tolerated, but upon rapid return to 5% CO_2 there was a concomitant decrease in CMAP amplitude of HypoKPP muscle (Fig. 4, A and C) and in maximal isometric force (Fig. 4, B and D). The precise temporal concordance of these changes is consistent with the notion that, as in episodic attacks of HypoKPP in vivo, the post-acidosis loss of force is caused by a transient reduction in fiber excitability.

Pharmacologic protection from acidosis-triggered loss of force

Carbonic anhydrase inhibitors have been used clinically to prophylactically reduce the severity and frequency of episodic weakness in HypoKPP (Resnick et al., 1968; Tawil et al., 2000). We tested the efficacy of pretreatment with acetazolamide (100 µM) to reduce the post-acidosis loss of force for the soleus muscle. Muscles from the same animal were tested in pairs, one drug exposed and the other not, to control for the variability of the force transients. Acetazolamide aggravated the loss of force for Na$_V$1.4-R669H soleus (Fig. 5 A), whereas partial protection of ~50% occurred for Ca$_V$1.1-R528H mice (Fig. 5 B). This divergent response has also been observed clinically, with a subset of Na$_V$1.4-HypoKPP patients (especially R672G or R672S) having a detrimental effect of acetazolamide and Ca$_V$1.1-HypoKPP individuals receiving benefit or no perceived change (Matthews

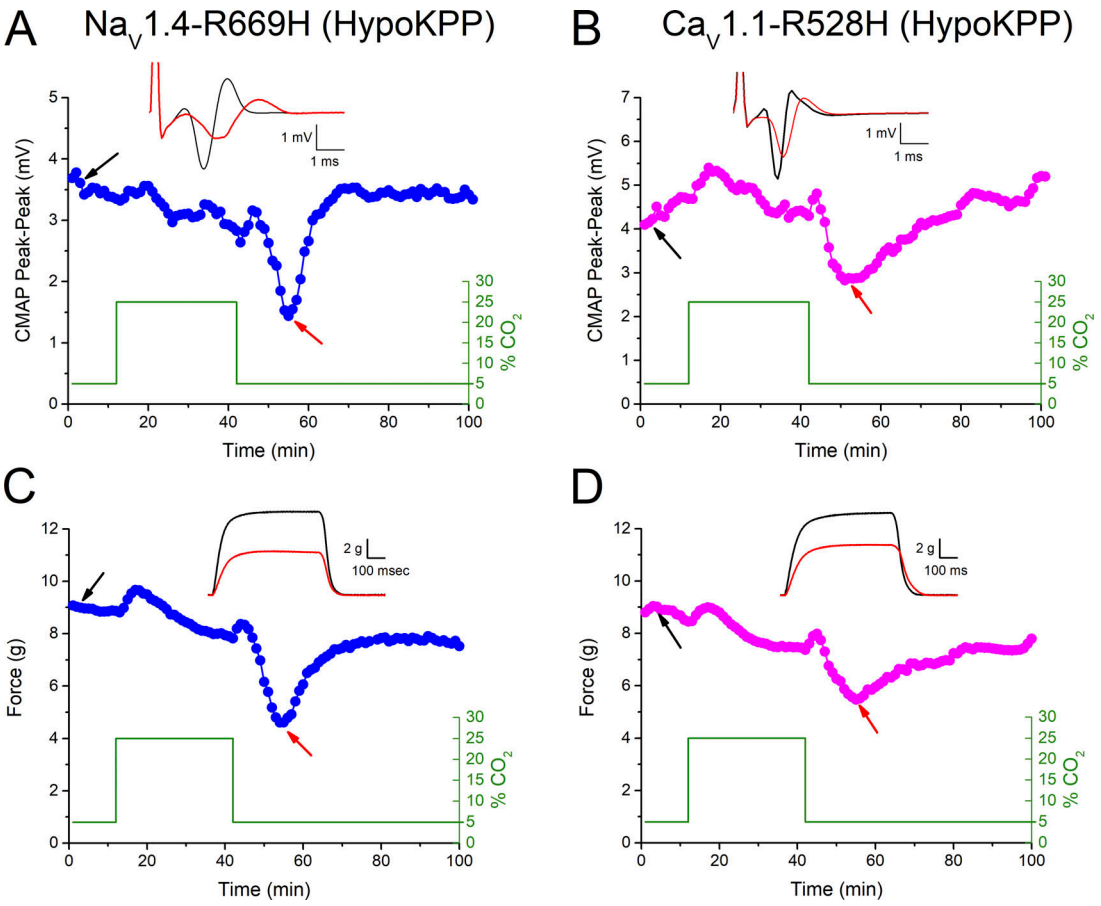

Figure 4. **HypoKPP muscle excitability is reduced during the transient loss of force. (A–D)** CMAP (A and B) and muscle force (C and D) were measured for a tetanic stimulation (0.5 ms at 100 Hz for 60 pulses) applied to the sciatic nerve ending once per minute. Insets in each panel show raw data (black trace) before the 25% CO_2 challenge (black arrow) and at the nadir of force (red trace, red arrow). The initial upward deflection for the CMAP is a shock artifact (clipped) from the stimulating electrode. The decrease in CMAP amplitude is coincident with the transient loss of force for both $Na_V1.4$-R669H (A and C) and $Ca_V1.1$-R528H HypoKPP soleus (B and D). Responses similar to these exemplary traces were observed for two additional trials with each genotype.

et al., 2011). More recently, inhibition of the NKCC1 co-transporter with bumetanide was shown to be highly effective at preventing or reversing the loss of force associated with a low-K^+ challenge (2 mM) in our mouse models of HypoKPP (Wu et al., 2013a,b). Consistent with this beneficial effect, pretreatment with 0.5 µM bumetanide completely prevented the post-acidosis loss of force for both $Na_V1.4$-R669H and $Ca_V1.1$-R528H soleus muscle (Fig. 5, C and D, respectively).

Implication of a chloride dependency for acidosis-induced loss of force

Loss of fiber excitability caused by a depolarized shift of V_{rest} was the final common pathway for episodic attacks of weakness in all forms of periodic paralysis. For HypoKPP, this depolarization occurred paradoxically in low extracellular K^+, where V_{rest} exhibited bistability (Cannon, 2010). In other words, HypoKPP fibers may have a normally polarized V_{rest} with preserved excitability or become depolarized and inexcitable (for the same low value of external K^+). The bias between these two possible states was strongly dependent on the chloride gradient because skeletal muscle has a very high resting chloride conductance (Geukes Foppen et al., 2002; Cannon, 2018). The normally low intracellular Cl^- (~3–4 mM) favored the hyperpolarized V_{rest} with normal excitability, whereas raised intracellular Cl^- (predicted to be 10 to 14 mM) promoted the depolarized V_{rest} and weakness. Indeed, the beneficial effect of bumetanide in preventing loss of force in a low-K^+ challenge for HypoKPP muscle is attributed to an effect on the Cl^- gradient (Wu et al., 2013a,b). Bumetanide inhibits the NKCC1 co-transporter and thereby blocks the major Cl^- influx pathway. The potent efficacy of bumetanide in also preventing post-acidosis loss of force (Fig. 5, C and D) suggests that this provocative trigger also involves a rise of intracellular Cl^-. We sought additional experimental evidence to test the notion that a rise of intracellular Cl^- underlies the transient loss of force after an acidosis challenge.

According to our hypothesis, the deleterious effect of a rise in myoplasmic Cl^- acts through the Cl^- conductance (ClC-1) to produce depolarization and loss of excitability in HypoKPP fibers. Blocking the ClC-1 with 9-anthracene carboxylic acid (9-AC) should, therefore, reduce the post-acidosis loss of force. Consistent with this prediction, pretreatment with 8 µM 9-AC attenuated the loss of force by ~50% for $Na_V1.4$-R669H HypoKPP soleus muscle (Fig. 6 A). The partial effect is

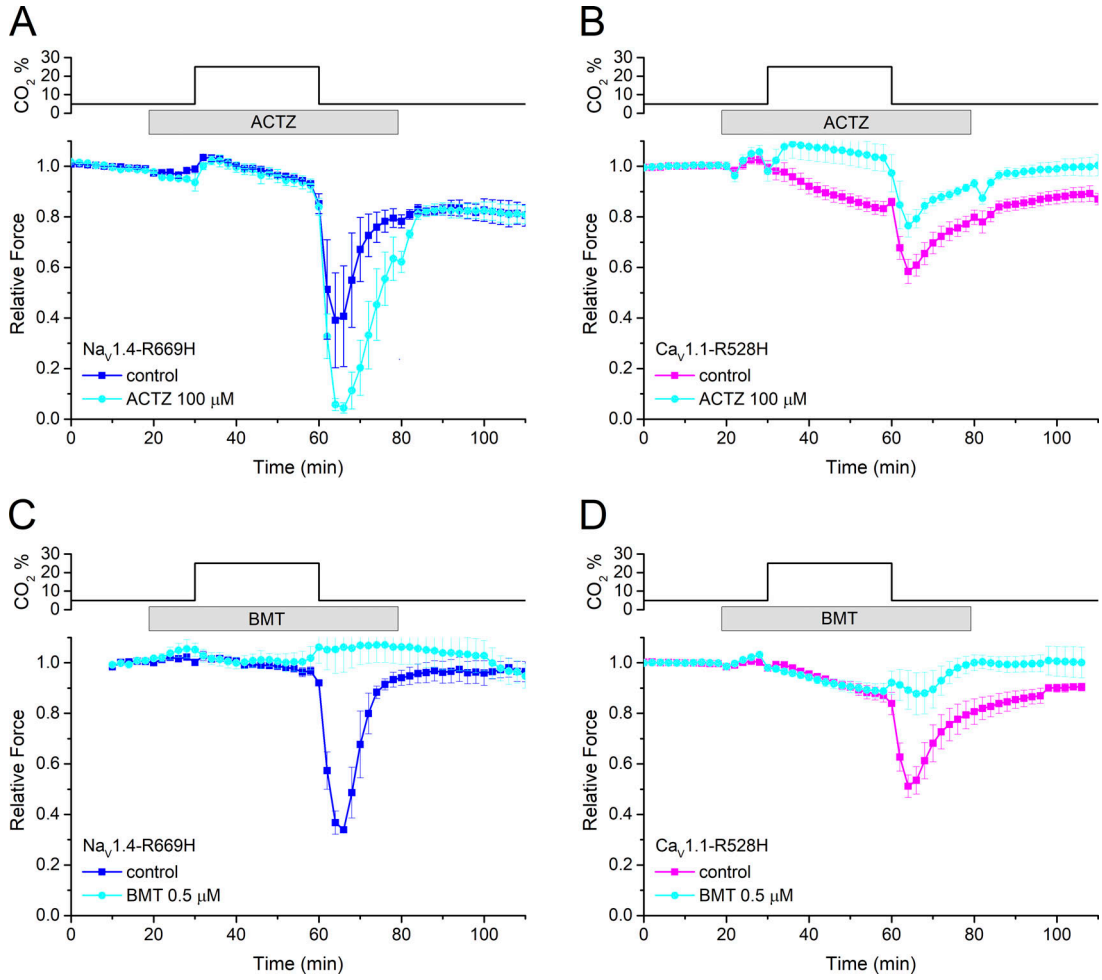

Figure 5. **Pharmacologic inhibition of the post-acidosis loss of force. (A and B)** Pretreatment with acetazolamide (ACTZ; 100 μM) accentuated the loss of force for $Na_V1.4$-R669H soleus (A; $P < 0.05$ from 64 to 70 min, $n = 3$) and partially reduced the loss of force for $Ca_V1.1$-R528H (B; $P < 0.01$ from 64 to 90 min, $n = 8$). **(C and D)** Bumetanide (BMT; 0.5 μM) completely prevented the post-acidosis loss of force for NaV1.4-R669H (C, $n = 3$) and CaV1.1-R528H soleus (D, $n = 3$).

likely attributable to only ~50% block of ClC-1 (IC_{50} ~7 μM; Astill et al., 1996), which was chosen to minimize a possible confounding effect from myotonia. This explanation is consistent with the very modest increase for the force relaxation time (~30%) in 8 μM 9-AC. An alternative approach is to remove Cl^- from the extracellular buffer and replace it with the impermeant anion methanesulfonate. As expected in Cl^--free conditions, the soleus muscle developed myotonia with a marked prolongation in relaxation of force at the end of a tetanic stimulation (Fig. 6 B, inset). Nevertheless, peak force was maintained, and so it was possible to perform a 25% CO_2 challenge. For $Ca_V1.1$-R528H HypoKPP muscle the tetanic force decreased during the period of acidosis, but more importantly, force quickly and fully recovered after a return to 5% CO_2 with no post-acidosis transient loss of force (Fig. 6 B).

The third line of evidence for ClC-1 involvement is based on the detection of myotonia as an indicator of a low-conductance ClC-1 state. Our $Na_V1.4$-M1592V mouse model of HyperKPP had electromyographic evidence of myotonia at baseline, but some provocative challenge (e.g., low Ca^{2+}) was required to cause a delay in muscle force relaxation (Hayward et al., 2008). Acidosis reduced the conductance of ClC-1 (Palade and Barchi, 1977), and a 25% CO_2 challenge elicited myotonia as expected from a decrease in G_{Cl} (Fig. 6 C), especially in combination with the $Na_V1.4$ gain of function caused by M1592V (Cannon and Strittmatter, 1993). Myotonia was fully reversible, as shown by the superimposed force transient after returning to 5% CO_2. We used the amplitude of the residual force 100 ms after the end the stimulus (t = 550 ms in the trace) as a measure of myotonia and, therefore, indirectly as a measure of reduced G_{Cl}. The time course for the development of and recovery from myotonia (reduced G_{Cl}) is shown in Fig. 6 D (red plot) for a 30-min challenge with 25% CO_2. A typical response for the post-acidosis loss of force in a HypoKPP muscle (Fig. 6 D, $Na_V1.4$-R669H, blue plot) is superimposed and shows the close temporal association for the recovery from myotonia (i.e., increase of G_{Cl} back to baseline) and the onset of the transient loss of force. These data support our proposal that the post-acidosis loss of force is chloride dependent and triggered by the rapid increase in G_{Cl} when the pH is quickly returned to the normal range.

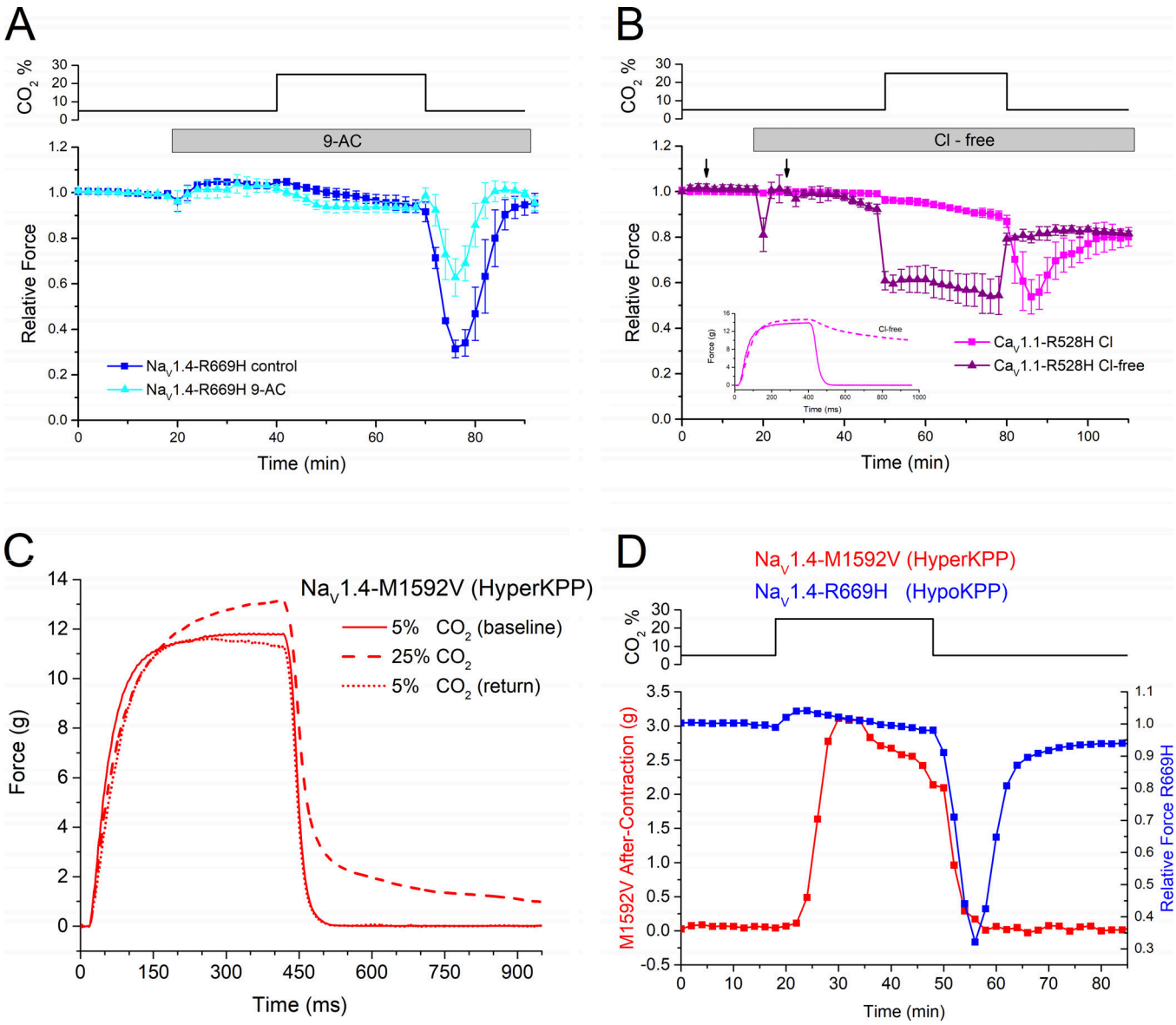

Figure 6. **The chloride conductance contributes to the post-acidosis loss of force in HypoKPP muscle. (A)** Partial block of ClC-1 with 8 µM 9-AC attenuated the transient loss of force ($Na_V1.4$-R669H, $n = 3$). **(B)** In a Cl^--free bath, peak isometric force was maintained (e.g., 20–50 min), and the force transient showed pronounced myotonia (inset shows superimposed responses at the times indicated by arrows). While there was a loss of force during the 25% CO_2 challenge compared with contralateral soleus muscle in a separate Cl^- bath (50–80 min), the post-acidosis loss of force did not occur (filled triangles, 80–100 min, $n = 3$). **(C)** Superimposed contractions show myotonia with delayed relaxation in 25% CO_2 for HyperKPP $Na_V1.4$-M1592V soleus that reverses upon return to 5% CO_2. **(D)** Overlay of the time course for acidosis-induced myotonia in HyperKPP soleus (red) and post-acidosis loss of force for HypoKPP $Na_V1.4$-R669H soleus (blue) shows the loss of force in HypoKPP muscle is coincident with the rapid recovery from myotonia in HyperKPP muscle.

Discussion

The relation between acidosis and susceptibility to periodic paralysis has been of interest for nearly 50 years, when it was proposed that the beneficial effect of carbonic anhydrase inhibitors on reducing the frequency and severity of attacks in HypoKPP may be a consequence of metabolic acidosis produced from bicarbonate loss in the urine (Griggs et al., 1970). Supporting evidence that systemic metabolic acidosis is a plausible mechanism for the beneficial effect of carbonic anhydrase inhibitors was obtained by inducing metabolic acidosis using a completely different mechanism (administering oral NH_4Cl), which also decreased the severity of HypoPP (Jarrell et al., 1976). Interpretation of how metabolic acidosis reduces the severity of HypoKPP in patients is confounded by multiple homeostatic mechanisms operating in vivo (Matthews and Hanna, 2010). For example, in individuals with HypoKPP, acidosis inhibits the shift of K^+ into muscle in response to a glucose plus insulin challenge (Vroom et al., 1975). So, is the beneficial effect of acidosis secondary to attenuating the decrease in extracellular K^+—a known trigger for weakness in HypoKPP—or some other mechanism by which acidosis preserves muscle fiber excitability? Our data provide additional information because extracellular $[K^+]$ was clamped in the ex vivo tissue bath. Consistent with these prior studies in patients, acidosis induced by

hypercarbia provides modest protection from loss of force in a controlled 2 mM K^+ challenge (Fig. 1 A). This observation implies some of the beneficial effect of acidosis occurs directly on muscle, independent from blunting the hypokalemia induced by carbohydrate ingestion.

The major finding of this study, however, is the marked loss of force in HypoKPP muscle that occurs after recovery from acidosis (Figs. 2, 3, 4, 5, and 6). This phenomenon is extremely robust. The loss of force induced by recovery from acidosis occurs more consistently than the canonical loss of force with a low-K^+ challenge (e.g., 2 mM). Put another way, from the results of a single trial with a 25% CO_2 challenge, it is possible to predict with near 100% accuracy whether the soleus muscle was from a HypoKPP or a WT mouse. The loss of force is biologically relevant. First, the magnitude is substantial and is therefore expected to adversely impact motor function. Second, the threshold for inducing a loss of force occurs within the range pH changes attainable in vivo. We have reported the change in external pH capable of inducing a loss of force (Fig. 2 C), whereas the pH_i is likely to be the relevant value (see below). The change of pH_i for muscle is typically 45% of the value imposed extracellularly by an increase in the percentage of CO_2 (de Paoli et al., 2007), and this estimate agrees with our observed 0.4-U intracellular decrease with a 25% CO_2 challenge (Fig. 2 B) corresponding to a 0.8-U decrease in extracellular pH. Exercise to exhaustion in humans produces a 0.5-U decrease of pH_i in active muscle (Sahlin et al., 1976), which is approximately equivalent to the 25% CO_2 challenge in our ex vivo experiments that produced an ∼60% loss of force (Figs. 2, 3, and 5).

The post-acidosis loss of force in HypoKPP muscle shares several features with the stereotypical episodes of impaired contractility that are triggered by a low-K^+ challenge. First, muscle excitability is reduced during the period of post-acidosis loss of force (Fig. 4). This observation is consistent with the central dogma that acute attacks of periodic paralysis are caused by sustained depolarization of V_{rest} that inactivates sodium channels and thereby reduces excitability (Rüdel et al., 1984; Cannon, 2015). Second, pharmacologic interventions that modify the loss of force in a low-K^+ challenge have similar effects on the post-acidosis loss of force. Pretreatment with bumetanide completely prevents the loss of force for both types of provocative challenge and in both $Ca_V1.1$-R528H and $Na_V1.4$-R669H HypoKPP muscle (Fig. 5, C and D). Moreover, acetazolamide exacerbates the post-acidosis loss of force in mouse $Na_V1.4$-R669H soleus muscle (Fig. 5 A) and worsens HypoKPP attack frequency and severity in patients with R/G or R/S mutations in S4DII of $Na_V1.4$ (Sternberg et al., 2001; Matthews et al., 2011). Conversely, with HypoKPP from $Ca_V1.1$-R528H acetazolamide partially protects against post-acidosis loss of force (Fig. 5 B) and reduces HypoKPP attacks in patients. As presented in the Introduction, acetazolamide is thought to act by promoting metabolic acidosis in the whole animal, and so it is remarkable that an effect was observed ex vivo. While acetazolamide does not have much of an effect on the steady-state pH gradient across the fiber in the organ bath, the inhibition of carbonic anhydrase will slow the rate of pH_i equilibration in response to a change in the percentage of CO_2 (Leem and Vaughan-Jones, 1998). As shown in Fig. 3, a slower rate of recovery from acidosis (and hence slower intracellular alkalization) reduces the risk of triggering a loss of force, and this is a plausible explanation for the modest protection in the case of $Ca_V1.1$-R528H (Fig. 5 B). On the other hand, the basis for an increased loss of force with acetazolamide for NaV1.4-R669H remains to be established. One marked difference between the two HypoKPP models is that $Na_V1.4$-R669H has a proton-selective gating pore leak, while the gating pore for CaV1.4-R528H is weakly selective for monovalent cations. Perhaps, in the presence of acetazolamide, a rapid bath exchange causes a transient pH gradient that increases the inward proton current in the $Na_V1.4$-R669H gating pore and thereby promotes a shift to the depolarized bistable state of V_{rest}? Additional experiments are needed to test these ideas. Taken together, however, these overlapping features that are shared for the loss of force provoked by low K^+ or by recovery from acidosis suggest that both triggers lead to a common pathological state of impaired muscle function.

We propose a mechanism by which recovery from acidosis produces a transient loss of force in HypoKPP muscle and present this notion in the context of exercise-induced attacks of weakness. Our proposed mechanism has two critical components. First, acidosis reduces the chloride conductance of the sarcolemma (Palade and Barchi, 1977; Pedersen et al., 2004, 2005). Second, in HypoKPP muscle with a bistable V_{rest}, low $[Cl]_{in}$ favors the hyperpolarized potential whereas high $[Cl]_{in}$ favors the depolarized state (Fig. 5 in Geukes Foppen et al., 2002; Cannon, 2018). This scheme is illustrated with the four-state diagram in Fig. 7. The top left quadrant represents the baseline resting state with a normal V_{rest}, G_{Cl}, and $[Cl]_{in}$. In Fig. 7, horizontal transitions represent a change in pH caused by exercise (rightward, producing acidosis) or rest (leftward, recovering from acidosis). Vertical transitions represent a change of $[Cl]_{in}$, with downward movement indicating an increase and upward being a decrease. We propose the pH changes are relatively fast (with abrupt changes in level of exercise or bath

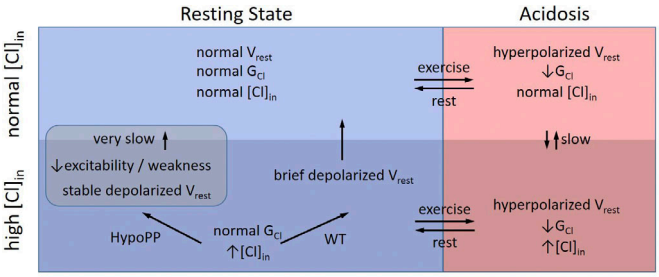

Figure 7. **Proposed scheme for post-acidosis loss of force in HypoKPP muscle.** Four states are depicted, with the basal conditions in the top left quadrant. Exercise promotes horizontal transitions to the right (acidosis), whereas rest favors a leftward shift back to the resting state. The build-up of susceptibility to post-acidosis weakness occurs on the right side, with a slow progressive shift to the bottom right quadrant as $[Cl]_{in}$ increases. Recovery from acidosis into the bottom left state (right to left transition in the bottom row) depolarizes V_{rest} because G_{Cl} recovers with a high $[Cl]_{in}$. In HypoKPP fibers, the bistable V_{rest} keeps the fiber depolarized and $[Cl]_{in}$ high. For WT fibers, V_{rest} repolarizes, and the large driving force on Cl^- through ClC-1 channels causes $[Cl]_{in}$ to rapidly return to the resting level.

exchange in our isolated muscle experiments) compared with the slow accumulation for $[Cl]_{in}$, which is limited by the influx rate of NKCC1 cotransporter and the surface area to volume for the myoplasm. Conversely, the washout of high $[Cl]_{in}$ after recovery to normal pH (and high G_{Cl}) is much faster because under a sufficient driving force through ClC-1 channels, Cl^- efflux rate is much higher.

Starting from the baseline top left quadrant in Fig. 7, exercise promotes a shift to the top right where acidosis rapidly decreases G_{Cl} and thereby causes a mild hyperpolarization of V_{rest} by a few millivolts because E_{Cl} is slightly depolarized from the normal V_{rest} (Aickin et al., 1989). If the exercise period is brief, then the process is quickly reversible and the muscle recovers back to the top left quadrant. With prolonged periods of exercise (e.g., tens of minutes), $[Cl]_{in}$ increases because Cl^- influx via NKCC1 exceeds efflux through ClC-1 with a reduced G_{Cl}. These changes are depicted as a slow shift down to the bottom right quadrant. If exercise is now abruptly stopped (i.e., the acidosis is rapidly reversed), then G_{Cl} rapidly increases. With the persistently high ratio of $[Cl]_{in}/[Cl]_{out}$, the depolarized E_{Cl} promotes a shift of V_{rest} to the depolarized state of the bistable system in HypoKPP muscle (bottom left quadrant). From this depolarized V_{rest}, sodium channels are inactivated, thereby causing reduced excitability and loss of force. This anomalous depolarization also keeps $[Cl]_{in}$ higher than baseline in HypoKPP fibers. In WT fibers, with G_{Cl} now increased back to its basal level, Cl^- efflux through ClC-1 now exceeds influx through NKCC1, and $[Cl]_{in}$ rapidly decreases until the baseline (top left) is reestablished and the net Cl^- flux is zero.

Several experimental observations presented herein are consistent with the mechanism proposed in Fig. 7. (1) Inhibition of NKCC1 with bumetanide prevents the post-acidosis loss of force (Fig. 5, C and D). Eliminating Cl^- influx via NKCC1 will prevent the build-up of $[Cl]_{in}$ in the low-G_{Cl} state (transition from top right to bottom right in Fig. 7). (2) Partial block of ClC-1 with 9-AC attenuates the loss of force (Fig. 6 A). When G_{Cl} remains low in 9-AC (independent of ΔpH), then the consequences of $[Cl]_{in}$ shifts on V_{rest} are attenuated. (3) Cl^--free conditions abolish the post-acidosis loss of force (Fig. 6 B). With no Cl^-, there can be no shift to the high-$[Cl]_{in}$ state (bottom row in Fig. 7). We propose the immediate loss of force (~40% drop) in 25% CO_2 in Fig. 6 B is likely due to a pH-dependent decrease of the inward rectifier conductance (Blatz, 1984). This G_{Kir} change minimally affects V_{rest} (and therefore force) when Cl^- is present because ClC-1 keeps V_{rest} near E_{Cl}. In the absence of Cl^-, however, then a modest decrease in G_{Kir} plus the gating pore leak of HypoKPP muscle produce depolarization and loss of force. (4) The kinetics for the onset of susceptibility to loss of force, the relatively fast drop in force, and duration of weakness are consistent with the model. The onset of susceptibility occurs very slowly, with a time constant of 33 min (Fig. 3 A), because the rate for the increase of $[Cl]_{in}$ is limited by the relatively small influx rate via NKCC1 cotransporter of ~20 pmol/(cm^2-s) (Gallaher et al., 2009). This is depicted by the slow transition from the top right to bottom right in Fig. 7. Conversely, the recovery from a transient loss of force occurs more quickly because the Cl^- efflux rate via the ClC-1 channel is much higher compared with the cotransporter (transition from bottom left to top left in Fig. 7). With an abrupt recovery of pH, the loss of force occurs relatively quickly with a large decrease in the first 2 min (Figs. 2 and 3). This rapid loss of force is consistent with our proposed rapid recovery of G_{Cl} (transition from bottom right to bottom left in Fig. 7), and is supported experimentally by the rapid loss of myotonia in HyperKPP muscle (Fig. 6 D). (5) The model is also consistent with the absence of a post-acidosis loss for force for HyperKPP muscle (Fig. 2 A). The importance of the Cl^- gradient occurs in the context of a bistable V_{rest} created by the presence of an anomalous gating pore conductance, which is the essential defect of mutant $Na_V1.4$ or $Ca_V1.1$ channels in HypoKPP (Cannon, 2018).

This initial report that a rapid recovery from acidosis triggers a robust loss of force in HypoKPP muscle has practical implications for the management of patients. First, these data clearly document the perils of resting motionless after a period of vigorous exercise—an activity notorious for eliciting an acute attack of HypoKPP. Second, they explain why a slow warm down after exercise may prevent triggering an attack of weakness. Moreover, the connection to pH suggests that simply increasing pCO_2 by rebreathing into a paper bag may ameliorate an impending attack. Third, this study supports the notion that mild acidosis may be part of the mechanism of action for carbonic anhydrase inhibitors in the prophylactic reduction of attack frequency and severity. While this study implicates recovery from acidosis as a likely contributing factor to postexercise weakness in periodic paralysis, additional mechanisms must be involved, for example, to account for the fact that postexercise weakness is also a prominent feature of HyperKPP.

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