pH buffering of single rat skeletal muscle fibers in the in vivo environment

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Tanaka Y, Inagaki T, Poole DC, Kano Y. pH buffering of single rat skeletal muscle fibers in the in vivo environment. Am J Physiol Regul Integr Comp Physiol 310: R926–R933, 2016. First published April 1, 2016; doi:10.1152/ajpregu.00501.2015.—Homeostasis of intracellular pH (pHi) has a crucial role for the maintenance of cellular function. Several membrane transporters such as lactate/H+ cotransporter (MCT), Na+/H+ exchange transporter (NHE), and Na+/HCO3− cotransporter (NBC) are thought to contribute to pHi regulation. However, the relative importance of each of these membrane transporters to the in vivo recovery from the low pHi condition is unknown. Using an in vivo bioimaging model, we pharmacologically inhibited each transporter separately and all transporters together and then evaluated the pHi recovery profiles following imposition of a discrete H+ challenge loaded into single skeletal muscle fibers by microinjection. The intact spinotrapezius muscle of adult male Wistar rats (n = 72) was exteriorized and loaded with the fluorescent probe 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxyethyl ester (10 µM). A single muscle fiber was then loaded with low-pH solution [piperazine-N,N′-bis(2-ethanesulfonic acid) buffer, pH 6.5, ∼2.33 × 10−3 µl] by microinjection over 3 s. The rats were divided into groups for the following treatments: 1) no inhibitor (CONT), 2) MCT inhibition (by α-Cyano-4-hydroxycinnamic acid; 4 mM), 3) NHE inhibition (by ethylisopropyl amiloride; 0.5 mM), 4) NBC inhibition (by DIDS; 1 mM), and 5) MCT, NHE, and NBC inhibition (All blockade). The fluorescence ratio (F500 nm/F445 nm) was determined from images captured during 1 min (60 images/min) and at 5, 10, 15, and 20 min after injection. The pHi at 1–2 s after injection significantly decreased from resting pHi (ΔpHi = −0.73 ± 0.03) in CONT. The recovery response profile was biphasic, with an initial rapid and more resistant to pH changes than many other cell types (an exception being CT26 colon cancer cells) (39). Within skeletal muscle, the pH consequences of metabolically produced H+ are constrained by intracellular buffering by bicarbonate (HCO3−) and proteins, such as carnosine, mitochondrial H+ utilization, and, crucially, by transport systems located on the sarcolemma that extrude H+ into the extracellular space. Skeletal muscle fibers have at least three transporters, which can extrude H+ from the fiber and contribute to pHi homeostasis: 1) lactate/H+ cotransporter (MCT) (1, 8, 19, 25, 34), 2) Na+/H+ exchange transporter (NHE) (23), and 3) Na+/HCO3− cotransporter (NBC) (26). Most studies associated with these transport systems have been performed under in vitro (no circulation) conditions, such that the contribution of these membrane transporters to the in vivo recovery process from low-pHi conditions has not been resolved. Using an in vivo bioimaging model, we documented the temporal profile of pHi recovery following imposition of a discrete H+ challenge loaded into single skeletal muscle fibers by microinjection and tested the impact of specific transporter inhibitors on that profile. Specifically, we tested the hypothesis that either one or all of these membrane transport system's contributions are crucial to pHi recovery.


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PRACTICALLY ALL PHYSIOLOGICAL processes are pH-sensitive, and pH perturbations can cripple cellular function (7, 12, 14, 31, 32, 39, 42, 43, 46). In physiological [heavy and severe-intensity exercise (37)] and pathological (e.g., local ischemia, tissue hypoxia, renal disease, diabetic ketoacidosis, and respiratory failure) conditions, skeletal muscle fibers undergo profound [H+] disturbances. Skeletal muscle intracellular pH (pHi) is maintained about 7.1 under resting noncontracted conditions (2, 3, 16, 48). H+ is produced by energy metabolism and, during severe-intensity exercise, pHi plummets due to accumulation of H+, consequent, in part, to activation of glycolysis and the powerful lactate dehydrogenase system that converts pyruvic to lactic acid, irrespective of the intracellular O2 availability (29, 38). Although not unequivocal (9, 10, 33, 48), acidosis is generally held to be an important factor in the etiology of skeletal muscle fatigue and exhaustion, and there is certainly evidence for low pHi reducing myofibrillar Ca2+ sensitivity at near-physiological temperatures and suboptimal Ca2+ conditions (43). Thus, there is a current and compelling mandate for elucidating pHi homeostatic mechanisms in as close-to-physiological conditions as possible in a mammalian system.
METHODS

Animals

Male Wistar rats (n = 72 rats, 10–14 wk of age; Japan SLC) were used in this study. Rats were maintained on a 12:12-h light-dark cycle and received food and water ad libitum. All experiments were conducted under the guidelines established by the Physiological Society of Japan and were approved by the University of Electro-Communications Institutional Animal Care and Use Committee. The rats were anesthetized using pentobarbital sodium (60 mg/kg ip), and supplemental doses of anesthesia were administered as needed. At the end of the experimental protocols, animals were killed by pentobarbital sodium overdose.

Muscle Preparation

All experimental techniques, including the spinotrapezius muscle preparation, were performed as described previously (15, 41). Briefly, the right spinotrapezius muscle was gently exteriorized with minimal blood loss and tissue/microcirculatory damage and attached to a wire horsehoe around the caudal periphery by six equidistant sutures placed around the muscle perimeter. The exposed muscle tissue was kept moist by superfusing with warmed Krebs-Henseleit Buffer (KHB; in mM: 132 NaCl, 4.7 KCl, 21.8 NaHCO3, 2 MgSO4, and 2 CaCl2) equilibrated with 95% N2-5% CO2 and adjusted to pH 7.4, at 37°C. The fluorescent pH indicator 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM, 5 mM; Dojindo Laboratories, Kumamoto, Japan) was dissolved in DMSO and dispersed into KHB solution at a final concentration of 10 μM. The muscles were incubated in BCECF-AM/KHB solution for 30–60 min on a 37°C hotplate. After incubation, muscles were rinsed with dye-free KHB solution to remove non-loaded BCECF-AM.

Image Analysis

The spinotrapezius muscles loaded with BCECF-AM were mounted on the 37°C glass hotplate (Kitazato Supply, Tokyo, Japan) and observed by fluorescence microscopy using a 10× objective lens (0.30 numerical aperture; Nikon, Shinagawa, Japan). After ensuring that the spinotrapezius muscle was not grossly damaged and could support robust capillary blood flow, a sampling area (~880 × 663 μm) was selected using branching vessels as landmarks, and bright-field images were captured. Thereafter, 445-nm and 500-nm wavelength excitation light was delivered using a Xenon lamp equipped with appropriate fluorescent filters, and pairs of fluorescence images were captured through the 535-nm emission wavelength filter for ratiometry. Fluorescence images were captured by a high-sensitivity digital camera (ORCA-Flash2.8; Hamamatsu Photonics, Hamamatsu City, Japan) using image-capture software (NIS-Elements Advanced Research, Nikon).

In Vivo Calibration of pH

In the in vivo intracelluar calibration was conducted according to Westerblad and Allen (47). Specifically, K+ calibration solution (130 mM KCl, 1 mM dextrose, 1 mM MgCl2, 10 mM KOH, 1 mM EGTA, 20 μM nigercin, 15 mM BDM, 10 mM HEPES, or 15 mM PIPES, pH 6.5–7.4), incorporating the K+/H+ ionophore (nigercin) was superfused continuously onto the muscle fiber surface. The calibration value was calculated from the stable fluorescence intensity ratio following application of the calibration solution. As shown in Fig. 1, there was a significant correlation between fluorescence ratio and superfused solution pH. The regression equation was R/R₀ = 0.4557 pH – 2.320 (r² = 0.92, P < 0.01). Subsequently, the pH was calculated from the ratio R (F500 nm/F445 nm) using this equation.

Experimental Protocols

Animals were divided randomly into four groups. Group 1: in vitro or in vivo control group. The resting in vitro control experiment was performed after surgically isolating the spinotrapezius muscle to interrupt its vascular and, therefore, oxygen supply and neural connections, and loading the muscle with BCECF-AM before microscopy. Sequential fluorescence images were captured every 5 min for 20 min. The in vivo control experiment was the same as for in vitro, except that the muscle was exteriorized with the principal vascular and neural pathways maintained intact. Group 2: in vivo injection of low-pH solution group. Capillary micropipettes were generated with a tip of ~1 μm in diameter, which was achieved by custom grinding and inserted into the selected single muscle fiber using a micromanipulator precision-controlled advancer (IM300, Narishige, Japan), a single muscle fiber was then loaded with a low-pH solution (in mM: 122 KCl, 11 dextrose, 1.0 MgCl2, 18 KOH, 15 PIPES, 1.0 EGTA, at 37°C and pH 6.5) by microinjection at 35 psi (24,000 Pa) for 1 s (~0.78 × 10⁻³ μl) or 3 s (~2.33 × 10⁻³ μl). In the inhibition groups, the spinotrapezius muscle was treated with each blocker and injected with the low-pH solution 5 min later. The fluorescence ratio was determined from images captured for 30 s (1 image/s) before injection, and at 1 (60 images/min), 5, 10, 15, and 20 min after injection. Group 3: in vivo low pH injection with carbonic anhydrase and transporter (MCT, NHE, and NBC) inhibition. This protocol was the same as for the 3-s injection (control, CONT) above, except that the KHB superfusate contained either the carbonic anhydrase (CA) inhibitor acetazolamide (0.5 mM) (45) or the pertinent inhibitors of the H+ membrane transporters. For inhibition of each membrane transporter (MCT, NHE, NBC), this protocol was applied using one or all (i.e., All blockade) of the following: α-Cyano-4-hydroxy-cinnamic acid (CHCA), 5-(N-Ethyl-N-isopropyl) amylid (EIPA), and 4′,4′-diisothiocyano2,2′-stilbenedisulfonic acid (DIDS). Each inhibitor was dissolved in DMSO and diluted with KHB. The dilution concentration was adjusted to either equal or surpass levels (CHCA: 4 mM, EIPA: 0.5 mM, and DIDS: 1 mM) reported to be effective previously (21, 24, 45). We performed the verification of substantial function of each inhibitor in in vivo conditions. As a result of these conditions, pH under the inhibition condition was transiently reduced compared with in vivo control condition (ΔpH, MCT: −0.32 ± 0.06, NHE: ΔpH, −0.43 ± 0.10, and NBC: ΔpH, −0.29 ± 0.08). The rat spinotrapezius muscle comprises both principal fiber types (32.5% type I and 66.5% type II) (15). The protein content of three transporters of the rat
spino-trapezius muscle is unknown. However, whereas the MCT content is dependent on the muscle fiber composition, NBC and NHE are present in all muscle fiber types without a known inter-fiber-type difference.

**Group 4: In vivo low-pH superfusion group.** This protocol was the same as for group 2 above, except that the low-pH solution (6.5) was continuously superfused onto the surface of a muscle fiber. The pH profile of that fiber was evaluated and compared with that of fibers adjacent to the directly injected fiber (i.e., as described in group 2).

**Modeling of pH Profiles**

Curve fitting was accomplished using KaleidaGraph software (version 4.1, Synergy Software, Reading, PA) and was performed on the pH data using a one-component model: $pHi(t) = pHi(b) + \Delta pHi \left[1 - e^{-\tau/(P - \text{time delay}, \text{TD})^{2}}\right]$, where $pHi(t)$ is a given time point, $pHi(b)$ is the base level just after the injection, and $\Delta pHi$ is the increase in $pHi$ from base level to the recovery values at 5 min.

To determine whether the one-component model would best describe the $pHi$ response for either muscle, the goodness-of-fit for the model was determined via three criteria: 1) the coefficient of determination (i.e., $r^{2}$), 2) the sum of the squared residuals term (i.e., $\chi^{2}$), and 3) visual inspection of the model fit to the data. Mean recovery $pHi$ was determined by integration over the 20-min postinjection observation period.

**Statistical Analysis**

All experimental data are expressed as means ± SE. All statistical analyses were performed in Prism version 6.0 (GraphPad Software, San Diego, CA). Comparisons among different groups were analyzed by either one-way or two-way ANOVA followed by Tukey’s multiple-comparison test or Bonferroni (groups 1 and 2) and Tukey (groups 3 and 4) post hoc test, respectively. The level of significance was set at $P < 0.05$ (two-tailed).

**RESULTS**

**Group 1: In Vitro or In Vivo Control Group**

Two-way repeated-measures ANOVA showed a significant effect of condition (in vivo vs. in vitro, $P = 0.0007$). Under the in vitro conditions, a significant fall in $pHi$ ($P < 0.05$) was observed at 10 min after starting the protocol and reached a low value of $7.05 \pm 0.04$ at the 20-min point (Fig. 2). In contrast, there was no significant change over the 20-min observation period for the in vivo muscles. These results justified selection of the in vivo muscle preparation for all subsequent $H^{+}$-loading protocols.

**Group 2: In Vivo Injection of Low-pH Solution Group**

The dynamics of in vivo $pHi$ following injection of the low-pH solution (1-s injection, or i-1s or 3-s injection, or i-3s) are displayed in the photomicrographs and the temporal profiles in Fig. 3. Two-way repeated-measures analyses showed a significant pH decrease ($P < 0.0001$) with the $pHi$ at 1–2 s after injection falling by $\Delta pHi = 0.48 \pm 0.03$ (i-1s)
and ΔpH, −0.73 ± 0.03 (i-3s). The recovery profile was biphasic with an initial very rapid pH recovery observed within the first 60 s after injection in both i-1s (−0.30 ± 0.03) and i-3s (−0.52 ± 0.05) conditions. Subsequently, there was a more gradual return toward baseline by 20 min (i-1s: −0.15 ± 0.03, i-3s: −0.35 ± 0.04). The recovery dynamics of both conditions were similar, but the i-3s recovery rate was significantly slower than for the i-1s.

Group 3: In Vivo Low-pH Injection with CA and Transporter (MCT, NHE, and NBC) Inhibition

We verified that each inhibitor functioned under in vivo conditions before low-pH injection. As shown in Fig. 4, a simple one-component exponential model with delay fit the pH recovery responses over the first 5 min after injection (CONT: \( r^2 = 0.990 \pm 0.004 \), MCT: \( r^2 = 0.990 \pm 0.221 \), NHE: \( r^2 = 0.987 \pm 0.009 \), NBC: \( r^2 = 0.996 \pm 0.002 \), All blockade: \( r^2 = 0.982 \pm 0.007 \), CA: \( r^2 = 0.990 \pm 0.005 \)). Two-way repeated-measures analyses identified a significant interaction (\( P < 0.0001 \)) with CA prolonging the rapid pH recovery response over the first 5 min after injection (Table 1). On the other hand, there was no detectable influence of the membrane H⁺ transport blockers in the rapid pH recovery response (Table 1 and Fig. 4). Specifically, the kinetics (TD, \( \tau \), MRT) and absolute extent of pH recovery were not significantly different across CONT and membrane H⁺ transport blockade conditions for the rapid initial phase of recovery. In contrast, for the more prolonged pH recovery phase (5–20 min), whereas neither MCT, NHE, nor NBC blockade individually impacted the response, All blockade abolished further recovery with pH at 10, 15, and 20 min being significantly lower than CONT (Fig. 4).

Group 4. In Vivo Low-pH Superfusion Group

Two-way repeated-measures analyses revealed a significant effect (\( P < 0.0001 \), Fig. 5) for the 3-s injection (i-3s) and the superfused conditions. For the i-1s condition, there was no significant pH change in the fibers surrounding the targeted fiber over the 20-min observation period (1 min: 7.27 ± 0.01, 20 min: 7.31 ± 0.01). In contrast, the i-3s condition significantly decreased pH in the surrounding fibers progressively up until the 20-min postinjection experimental endpoint (1 min: 7.21 ± 0.01, 20 min: 7.08 ± 0.02). Superfusion directly onto the surface of the muscle fiber also decreased pH until the 20-min point evaluated (1 min: 7.16 ± 0.01, 20 min: 7.11 ± 0.03, \( P < 0.05 \)).

Table 1. Profiles of recovery pH kinetics after injection of low pH solution in CONT CA and transporter (MCT, NHE, and NBC) inhibition; pH recovery responses at first 5 min after injection

<table>
<thead>
<tr>
<th></th>
<th>CONT (i-3s)</th>
<th>CA</th>
<th>MCT</th>
<th>NHE</th>
<th>NBC</th>
<th>All Blockade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak ΔpH</td>
<td>−0.70 ± 0.04</td>
<td>−0.88 ± 0.03</td>
<td>−0.77 ± 0.19</td>
<td>−0.73 ± 0.07</td>
<td>−0.81 ± 0.07</td>
<td>−0.81 ± 0.04</td>
</tr>
<tr>
<td>Recovery ΔpH</td>
<td>0.40 ± 0.04</td>
<td>0.56 ± 0.05</td>
<td>0.40 ± 0.11</td>
<td>0.41 ± 0.10</td>
<td>0.56 ± 0.05</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>TD, s</td>
<td>2.7 ± 1.2</td>
<td>1.8 ± 1.3</td>
<td>0.4 ± 0.3</td>
<td>2.1 ± 1.0</td>
<td>1.9 ± 0.7</td>
<td>4.1 ± 2.4</td>
</tr>
<tr>
<td>( t, ) s</td>
<td>60.0 ± 7.9</td>
<td>137.3 ± 28.8*</td>
<td>56.8 ± 14.1</td>
<td>48.1 ± 7.0</td>
<td>47.3 ± 10.9</td>
<td>39.9 ± 14.6</td>
</tr>
<tr>
<td>MRT, s</td>
<td>62.7 ± 8.1</td>
<td>139.1 ± 28.2*</td>
<td>57.2 ± 14.1</td>
<td>50.3 ± 7.2</td>
<td>49.1 ± 10.6</td>
<td>44.0 ± 13.3</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. pH, intracellular pH; TD, time delay; \( t \), time constant, MRT, mean response time (TD + \( t \)); CONT, control, no inhibitor; CA, carbonic anhydrase; MCT, lactate/H⁺ cotransporter; NHE, Na⁺/H⁺ exchange transporter; NBC, Na⁺/HCO₃⁻ cotransporter; i-3s, 3-s injection. *Significant difference (\( P < 0.05 \)) vs. CONT (i-3 s).
DISCUSSION

This study determined that the three membrane transporters MCT, NBC, and NHE in toto play an essential role in pH recovery from a measured H⁺ load delivered by microinjection into single mammalian muscle fibers in vivo. The principal original findings included identifying two discrete components of the recovery dynamics; an initial rapid and close-to-exponential phase, and a subsequent more gradual phase. The kinetics and magnitude of the rapid phase were independent of membrane transporter function, which coheres with a HCO₃⁻ and protein-buffering etiology. In marked contrast, the gradual recovery phase was abolished by inhibiting all three membrane transporter systems simultaneously. Interestingly, there was a significant decrease in pH throughout this period in surrounding muscle fibers, which supports that H⁺ uptake by neighboring fibers can help alleviate the pH consequences of myocyte H⁺ exudation on extracellular and vascular pH. This observation also coheres with the recent discovery that some muscle cells are better at accepting H⁺ than others at least in the C2C12 line (39). Overall, these results identify crucial in vivo properties of skeletal muscle that contribute to pH homeostasis by coupling cytoplasm-interstitial tissue-blood H⁺ transport via the membrane transporter system.

pH Recovery Dynamics from Acidosis

The kinetics analyses together with the selective effects of All blockade on the prolonged but not on the rapid pHᵢ recovery support that 1) there are two discrete phases of pHᵢ recovery and 2) these phases are controlled by different mechanisms. The pHᵢ τ of 40–137 s across conditions presented in Table 1 indicates that the rapid recovery phase will be >83% complete by 5 min and, therefore, unable/unlikely to contribute further to pHᵢ recovery.

As mentioned above, myocyte pHᵢ homeostasis is regulated by the combined influence of cytoplasmic buffering by HCO₃⁻ and other H⁺ acceptors, such as the dipeptide carnosine, H⁺ utilization by mitochondria and H⁺ efflux across the sarcolemma by the transmembrane transporters (45). As shown in Fig. 3, and in agreement with previous in vitro studies in isolated alveolar type II cells (45), the recovery of pHᵢ from acidification consisted of two phases (rapid followed by gradual increase). Also, Leem and Vaughan-Jones (27) reported that there are two kinetic phases of pHᵢ recovery from metabolic alkali load in heart muscle.

Rapid recovery phase. That the rapid pHᵢ recovery phase is unaffected by membrane transporter pharmacological inhibition implicates alternative sources of H⁺ buffering and removal (Fig. 5, Table 1). In this regard, it is well known that anions such as diprotonated phosphate (HPO₄²⁻) and bicarbonate (HCO₃⁻), as well as carnosine, serve to buffer H⁺ in the intracellular compartments (5, 13, 49). Specifically, carbonic anhydrase (CA) in the cytoplasm catalyzes the rapid reversible chemical reaction: H₂O + CO₂ ↔ HCO₃⁻ + H⁺ (4, 18). The present investigation demonstrated that compromising the bicarbonate-buffering action with CA significantly constrained the rapid phase of the pH recovery. With respect to carnosine content, rat mixed-fiber type muscles contain a modest 11–16 mM/kg dry wt (17). This compares to 54, 85, and 180 mM/kg dry wt for the horse musculus gluteus medius type I, type IIa, and type IIb fibers, respectively (40), which may relate to their prodigious lactic acid production, which increases blood lactate concentration up to 30 mM within 2–4 min of exercise (36). By process of elimination (vide supra), these H⁺ removal/buffering systems, in addition to any H⁺ dispersion along the fiber, determine the time constant (τ) of the rapid pHᵢ recovery seen after H⁺ injection whether or not the membrane transporters were inhibited.

Gradual recovery phase. As shown in Fig. 4, simultaneous application of the three pharmacologic transporters (MCT, NHE, and NBC) inhibitors completely abolished the pHᵢ recovery from 5 to 20 min. On the other hand, no significant reduction of the buffering potential was found in any single inhibition condition. These results suggested that several transporters cooperatively provide the intramyocyte H⁺ loading curve, such that of acid loading. Acid-sensitive myocytes demonstrated a downward shift in their H⁺ extrusion curve combined with an upward shift in their H⁺ loading curve, such that their steady-state pH shifted to a lower value than seen initially (see Fig. 11C of Ref. 39). Moreover, close to half of the myocytes adapted to this challenge, such that their response to a second identical HCO₃⁻ reduction was either markedly reduced or, alternatively, they became alkalinized, whereas 25% maintained their original responses and 29% decompensated. This cluster of behaviors may help explain how steady-
state pHi after low-pH solution injection shifts to a value that is lower than the baseline condition in some fibers. Certainly, it was not related to any myocyte damage consequent to the injection per se, as we confirmed that pHi was restored to resting values (7.21 ± 0.04) after injection of the neutral pH 7.2 solution (data not shown in Fig. 3).

The present in vivo and previous in vitro studies provide evidence that H+ extrusion via NHE and HCO3− entry via NBC is primarily responsible for the gradual pHi recovery observed in this phase. That complete blockade of any pHi recovery in the gradual phase is achieved under the All blockade condition supports that mitochondrial or other means of H+ removal is minimal. It is also pertinent that the MCT transporters will likely assume a proportionately greater role in removing H+ from the myocyte by extrusion across the sarcolemma and also into the mitochondria when the pHi is reduced by the lactic acidosis of intense contractions/exercise. This remains to be verified experimentally.

Acidification of Adjacent Muscle Fibers

pHi homeostasis is accomplished by several systems, including cytoplasmic H+ buffering, mitochondrial H+ utilization, and H+ excretion by transporters working in concert. Elucidation of the role of these systems has relied extensively upon in vitro experimental approaches using isolated muscle fibers. Thus, real-time in vivo evaluation as employed herein is absolutely necessary to resolve the mechanistic bases for pHi homeostasis and establish the participation of, and interrelationships among, the predominant H+ removal systems. Interestingly, significant reduction of pHi was observed in not only the injected fiber but also in the neighboring fibers. Note in Fig. 5 that pHi reduction of the H+-superfused fibers was more rapid than that seen in those muscle fibers adjacent to a low-pH-injected fiber. This occurrence is likely to reflect the additional inertia associated with the H+ extrusion step that is avoided when the acid load is superfused directly onto the fibers in question.

Lactate was once thought to be a dead-end metabolite before radiolabeled tracers were used to demonstrate its uptake (presumably in conjunction with H+) by muscle fibers and metabolism in the Kreb’s cycle (11). Thus, it is now known (39), and evident herein, that H+ can be taken up from the extracellular space by the membrane transporters, potentially for consumption by the mitochondria. Moreover, different muscle fibers (and potentially fiber types) may preferentially release or uptake H+ from the intracellular space, although this possibility remains to be verified. Irrespective of whether or not a fiber-type-selective activity of the membrane transporters is present, this behavior relieves the H+ load that must be buffered in the interstitial space and vascular compartment, especially during severe-intensity exercise (44). Intriguingly, Salameh et al.’s (39) data suggest the presence of an ion channel that promotes H+ influx as an additional method to deal with the contracting muscle H+ load (39). The molecular mechanisms for this effect remain to be determined.

Methodological Considerations

In the present investigation, it was crucial that the entire microcirculation and especially the capillaries sustained red blood cell flow throughout the experimental period, as this characterizes the healthy skeletal muscle microcirculation (6, 35). pHi homeostasis cannot be sustained in the absence of adequate blood flow, and ischemia and the resultant tissue hypoxia drive a metabolic acidosis (20, 22, 28). This condition cannot be corrected effectively by exposing in vitro tissue to hyperoxic gases, as this leads to unphysiologically high O2 partial pressures at the tissue-gas interface and the likelihood of hypoxia/anoxia remains in the tissue core. Therefore, it is necessary to elucidate homeostatic mechanisms for pHi maintenance in close-to-physiological conditions. We have previously observed that calcium ion homeostasis is sustained for at least 90 min in the spinotrapezius muscle in vivo model employed herein (15, 41). Accordingly, as evident in Fig. 2, in the absence of acidic challenge, pHi was maintained close to control (i.e., initial) values over the entire 20-min period for the in vivo data collection. In marked contrast, the in vitro (total ischemia) condition (Fig. 2) reduced pHi significantly within 10 min and further up to 20 min. This finding coheres with the report from Marcinek et al. (28) that pH, estimated from 31P-magnetic resonance spectra, fell from 7.0 to 6.7 during 20 min of ischemia in the mouse hindlimb (28). 

Previous studies have suggested that the three membrane transporters that we tested in this study have a primary role in mammalian skeletal muscle intramyocyte H+ regulation. Our data demonstrate that, at least for the H+ load investigated herein, no single one of these transporters is essential for the gradual pH recovery observed, whereas inhibition of all three abolished this phase of recovery. This does not mean that there are not additional H+ transport mechanisms across the sarcolemma; such as the voltage-gated proton (Hv1) channel described recently by Salameh et al. (39). However, what we can say is that during inhibition of NHE, NBC, and MCT transporters, we were unable to detect any pHi recovery from 5 to 20 min (Fig. 4). Moreover, our inability to detect any effect of single inhibitors does not constitute evidence that recovery from a greater H+ challenge does not require the action of all three transporters.

Perspectives and Significance

Using a circulation-intact in vivo bioimaging model, we challenged intramyocyte homeostasis by loading H+ into single fibers by microinjection. We modeled the rapid exponential pHi recovery dynamics and determined that the kinetics (i.e., mean response time) of this phase is not dependent upon H+ extrusion across the sarcolemma, as it remains intact after the three principle H+ membrane transporters (MCT, NHE, and NBC) have been inhibited. This suggests that this rapid recovery phase is determined primarily by H+ buffering/removal in the myocyte by HCO3−, carnosine, and any mitochondrial H+ usage. Of these, we demonstrated that HCO3− is an essential component of the rapid pH recovery in the rat spinotrapezius. In marked contrast, the subsequent gradual recovery phase of pHi was unimpeded by single blockade of the MCT, NHE, and NBC transporters but prevented by blockade of all transporters. We also noted that H+ was removed from the interstitial compartment by adjacent muscle fibers, suggesting that they play an important role in sharing the H+ load, thereby limiting the pH consequences of an intramuscular acidosis within the interstitial and blood compartments. This behavior may be contingent on the action of a recently described H+ ion channel.

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that supports $H^+$ intrusion (39). We contend that this circulation-intact model offers a unique opportunity to investigate the consequences of experimental and physiological manipulations of $H^+$ on muscle pH dynamics under close-to-physiological conditions. Key pressing questions include how the concentrations and fluxes of, for example, $H^+$, $Na^+$, $Cl^-$, $Ca^{2+}$, and $CO_2$ change in the intracellular, interstitial, and blood compartments during and after intense skeletal muscle contractions.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS

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