Effects of intracellular acidosis on Ca\textsuperscript{2+} activation, contraction, and relaxation of frog skeletal muscle

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Baker, A. J., R. Brandes, and M. W. Weiner. Effects of intracellular acidosis on Ca\textsuperscript{2+} activation, contraction, and relaxation of frog skeletal muscle. Am. J. Physiol. 268 (Cell Physiol. 37): C55–C63, 1995.—The goal of this study was to determine the effects of intracellular acidosis (pH ~ 6.3) of frog skeletal muscle on force and on intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}; measured at 20°C using indo 1 fluorescence). Acidosis reduced tetanic force by only 11 ± 2% (mean ± SF, n = 8) but increased tetanic [Ca\textsuperscript{2+}]\textsubscript{i} by 33 ± 6%, suggesting that acidosis reduced the maximum Ca\textsuperscript{2+}-activated force. During relaxation, the [Ca\textsuperscript{2+}]\textsubscript{i} at half-maximal force was doubled with acidosis, suggesting that acidosis altered the Ca\textsuperscript{2+}–force relationship. Acidosis markedly slowed force relaxation and [Ca\textsuperscript{2+}]\textsubscript{i} decline (time constants fitted to force and [Ca\textsuperscript{2+}]\textsubscript{i}; during relaxation increased by 133 ± 20 and 68 ± 13%, respectively, with acidosis), suggesting that slowed force relaxation with acidosis may arise from slowed Ca\textsuperscript{2+}–cytosol. Late in relaxation, at ~ 30% of initial force, there was a transient phase of [Ca\textsuperscript{2+}]\textsubscript{i} increase that was delayed with acidosis in proportion to the slowing of force relaxation. This is consistent with previous suggestions that dissociation of cross-bridges from the thin filament during relaxation promotes Ca\textsuperscript{2+}–cytosol. This study concludes that in skeletal muscle acidosis has little effect on tetanic force and that the major effects are decreased Ca\textsuperscript{2+} sensitivity and slower relaxation.

pH, indo 1, fatigue, excitation-contraction coupling

With fatiguing exercise, intracellular pH (pH\textsubscript{i}) may fall to ~ 6.3 because of anaerobic glycolysis (4). Low pH\textsubscript{i} is thought to impair contraction and relaxation (8, 12, 13, 24, 26, 29). However, the contribution of decreased pH\textsubscript{i} to decreased force is uncertain. Measurement of pH\textsubscript{i} using \textsuperscript{31}P nuclear magnetic resonance (NMR) found that during fatigue decreased pH\textsubscript{i} correlated with decreased force (4, 13, 22), suggesting that increased [H\textsuperscript{+}] may impair contraction. Consistent with this, the force produced by skinned muscle fibers was inhibited by low pH\textsubscript{i} (10, 11). However, in living muscle after fatiguing exercise, force recovered much more rapidly than pH\textsubscript{i} (4, 6). This finding was not consistent with a role for H\textsuperscript{+} in regulating force and suggested that in vivo the relationship between force and pH\textsubscript{i} found during fatigue may have been coincidental.

Intracellular acidosis may be experimentally induced by hypercapnia. However, the effects of hypercapnic acidosis on force production have varied. Some studies found that hypercapnia decreased developed force (12, 24, 26, 29); more recently, both mild and severe hypercapnic acidosis caused very little decrease in force (1, 21, 27).

 Despite the differences observed for the effects of acidosis on force, a more consistent finding has been that acidosis, due either to fatigue or hypercapnia, was associated with slower force relaxation (1, 12, 13, 21, 24, 26, 27, 29). Slowed relaxation with acidosis could arise from slowed cross-bridge cycling (14, 27). Slowed cross-bridge cycling with acidosis has been inferred from the decreased adenosinetriphosphatase (ATPase) and shortening velocity of skinned fibers at low pH (10, 11). Alternatively, slowed relaxation could arise from slowed clearance of Ca\textsuperscript{2+} from the cytosol at the end of contraction (14, 27). With a moderate reduction of pH\textsubscript{i} induced by hypercapnia, impaired clearance of Ca\textsuperscript{2+} was inferred (2, 27). However, this was not thought to be responsible for the slowed force relaxation (27).

Given the uncertainties of the role of [H\textsuperscript{+}] as a regulator of force production and relaxation, the goal of this study was to determine the effects of severe intracellular acidosis (pH ~ 6.3) in skeletal muscle on Ca\textsuperscript{2+} activation, contraction and relaxation. The experimental approach was to monitor intracellular Ca\textsuperscript{2+} levels (using the Ca\textsuperscript{2+} indicator indo 1) and force during contractions of intact bullfrog semitendinosus muscle at physiological pH and after acidosis was induced by hypercapnia. These experiments extend previous studies by examining the effects of severe acidosis (pH ~ 6.3) on both force and Ca\textsuperscript{2+}. Because the indo 1 dissociation constant for Ca\textsuperscript{2+} and fluorescence spectra are markedly affected by intracellular protein and acidosis (3), calibration constants derived in the presence of protein and appropriate pH were used.

METHODS

Force and Ca\textsuperscript{2+} signals were monitored at 20°C during twitch and tetanic contractions in whole bullfrog muscles before and after induction of acidosis. Methods for loading muscles with indo 1 and recording force and Ca\textsuperscript{2+} signals were previously described in detail (5).

Muscle Preparation and Indo 1 Loading

Bullfrogs (Rana catesbeiana; 4–5 in. long) were double pithed. The ventral head of the semitendinosus muscle was used (weight = 0.36 ± 0.03 g; length = 4.4 ± 0.12 cm; cross section was approximated as an ellipse with a major axis width = 4.8 ± 0.2 mm and minor axis width = 3.3 ± 0.2 mm; mean ± se, n = 8). The muscle artery was cannulated and the muscle was removed to a bath of oxygenated Ringer solution [contents in mM: 111 NaCl, 2.5 KCl, 2 CaCl\textsubscript{2}, 10 tris(hydroxymethyl)aminomethane; pH set to 7.2 with HCl] at 30°C. The muscle was perfused for 40 min at 4 ml/h with an oxygenated loading solution consisting of Ringer solution, 5 \textmu M indo 1 acetoxymethyl ester (AM; Molecular Probes, Eugene, OR), 5% calf serum, 0.1 mM probenecid, 0.2% Pluronic F127, and 1%
dimethyl sulfoxide. After loading, muscles were washed in oxygenated Ringer solution, and perfused with oxygenated Ringer solution for 1 h at 0°C.

**Physiological Methods**

Loaded muscles were mounted in a bath of oxygenated bicarbonate buffer (contents in mM: 118 NaCl, 3 KCl, 1 MgSO₄, 1.8 CaCl₂, 25 NaHCO₃, 1 NaHPO₄, and 5 glucose) and arterially perfused with the same solution. Initially, the pH of the buffer (pH₀) was set to pH₅ 7.3 ± 0.01 (n = 8) by equilibration with a gas mixture of 95% O₂-5% CO₂. Later, pH₀ was reduced by raising the CO₂ level in the gas mixture to 80% CO₂-20% O₂, which lowered the extracellular pH to pH₅ 6.2 ± 0.005 (n = 8). This acidification is expected to decrease to pH₅ ~6.3 based on the relationship between pH₀ and pH₅ previously determined in cat fast-twitch muscle using ³¹P-NMR (1). Muscles were anchored at one end and tied onto a force transducer at the other (Cambridge Technology model 300B). A light guide was placed over the surface of the muscle for monitoring Ca²⁺ signals from indo 1 fluorescence. Muscles were electrically stimulated (0.1-ms pulse duration, voltage twice that needed for maximum force) through parallel platinum wire electrodes placed on either side of the muscle. Muscle length was adjusted to maximize the force (resting tension was 2.5 ± 0.8% of tetanic). Force and Ca²⁺ signals were measured during a twitch in response to a single stimulus and during a tetanus (200-ms duration, 100-Hz stimulus frequency).

During relaxation, the rate of force loss reached a maximum; thereafter relaxation was closely described by an exponential decay. The time constant of the decline in force (τₚ) was calculated by a least-squares fit of the exponential fall of force according to

$$P_t = (P_o - 0) \cdot e^{-t/\tau_p} + b$$

where P₀ and Pₚ are the forces at the start of the exponential fall of force and after time t, respectively, and b is the baseline level.

**Optical Measurements**

Ca²⁺ signals were recorded using a modified SLM 48000S spectrofluorometer (SLM Instruments, Urbana, IL). The common arm of a bifurcated quartz fiber-optic cable was placed over the muscle to provide illumination and to collect fluorescent light. During each contraction the muscle was briefly illuminated with light filtered at 350 nm (10-nm bandwidth). Fluorescent light from the muscle was split with a dichroic mirror, and each beam filtered (at 400 or 470 nm; 10-nm bandwidth). Changes in [Ca²⁺]i during a tetanus (200-ms duration, 100-Hz stimulus frequency) were recorded during 750 ms of relaxation, and fitting was performed over the first 50 ms and last 300 ms.

**Calibration Methods**

The ratio (R) of fluorescence intensities measured at 400 and 470 nm was related to [Ca²⁺] according to Grynkiewicz et al. (16)

$$[Ca^{2+}] = K_d \cdot S_{470} \cdot (R - R_{min})/(R_{max} - R)$$

where Rₘᵢₘ and Rₘₐₓ are the ratios at zero and saturating [Ca²⁺], respectively, S₄₇₀ is the ratio of fluorescence at 470 nm at zero and saturating [Ca²⁺], and K₅ is the dissociation constant. [Ca²⁺] was calculated from in vivo fluorescence by the method of Brandes et al. (7), deriving in vivo Rₘᵢₘ and Rₘₐₓ according to

$$R_{max} = [(1 - S_{470})/(1 - S_{400})] / bH$$

$$R_{min} = R_{max} \cdot S_{400} / S_{470}$$

where bH is the slope of the relationship between the intensity measured in vivo at 470 nm vs. the intensity at 400 nm and S₄₀₀ is the ratio of fluorescence at 400 nm at zero and saturating [Ca²⁺]. We have previously made a detailed characterization of the effects of frog muscle proteins and acidosis on in vivo fluorescence spectra and K₅ (3). In frog muscle proteins at pH 7.3, S₄₀₀, S₄₇₀, and K₅ were previously determined to be 0.158, 2.567, and 788 nM, respectively, and at pH 6.3, these values were 0.184, 2.509, and 1,696 nM, respectively (3). The SE of these estimates was between 1.1 and 3.5% (3).

**Statistics**

All measurements were expressed as means ± SE (n = 8). The Student’s t-test and correlation coefficients were used to compare different data sets, using a significance level of P < 0.05.

**RESULTS**

**Changes in [Ca²⁺] During Contraction**

Figure 1 shows typical records during a tetanus of background-corrected indo 1 fluorescence intensities measured at wavelengths of 400 (I₄₀₀) and 470 nm (I₄₇₀), fluorescence ratio R of I₄₀₀/I₄₇₀, and calculated [Ca²⁺]. The changes in [Ca²⁺] were similar to those found in previous studies of single fibers (2, 20) or whole muscle (5). Within a few milliseconds after the start of stimulation, [Ca²⁺] reached an almost steady tetanic level, which tended to slowly rise throughout stimulation. During relaxation, [Ca²⁺] declined in three distinct phases similar to those described by Cannell (9) and others (2, 5, 20). In phase 1, [Ca²⁺] declined rapidly. In phase 2, the [Ca²⁺] decline was markedly slowed, and in some experiments, such as that shown in Fig. 1, the [Ca²⁺] decline was briefly reversed. Finally, in phase 3 there was a very gradual [Ca²⁺] decline, in which [Ca²⁺] remained significantly elevated above the initial resting level for several seconds.
Fig. 1. Recordings during a tetanus of indo 1 fluorescence intensity at 400 (I_{400}) and 470 nm (I_{470}), ratio (R) of I_{400}/I_{470}, and calculated intracellular Ca^{2+} concentration ([Ca^{2+}]). Horizontal bar shows period of tetanic stimulation. Three phases of [Ca^{2+}] decline during relaxation are indicated, a rapid initial decline (phase 1), a transient rise of [Ca^{2+}] (phase 2), and a final slow decline (phase 3) (precontraction level indicated by a horizontal line near end of record). [Ca^{2+}] measured during a twitch contraction has been superimposed (dashed line).

[Ca^{2+}], measured during a twitch contraction has been superimposed (dashed line). Maximum [Ca^{2+}] during a twitch was ~85% of that during a tetanus. In contrast to the complex time course of [Ca^{2+}] decline after a tetanus, [Ca^{2+}] decline after the twitch was approximately monoeponential. This finding was also found in a previous study using the same preparation (5). Furthermore, after a twitch, [Ca^{2+}] did not remain appreciably elevated above the precontraction level.

Effects of Acidosis on Force and [Ca^{2+}]

The effects of acidosis on force and [Ca^{2+}], are illustrated for a typical experiment in Fig. 2 and summarized for all experiments (n = 8) in Table 1. Figure 2 shows measurements of [Ca^{2+}], and force during twitch and tetanic contractions at pH 7.3 (solid lines) and pH 6.3 (dashed lines).

Acidosis caused a relatively small decrease in tetanic force (11 ± 2%) from 1.95 ± 0.15 kg/cm^2 at pH 7.3 to 1.73 ± 0.11 at pH 6.3 (n = 8, P < 0.01). In contrast, the twitch force actually increased (24 ± 7%) from 0.62 ± 0.1 kg/cm^2 at pH 7.3 to 0.77 ± 0.1 at pH 6.3 (P < 0.01). Acidosis was also associated with considerable slowing of force relaxation after both the twitch and tetanus (see below).

With acidosis, tetanic R was slightly but significantly decreased (6.3 ± 1.6%, P < 0.01). In contrast, the calculated tetanic [Ca^{2+}], increased with acidosis from 3.0 ± 0.4 μM at pH 7.3 to 4.0 ± 0.49 at pH 6.3 (P < 0.01). This increase of [Ca^{2+}], despite a slightly reduced R, arose because acidosis increases the K_d of indo 1 for Ca^{2+} and changes indo 1 fluorescence (3) (see METHODS). The rise of tetanic [Ca^{2+}], with severe acidosis is consistent with previous findings with moderate acidosis (2, 20, 27), indicating that the small decrease in tetanic force was not due to decreased tetanic [Ca^{2+}],

The increased twitch force observed with acidosis was paralleled by an increased peak [Ca^{2+}], which rose from 2.6 ± 0.32 μM at pH 7.3 to 3.8 ± 0.57 at pH 6.3. The peak [Ca^{2+}], during the twitch was large compared with tetanic [Ca^{2+}], (~85% tetanic at pH 7.3). This was similar to a previous report (5). In contrast, twitch force was much smaller relative to tetanic force (~31% of tetanic). Acidosis also resulted in considerable elevation of [Ca^{2+}], in resting muscle: the fluorescence ratio rose 17 ± 4% with acidosis and [Ca^{2+}], rose from 0.42 ± 0.03 μM at pH 7.3 to 0.95 ± 0.08 at pH 6.3 (P < 0.01). This increase in [Ca^{2+}], was not sufficient to activate force development. Similar reports of elevated resting [Ca^{2+}], have been made with moderate acidosis (2, 27) and after fatiguing stimulation (2, 5, 20).

Changes in [Ca^{2+}], and Force During Relaxation

Figure 3 shows records of [Ca^{2+}], and force during relaxation after a tetanus at pH 7.3 and 6.3. The effects of acidosis on the three phases of [Ca^{2+}], decline and on force relaxation are described below. At either pH, the decline of [Ca^{2+}], during relaxation was biexponential. There was an initial rapid [Ca^{2+}], decline in phase 1. Phase 2 of [Ca^{2+}], decline occurred at the transition from the rapid [Ca^{2+}], decline in phase 1 to a much slower [Ca^{2+}], decline in phase 3. The time constant of [Ca^{2+}], decline (τ_{Ca}) during phase 3 was ~50 times longer than that of phase 1 (pH 7.3). During phase 3, [Ca^{2+}], remained appreciably above the precontraction level.

Table 1. Effect of acidosis on force and [Ca^{2+}]

<table>
<thead>
<tr>
<th>pH</th>
<th>Twitch Force, kg/cm²</th>
<th>Tetanic Force, kg/cm²</th>
<th>Twitch [Ca^{2+}], μM</th>
<th>Tetanic [Ca^{2+}], μM</th>
<th>Rest [Ca^{2+}], μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3</td>
<td>0.62 ± 0.1</td>
<td>2.6 ± 0.32</td>
<td>1.95 ± 0.15</td>
<td>3.0 ± 0.40</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>6.3</td>
<td>0.77 ± 0.1</td>
<td>4.0 ± 0.49</td>
<td>1.73 ± 0.11</td>
<td>3.8 ± 0.57</td>
<td>0.95 ± 0.08</td>
</tr>
</tbody>
</table>

% Change: +24 ± 7% * for twitch force; +46 ± 11% * for tetanic force; +33 ± 6% * for twitch [Ca^{2+}]; +126 ± 15% * for tetanic [Ca^{2+}]. Values are means ± SE for summary of measures of force and intracellular Ca^{2+} concentration ([Ca^{2+}]) at pH 7.3 and 6.3 and percentage changes with acidosis within each experiment; n = 8. *P < 0.01 for paired t-test comparison of data at pH 7.3 and 6.3.
the time course of [Ca\(^{2+}\)] decline during phases 1 and 3 (dotted curves in Fig. 3, left) could be closely fitted to a biexponential function (solid curves). In contrast, during phase 2, [Ca\(^{2+}\)] was significantly higher than the fitted values. This was consistent with previous suggestions that phase 2 of [Ca\(^{2+}\)] decline may arise due to additional Ca\(^{2+}\) release to the cytosol during relaxation (9, 20). The time course of changes in [Ca\(^{2+}\)], during phase 2 was examined from the difference between the data and the biexponential function fitted to phases 1 and 3. Figure 3 shows that this difference function appears as a transient rise and fall of [Ca\(^{2+}\)], during phase 2 (curves near baseline).

The time course of tetanic force relaxation was simpler compared with the time course of tetanic [Ca\(^{2+}\)] decline. As previously described (5), after a period during which the rate of force loss increased, force relaxation could be closely fitted to a single exponential function. The phase 2 of [Ca\(^{2+}\)] decline was not associated with a similar phase in the force decline (2, 5, 20).

**Effect of Acidosis on Force Relaxation and [Ca\(^{2+}\)] Decline**

Acidosis had several effects on relaxation that are evident in Figs. 2 and 3 and summarized for all experiments (n = 8) in Table 2. The rapid (Ca\(^{2+}\)) decline, phase 1, was slowed with acidosis: \(\tau_{Ca}\) during phase 1 rose from 36.8 ± 3.5 ms at pH 7.3 to 61.9 ± 3.6 ms at pH 6.3 (\(P < 0.01\)). This finding is in contrast to a recent report in which milder acidosis did not appear to slow [Ca\(^{2+}\)] decline (27). In contrast to the increased \(\tau_{Ca}\) during phase 1, the amplitude of phase 1 of [Ca\(^{2+}\)] decline (see METHODS) was not affected by acidosis and equalled \(\sim 2.2 \mu M\) at both pH levels. Thus, at the start of relaxation, the elevation of [Ca\(^{2+}\)] associated with phase 1 of relaxation was similar at both pH levels.

Acidosis had several effects on phase 2. Because this phase may represent addition of Ca\(^{2+}\) to the cytosol during relaxation rather than Ca\(^{2+}\) removal, it is discussed separately below.

In contrast to acidosis causing prolongation of \(\tau_{Ca}\) during phase 1, acidosis did not alter \(\tau_{Ca}\) during phase 3 of [Ca\(^{2+}\)] decline. With acidosis, there was a slight nonsignificant change of \(\tau_{Ca}\) during phase 3 from 1.9 ± 0.18 s at pH 7.3 to 1.7 ± 0.3 at pH 6.3 (\(P > 0.05\)). Furthermore, in contrast to the effects of acidosis on phase 1 (in which the amplitude of phase 1 was unchanged with acidosis) acidosis resulted in a considerable rise in the amplitude of phase 3. The amplitude of phase 3 increased from 0.35 ± 0.06 \(\mu M\) at pH 7.3 to 0.9 ± 0.17 at pH 6.3 (\(P < 0.01\)). Thus, at the start of relaxation, the elevation of [Ca\(^{2+}\)] associated with phase 3 of relaxation was greater at pH 6.3 than at pH 7.3.

The effect of acidosis on \(\tau_{F}\) was similar to the effect of acidosis on \(\tau_{Ca}\) during phase 1. Figure 4 shows the relationship between \(\tau_{F}\) and \(\tau_{Ca}\) (phase 1). Both \(\tau_{F}\) and \(\tau_{Ca}\) (phase 1) increased with acidosis, and there was a significant correlation between \(\tau_{F}\) and \(\tau_{Ca}\) (phase 1) for all experiments (\(r = 0.685, P < 0.01\)). The slope of the regression relation was 1.16, and the relationship extrapolated to the origin. These proportional increases in \(\tau_{F}\) and \(\tau_{Ca}\) with acidosis are consistent with the view that slowed force relaxation with acidosis may be influenced by the slowed decline of [Ca\(^{2+}\)] during phase 1. In contrast, Fig. 4 also shows that at each pH \(\tau_{F}\) and \(\tau_{Ca}\)

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### Table 2. Effect of acidosis on force and [Ca\(^{2+}\)], decline during relaxation

<table>
<thead>
<tr>
<th></th>
<th>(\tau_{F}) ms</th>
<th>(\tau_{Ca}) ms</th>
<th>(\tau_{Ca}) Amplitude, (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phase 1</td>
<td>Phase 3</td>
<td>Phase 1</td>
</tr>
<tr>
<td>pH 7.3</td>
<td>32.2 ± 1.8</td>
<td>36.8 ± 3.5</td>
<td>1.888 ± 178</td>
</tr>
<tr>
<td>pH 6.3</td>
<td>74.5 ± 6.6</td>
<td>61.9 ± 3.6</td>
<td>1.668 ± 303</td>
</tr>
<tr>
<td>% Change</td>
<td>+131 ± 20°</td>
<td>+65 ± 13°</td>
<td>-12 ± 10°</td>
</tr>
<tr>
<td></td>
<td>0 ± 6°</td>
<td>157 ± 17°</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE for summary of fit of exponential functions to force and [Ca\(^{2+}\)], decline during relaxation for data at pH 6.3 and 7.3 and percentage changes; \(n = 8\). For each experiment (\(n = 8\)) values obtained at pH 7.3 (○) are connected to corresponding values obtained after inducing acidosis (pH 6.3, ■). Regression through entire data set is shown.
EFFECTS OF ACIDOSIS ON SKELETAL MUSCLE

were not closely correlated. This finding is not consistent with the view that $\tau_f$ is determined simply by $\gamma_{Ca}$.

$Ca^{2+}$ Release to Cytosol During Relaxation and Effect of Acidosis

As described above, Fig. 3 shows that after a tetanus the time course of $[Ca^{2+}]_i$ decline during phases 1 and 3 could be closely fitted to a biexponential function. Furthermore, the difference between the data and fitted values corresponds to a brief rise and fall of $[Ca^{2+}]_i$ during phase 2 of relaxation. Acidosis had two effects on this difference function. First, compared with at pH 7.3, Fig. 3 shows a larger and broader difference function with acidosis (also evidenced by a greater divergence between data and fit). For all experiments, the integral of the difference function with respect to time approximately doubled with acidosis: from 16.6 ± 3.2 μM·s at pH 7.3 to 34.6 ± 9.9 at pH 6.3 ($P < 0.05$). Second, the peak of the difference function occurred later with acidosis. The time to the peak of the difference function increased from 96 ± 4 ms at pH 7.3 to 166 ± 12 at pH 6.3 ($P < 0.01$).

Phase 2 has been suggested to represent $Ca^{2+}$ release to the cytosol during relaxation, possibly due to $Ca^{2+}$ unbinding from troponin C late in relaxation (9, 20). Two findings in the present study were consistent with this. First, when the force data and $Ca^{2+}$ difference functions from Fig. 3 were replotted with their peak values normalized to 1 (Fig. 5), the time to peak of the difference function and the time course of force relaxation appeared similarly delayed with acidosis. Second, the peak of the difference function occurred at a similar level of force (~30% of maximum) at both pH 7.3 and 6.3. These findings were consistent with a transient process of $Ca^{2+}$ release to the cytosol that peaked late during force relaxation. These findings were examined in more detail using the entire data set. First, Fig. 6A shows that there was a linear relationship between the time to peak of the difference function and the force relaxation time (expressed as time to 50% force decline; $r = 0.962$, $P < 0.01$). This relationship demonstrated that slowed relaxation of force with acidosis was associated with a proportional delay in the timing of phase 2 of $[Ca^{2+}]_i$ decline. Second, Fig. 6B shows the relationship between the time to the peak of the difference function and the corresponding force level at the time of the peak of the difference function. Figure 6B shows that the peak of the difference function consistently occurred late in relaxation at a time when force had fallen to between 35 and 15% of maximum. The correlation between the variables in Fig. 6B did not reach statistical significance ($r = 0.47$, $P > 0.05$); however, taken as a group, at pH 6.3 the mean force remaining at the peak of the difference function (30 ± 15%) was higher than at pH 7.3 (21.4 ± 2.3%; $P < 0.01$).

Effect of Acidosis on Responsiveness of Force Development to $[Ca^{2+}]_i$

The effects of acidosis on the relationship between steady-state $[Ca^{2+}]_i$ and force was not determined. The high $[Ca^{2+}]_i$ during a twitch, compared with a tetanus, precluded defining the $Ca^{2+}$-force relationship over low $Ca^{2+}$ levels by simply using low-frequency tetanic stimulation. However, several studies suggest that analysis of the instantaneous relationship between force and $[Ca^{2+}]_i$ during relaxation may reflect on the true steady-state $Ca^{2+}$-force relationship (23, 27). During relaxation,
changes in $[Ca^{2+}]$, and force may be slow enough for there to be a quasi equilibrium between them.

Figure 7 shows the relationship between instantaneous force and $[Ca^{2+}]$ during relaxation at pH 7.3 and 6.3. During relaxation there was a sigmoidal relationship between the fall of force and $[Ca^{2+}]$. Figure 7 shows that with acidosis the relationship between instantaneous force and $[Ca^{2+}]$ was shifted to the right to higher values of $[Ca^{2+}]$. With acidosis, the $[Ca^{2+}]$ at half-maximal force doubled from $1.2 \pm 0.1$ µM at pH 7.3 to $2.4 \pm 0.3$ at pH 6.3 ($P < 0.01$).

The relationships between instantaneous force and $[Ca^{2+}]$, in Fig. 7 are steeper and span narrower ranges of $[Ca^{2+}]$ compared with the steady-state $Ca^{2+}$-force relationship found in studies of skinned fibers. Consistent with this, Gao et al. (15) recently suggested that skinned fibers may be less sensitive to $Ca^{2+}$ than living muscle. The steady state $Ca^{2+}$-force relationship of living rat cardiac trabeculae was steeper and spanned a narrower range of $[Ca^{2+}]$ compared with the same preparation after skinned (15).

**DISCUSSION**

The major findings of this study were, first, that severe intracellular acidosis in skeletal muscle at 20°C caused only a small reduction (~10%) of tetanic force but a 33% increase of tetanic $[Ca^{2+}]$. These findings, consistent with a recent study of milder acidosis (27), suggest that the small force reduction with acidosis was due to a decrease in the maximum activated force. Second, acidosis caused markedly slowed force relaxation, which was paralleled by an equivalent slowing of the decline of $[Ca^{2+}]$. This finding, in contrast to a recent study of milder acidosis (27), suggests that slowed force relaxation with severe acidosis may arise from slowed $Ca^{2+}$ removal from the cytosol. Third, phase 2 of $[Ca^{2+}]$, decline after a tetanus, in which $[Ca^{2+}]$ tended to increase, was delayed, with acidosis in proportion to the slowing of force relaxation. Furthermore, phase 2 of $[Ca^{2+}]$, decline always occurred toward the end of relaxation, when force had fallen to 20–30% of initial. These novel findings indicate that the processes involved in phase 2 of $[Ca^{2+}]$, decline are closely associated with the final stage of force decline. They are also consistent with a previous suggestion that during force relaxation cross-bridge dissociation from the thin filament promotes $Ca^{2+}$ release to the cytosol due to dissociation of $Ca^{2+}$ from troponin on the thin filament (9). Finally, with acidosis, the instantaneous relationship between $[Ca^{2+}]$, and force was shifted to higher values of $[Ca^{2+}]$. This finding is consistent with previous studies suggesting that acidosis reduces the sensitivity of the contractile process to $[Ca^{2+}]$. Together these findings suggest that acidosis in skeletal muscle has little effect on maximum tetanic force and that the major effects may be to cause slower relaxation and decreased $Ca^{2+}$ sensitivity.

**Effects of Acidosis on Tetanic Force and Relaxation**

The finding in this study that force development of frog skeletal muscle was not appreciably decreased by severe hypercapnic acidosis was similar to that found with hypercapnic acidosis of mammalian skeletal muscle (1, 27). The findings was also consistent with several studies of fatigue of human and animal muscle, suggesting that decreases of pH, may not influence force; e.g., after exercise, muscle force almost fully recovered before appreciable recovery of pH, which was monitored with $^{31}P$-NMR (4, 6). Taken together the findings from these previous studies and the present study suggest that in living muscle decreased pH, may not appreciably impair force development.

In contrast, several previous studies found that hypercapnic acidosis significantly decreased force development (12, 24, 26, 29). Adams et al. (1) suggested that some studies may have been complicated by the presence of hypoxia. The present study and the studies of Adams et al. (1) minimized the potential for hypoxia by arterially perfusing muscles.

The findings in intact muscle that acidosis had little effect on force represent an interesting contrast to findings from skinned fibers, in which acidosis considerably inhibited force development (10, 11). The cause for a difference between the effect of acidosis on force in intact muscle and on skinned fibers is unclear.

In contrast to differences between studies concerning the effect of acidosis on force development in living muscle, a consistent finding in most studies has been that acidosis markedly impaired force relaxation (1, 2, 8, 12, 24, 26, 27, 29). Two explanations for impaired relaxation have been recognized. First, acidosis may impair relaxation due to slowed $Ca^{2+}$ removal from the cytosol. In support of this, in the present study the slowing of force relaxation with acidosis was closely matched to an equivalent slowing of $[Ca^{2+}]$, decline. Alternatively, impaired relaxation could arise from slowed cross-bridge cycling (27). Consistent with this suggestion, slowed cross-bridge cycling with acidosis has been evidenced from a decreased speed of shortening (10, 11).

The findings of the present and recent studies (1, 19, 27) are relevant in relationship to the process of fatigue.
The findings suggest that the acidosis which may occur during fatiguing contractions would contribute little to the decreased force. However, acidosis may contribute significantly to the marked slowing of relaxation during fatigue.

Effects of Acidosis on \([\text{Ca}^{2+}]\)

As noted in previous reports for mild acidosis (2, 20, 27), severe acidosis caused increased \([\text{Ca}^{2+}]\), both in resting muscle and during a tetanus. However, in contrast to a recent report (27), the present study also found that the decline of \([\text{Ca}^{2+}]\) during relaxation was slowed with acidosis.

\([\text{Ca}^{2+}]\) in resting muscle. Elevated \([\text{Ca}^{2+}]\) in resting muscle has previously been noted with moderate acidosis (2, 27) and after fatiguing stimulation (2, 5, 21). The \([\text{Ca}^{2+}]\) in resting muscle involves a balance between \(\text{Ca}^{2+}\) leakage to and \(\text{Ca}^{2+}\) removal from the cytosol. Elevated resting \([\text{Ca}^{2+}]\) with acidosis could therefore arise from increased \(\text{Ca}^{2+}\) leakage into the cytosol or impaired \(\text{Ca}^{2+}\) removal from the cytosol (5, 27). Several studies suggested that leakage of \(\text{Ca}^{2+}\) into the cytosol was decreased with acidosis (19, 25); thus increased resting \([\text{Ca}^{2+}]\) with acidosis is likely to arise from impaired \(\text{Ca}^{2+}\) removal from the cytosol. This suggestion was recently made by Westerblad and Allen (27) in a study of the effects of mild acidosis on \([\text{Ca}^{2+}]\) and force in mouse muscle. Consistent with this suggestion, the present study found that during relaxation the rate of \([\text{Ca}^{2+}]\) decline during phase 1 was slowed with acidosis. This finding suggests that \(\text{Ca}^{2+}\) removal from the cytosol was impaired with acidosis.

Tetanic \([\text{Ca}^{2+}]\). The increased tetanic \([\text{Ca}^{2+}]\), found in this study with severe acidosis was consistent with the increased \([\text{Ca}^{2+}]\); reported in several previous studies with moderate acidosis (2, 20, 27). Increased tetanic \([\text{Ca}^{2+}]\) with acidosis could arise through several mechanisms. First, \(\text{Ca}^{2+}\) release to the cytosol may be increased. With acidosis, depolarization was previously shown to produce close to the maximal activated force in skinned toad muscle fibers, suggesting that \(\text{Ca}^{2+}\) release to the cytosol was not impaired with acidosis (19). However, whether increased \(\text{Ca}^{2+}\) release to the cytosol occurs with acidosis is not known. Second, increased tetanic \([\text{Ca}^{2+}]\); with acidosis could arise through impaired \(\text{Ca}^{2+}\) removal from the cytosol. The findings of the present study were consistent with this mechanism. Finally, decreased buffering of \(\text{Ca}^{2+}\) in the cytosol with acidosis could also increase tetanic \([\text{Ca}^{2+}]\). The findings of the present study were also consistent with this mechanism (described below).

\([\text{Ca}^{2+}]\); decline. The effects of acidosis on \([\text{Ca}^{2+}]\); decline were complex. The decline of \([\text{Ca}^{2+}]\) during phase 1 was significantly slowed with acidosis. This finding was in contrast to a recent report (27) showing that with moderate acidosis (pH 6.8) in mouse muscle fibers the early \([\text{Ca}^{2+}]\); decline was not slowed. The source of this difference is unclear; there may possibly be a fundamental difference between mouse and frog muscle; or, alternatively, a slowed \([\text{Ca}^{2+}]\); decline may be more apparent in the present studies due to the more severe acidosis used and possibly due to the higher signal to noise ratio available from a whole muscle preparation. In the present study, the increase of \(\tau_{\text{Ca}}\) in phase 1 with acidosis was similar to the increase of \(\tau_{r}\), suggesting that slowed force relaxation with acidosis may derive from a slowed \(\text{Ca}^{2+}\) removal from the cytosol. However, the lack of a relationship between \(\tau_{\text{Ca}}\) and \(\tau_{r}\) noted previously with milder acidosis (27) suggests that acidosis may have a direct effect on force decline independent of a prolonged \([\text{Ca}^{2+}]\); decline. Increased \(\tau_{\text{Ca}}\) could arise if the sarcoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase was impaired. Alternatively, if \(\text{Ca}^{2+}\) binding to intracellular proteins significantly contributed to the \([\text{Ca}^{2+}]\); decline (such as parvalbumin) (17), then decreased \(\text{Ca}^{2+}\) buffering in the cytosol with acidosis could also impair \([\text{Ca}^{2+}]\); decline.

In contrast to phase 1, during phase 3, \(\tau_{\text{Ca}}\) was unchanged with acidosis; however, the amplitude of phase 3 \((A_3)\) increased significantly from 0.35 to 0.9 \(\mu\text{M} \,[\text{Ca}^{2+}]\), and the contribution of phase 3 to total \([\text{Ca}^{2+}]\); elevation (i.e., \(A_0/[A_1 + A_3]\)) increased therefore from ~15 to 29% with acidosis. A slowly declining tail of \([\text{Ca}^{2+}]\), has previously been observed by others (9, 18, 27, 28) and attributed to an equilibrium between the gradual release of \(\text{Ca}^{2+}\) to the cytosol from \(\text{Ca}^{2+}\)-binding sites on proteins vs. \(\text{Ca}^{2+}\) uptake to the sarcoplasmic reticulum. The increased amplitude of phase 3, with acidosis in the present study, may be due to decreased \(\text{Ca}^{2+}\) buffering in the cytosol. Thus, during relaxation, acidosis may facilitate unloading of \(\text{Ca}^{2+}\) from intracellular \(\text{Ca}^{2+}\) buffers.

Phase 2 occurred at the transition between 2 exponential phases of \([\text{Ca}^{2+}]\); decline. Phase 2 was characterized by a marked slowing or reversal of the \([\text{Ca}^{2+}]\); decline (5, 9, 20). Phase 2 has been suggested to represent \(\text{Ca}^{2+}\) release to the cytosol due to \(\text{Ca}^{2+}\) unbinding from troponin (9). Two findings in the present study support this view. First, for all experiments, the time of the peak of the \(\text{Ca}^{2+}\) release process during phase 2 was highly correlated with the half time of force relaxation. Second, phase 2 consistently occurred at a time when force had fallen to ~20–30% of the tetanic level. These findings are consistent with the view that cross-bridge dissociation from the thin filament promotes dissociation of remaining \(\text{Ca}^{2+}\) bound to troponin. This view is further supported by the finding that phase 2 was absent in muscles that were stretched to long sarcomere lengths to prevent cross-bridge interactions (discussed in Ref. 20).

Effects of Acidosis on Responsiveness to \(\text{Ca}^{2+}\)

Acidosis has previously been shown in skinned fibers to decrease \(\text{Ca}^{2+}\) sensitivity in terms of an increase in the \([\text{Ca}^{2+}]\); at 50% of maximum force. Consistent with this, the present studies found that the relationship between instantaneous levels of \(\text{Ca}^{2+}\) and force during relaxation was shifted by acidosis such that the \([\text{Ca}^{2+}]\);
at half-maximal force was increased. This may reflect a change in the true steady-state Ca\textsuperscript{2+}-force relationship.

**Effects of Acidosis on Twitch Contraction**

At pH 7.3, the twitch force was small (~30% of tetanic) despite a relatively large twitch [Ca\textsuperscript{2+}], (~85% tetanic). This may have arisen because the Ca\textsuperscript{2+} transient was short lived, thus [Ca\textsuperscript{2+}], declined before appreciable activation of force occurred (5).

Acidosis increased both maximum force and [Ca\textsuperscript{2+}], during a twitch contraction. The higher twitch force with acidosis may be due to the higher peak [Ca\textsuperscript{2+}], and/or to the slowed [Ca\textsuperscript{2+}], decline during relaxation (effectively maintaining [Ca\textsuperscript{2+}], elevated for longer).

Phase 2 of [Ca\textsuperscript{2+}], decline was absent in the twitch contraction. [Ca\textsuperscript{2+}], declined monoeXponentially to close to the initial level. The absence of a phase 2 may reflect a lower loading of Ca\textsuperscript{2+} to troponin during a brief twitch contraction. The absence of a slowly declining tail of [Ca\textsuperscript{2+}], may reflect a lower loading of Ca\textsuperscript{2+} to cytoplasmic buffers during the twitch (9).

**Problems and Limitations**

Several uncertainties limit the findings of this study. Calibration of [Ca\textsuperscript{2+}], from indo 1 fluorescence is hampered by uncertain effects of the intracellular environment on indo 1 Ca\textsuperscript{2+} binding and fluorescence spectra. This study used calibration constants which accounted for the effects of frog muscle proteins and acidosis on indo 1.

pH was not measured; muscle pH was assumed to decline with hypercapnia as found in previous studies. Calculated [Ca\textsuperscript{2+}], is directly proportional to $K_d$ (Eq. 1), and $K_d$ increased with acidosis (pH 6.3) (3). Thus [Ca\textsuperscript{2+}], will be overestimated with acidosis if pH\textsubscript{w} was higher than pH 6.3.

Problems arise in extrapolating from measurements on a multicellular preparation to mechanisms in single cells. For example, only ~5% of muscle fibers were sampled by epifluorescence measurements, whereas all fibers contributed to measured force. However, frog muscle consists almost entirely of fast-twitch fibers; therefore surface fibers would have fiber types representative of the whole muscle. Furthermore, if fibers are not synchronized during relaxation then time-dependent measures may be distorted (e.g., time constants may be overestimated, and amplitude of phase 2 may be underestimated).

Finally, indo 1 was loaded in the AM form; thus some indo 1 may load into noncytosolic compartments such as mitochondria or sarcoplasmic reticulum. However, in frog muscle the mitochondrial content is very low. Furthermore, indo 1 in the sarcoplasmic reticulum would remain saturated with Ca\textsuperscript{2+} and therefore should not vary in fluorescence with muscle stimulation.

In conclusion, this study suggests that acidosis in skeletal muscle has little effect on maximum tetanic force and that the major effects may be to cause slower relaxation and decreased Ca\textsuperscript{2+} sensitivity.

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