Modulation of ventricular transient outward $K^+$ current by acidosis and its effects on excitation-contraction coupling

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Saegusa N, Garg V, Spitzer KW. Modulation of ventricular transient outward $K^+$ current by acidosis and its effects on excitation-contraction coupling. Am J Physiol Heart Circ Physiol 304: H1680–H1696, 2013.—The contribution of transient outward current ($I_{to}$) to changes in ventricular action potential (AP) repolarization induced by acidosis is unresolved, as is the indirect effect of these changes on calcium handling. To address this issue we measured intracellular pH (pHi), $I_{to}$, L-type calcium current ($I_{Ca,L}$), and calcium transients (CaTs) in rabbit ventricular myocytes. Intracellular acidosis [pHi 6.75 with extracellular pH (pHo) 7.4] reduced $I_{to}$ by ∼50% in myocytes with both high (epicardial) and low (papillary muscle) $I_{to}$ densities, with little effect on steady-state inactivation and activation. Of the two candidate α-subunits underlying $I_{to}$, human (h)Kv4.3 and hKv1.4, only hKv4.3 current was reduced by intracellular acidosis. Extracellular acidosis (pHo 6.5) shifted $I_{to}$ inactivation toward less negative potentials but had negligible effect on peak current at +60 mV when initiated from −80 mV. The effects of low pH-induced inhibition of $I_{to}$ on AP repolarization were much greater in epicardial than papillary muscle myocytes and included slowing of phase 1, attenuation of the notch, and elevation of the plateau. Low pHi increased AP duration in both cell types, with the greatest lengthening occurring in epicardial myocytes. The changes in epicardial AP repolarization induced by intracellular acidosis reduced peak $I_{Ca,L}$, increased net calcium influx via $I_{Ca,L}$, and increased CaT amplitude. In summary, in contrast to low pHi, intracellular acidosis has a marked inhibitory effect on ventricular $I_{to}$, perhaps mediated by Kv4.3. By altering the trajectory of the AP repolarization, low pHi has a significant indirect effect on calcium handling, especially evident in epicardial cells.

transient outward current; acidosis; excitation-contraction coupling; rabbit ventricular myocytes

**TRANSPORT OUTWARD K+ CURRENT ($I_{to}$)** is the major repolarizing current flowing during the early repolarization phase of the cardiac action potential (AP) in a variety of cell types including human and rabbit ventricular myocytes and is largely responsible for phase 1 and the “spike-and-dome” configuration (4, 12, 17, 20, 22, 31, 48). By modulating the initial voltage trajectory of the AP, $I_{to}$ importantly influences the overall AP waveform through voltage-dependent effects on other ionic currents such as L-type calcium current ($I_{Ca,L}$), Na$^+$/Ca$^{2+}$ exchange (NCX) current, and delayed rectifier currents (34). In this regard, several studies have shown that inhibition of $I_{to}$ markedly affects excitation-contraction (E-C) coupling by altering transsarcolemmal Ca$^{2+}$ flux via $I_{Ca,L}$ and NCX, with secondary effects on calcium loading of the sarcoplasmic reticulum (SR) (5, 6, 11, 13, 27, 42, 43, 53).

The expression and magnitude of cardiac $I_{to}$ are regulated by a variety of factors and conditions including hormones, metabolic inhibition, and hypertrophy/failure with consequent effects on AP configuration (32, 36). In addition, previous work has shown that $I_{to}$ in rat ventricular myocytes, measured at room temperature, is altered by extracellular acidosis [low extracellular pH (pHo)] (50), intracellular acidosis [low intracellular pH (pHi)] (58, 59), and simultaneous reductions in pHo and pHi (combined acidosis) (24). Low pHi elicits right shifts in inactivation (toward less negative potentials) causing increased $I_{to}$ at voltages of approximately +50 mV to +60 mV when initiated from depolarized potentials but has little or no effect when initiated from voltages near the normal resting potential (50). In contrast, intracellular acidosis (pHo 7.4), induced by pipette acid loading, is reported to decrease $I_{to}$ over the voltage range of −30 mV to +60 mV (58, 59). However, pHo was not measured in these reports, and subsequent work has demonstrated that accurate pHi dialysis via a suction pipette is very difficult to achieve, given the high intracellular H$^+$ buffering and low H$^+$ mobility (52, 61). Thus the quantitative relationship between ventricular pHi and $I_{to}$ remains unresolved. To address this issue, in the present study we measured pHi and $I_{to}$ at 37°C in rabbit ventricular myocytes, a preparation with a more humanlike AP than rat ventricle, using a more reliable technique for selectively reducing pHi.

Also unresolved is the role of $I_{to}$ in mediating pH-induced changes in AP repolarization and calcium handling. Using a mixture of rabbit myocytes from the entire left ventricle, we recently reported that a selective reduction in pHi (pHo 7.4) slowed the rate of phase 1 repolarization, reduced the notch, elevated the AP plateau, and prolonged AP duration (APD). These AP changes are consistent with $I_{to}$ inhibition and may indirectly contribute to the effects of low pHi on ventricular E-C coupling. However, because $I_{to}$ exhibits a large transmural density gradient (epicardial > endocardial) (4, 9, 17, 18, 31), our use of a mixture of cells from the entire left ventricle meant that phase 1 and the notch varied considerably from one cell to another. Thus it remains unclear how pHi-induced changes in $I_{to}$ modulate the AP, $I_{Ca,L}$, and the calcium transient (CaT). Here we address this question in rabbit ventricular myocytes displaying both high [epicardial (Epi)] and low [papillary muscle (PM)] $I_{to}$ densities, using AP voltage clamps and fluo-4 fluorescence.

Collectively the results demonstrate that intracellular acidosis markedly reduces ventricular $I_{to}$, which significantly contributes to the accompanying changes in early AP repolarization, resulting in indirect modulation of E-C coupling.

**MATERIALS AND METHODS**

Myocyte Isolation

The experiments were performed on left ventricular myocytes isolated from adult rabbits of both sexes by enzymatic digestion. To minimize transmural differences in $I_{Ca,L}$ density (37), only male rabbits were used for the measurements of $I_{Ca,L}$ and CaTs. All
procedures involving animals were approved by the Animal Care and Use Committee of the University of Utah and complied with the American Physiological Society’s “Guiding Principles for the Care and Use of Vertebrate Animals in Research and Teaching.” Rats were anesthetized with pentobarbital sodium (50 mg/kg iv), and the excised heart was attached to an aortic cannula and perfused with solutions gassed with 100% O2 and held at 37°C, pH 7.3. The heart was first perfused for 5 min with a Ca2+-free solution at 37°C containing (in mM) 92.0 NaCl, 4.4 KCl, 11.0 dextrose, 5.0 MgCl2, 20.0 taurine, 5.0 creatine, 5.0 sodium pyruvate, 1.0 NaH2PO4, 24.0 HEPES, and 12.5 NaOH (pH 7.3). This was followed by 15 min of recirculation with the same solution containing 0.15 mg/ml collagenase P (Roche Diagnostic, Mannheim, Germany), 0.05 mg/ml protease (type XIV, Sigma Chemical), and 0.05 mM CaCl2. The heart was then perfused for 5 min with the same solution containing no enzymes. The ventricle was isolated and minced, shaken for 10 min, and then filtered through a nylon mesh. Cells were stored at room temperature in the normal control bathing solution. To obtain cells with both low and high I0 densities, myocytes were isolated from both PMs and the epicardial layers (Ep) of the left ventricle, respectively (17). All cells used in this study were rectangular, had well-defined striations, and did not spontaneously contract. All experiments were conducted within 10 h of isolation.

Cell Culture

Human embryonic kidney cells (HEK 293T) were cultured in DMEM supplemented with 10% fetal bovine serum, l-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 µg/ml). HEK 293T cells were plated onto 35-mm culture dishes and transfected with human (h)Kv1.4 (pcDNA3) along with green fluorescent protein (pEGFP) or hKv4.3 (pIRES-GFP) with Lipofectamine (Invitrogen). CE experiments were conducted within 1–2 days after transfection.

Electrophysiological Techniques

All electrophysiological measurements in both myocytes and HEK 293T cells were made with whole cell ruptured patch pipettes. Pipettes (Corning 8250 glass) for myocytes and HEK 293T cells had resistances of 1–2 MΩ and 2.5–4.0 MΩ, respectively, when filled. Myocyte APs were recorded with an Axoclamp-2A amplifier system (Axon Instruments) in bridge mode, and voltage clamping (step clamps and AP clamps) was achieved with an Axopatch 200B clamp system using a CV203B headstage. APs were triggered with brief (2–3 ms) square pulses of depolarizing intracellular current (~2 nA), and APD was measured at 90% repolarization (APD90). Membrane potential (Vm) and membrane current (Im and IcL) were filtered at 5 kHz, digitized at 50 kHz with a 16-bit A/D converter (Digidata 1322A), and analyzed with pCLAMP 8 software (Molecular Devices). The reference electrode was a flowing 3 M KCl bridge. Compensation for series resistance (75–80%) and capacitance were performed elec-
tronic. Membrane currents were normalized for cell capacitance (pA/pF).

Current-voltage (I-V) relationships for \( I_{\text{Ca,L}} \) were determined by applying test pulses from \(-80 \text{ mV} \) to \(+60 \text{ mV} \) (400-ms duration) at a cycle length (CL) of 5 s. Typically, each test pulse was preceded by a prestep from a holding potential of \(-80 \text{ mV} \) to \(-40 \text{ mV} \) (duration 40 ms) to inactivate sodium current. Zero-sodium bathing solution was used in some experiments to block sodium current. For each test pulse, the amplitude of \( I_{\text{Ca,L}} \) was measured as the difference between peak outward current and the current level at the end of the depolarizing clamp pulse. This clamp protocol was applied first in the control solution and then after 2 min in the acidic test solution. Each cell was exposed only once to an acidic test solution.

I-V relationships for \( I_{\text{Ca,L}} \) in myocytes were determined with the same protocol (CL = 5 s). At the end of the experiment the protocol was repeated in the presence of 200 \( \mu \text{M} \) CdCl\(_2\) and 10 \( \mu \text{M} \) nifedipine to correct for background current.

I-V relationships for hKv4.3 and hKv1.4 expressed in HEK 293T cells were determined by holding the cell at a potential of \(-80 \text{ mV} \) and applying test pulses from \(-40 \text{ mV} \) to \(+60 \text{ mV} \) (500-ms duration) at CL of 5 s and 30 s, respectively, and the clamp protocol was applied first in the control solution and then after 1 min in the acidic test solution.

The steady-state voltage dependence of \( I_{\text{Ca,L}} \) activation in myocytes was determined with results obtained from the I-V curve clamp protocol. Relative conductance (\( G_{\text{max}} \)) was calculated as \( G = I_{\text{Ca,L}}/(V_m - V_{\text{rev}}) \), where \( V_m \) is the clamp potential and \( V_{\text{rev}} \) is the estimated reversal potential (\(-76 \text{ mV} \)) used as the estimated reversal potential, \( V_{\text{rev}} \) was calculated by Nernst equation and liquid junction potential. The relationship was fit to a Boltzmann function, and \( G_{\text{max}} \) was estimated by extrapolation of the curve to more positive potential. The resulting normalized G-V relationships were again fit with a Boltzmann function: \( G/G_{\text{max}} = 1/[1 + \exp(V_m - V_1/2)/k] \), where \( V_{1/2} \) and \( k \) are the half-maximal activation potential and the slope (mV) of the curve, respectively.

The steady-state voltage dependence of \( I_{\text{Ca,L}} \) inactivation in myocytes was determined with a double-pulse protocol. Conditioning clamp pulses ranging from \(-80 \text{ mV} \) to 0 mV (500-ms duration) were initiated from a holding potential of \(-80 \text{ mV} \) at a CL of 5 s. At the end of each conditioning pulse \( V_m \) was stepped to \(-40 \text{ mV} \) for 10 ms before application of the test pulse to \(+60 \text{ mV} \). \( I_{\text{Ca,L,1}} \) is negligible at \(+60 \text{ mV} \) and thus is unlikely to contaminate measurements of \( I_{\text{Ca,L}} \). Peak currents elicited by the test pulses were normalized as \( I_{\text{Ca,L,0}}/I_{\text{Ca,L,max}} \) and plotted as a function of conditioning \( V_m \). The curves were fit with a Boltzmann function: \( I_{\text{Ca,L,0}}/I_{\text{Ca,L,max}} = 1/[1 + \exp(V_m - V_1/2)/k] \), where \( V_m \) is the conditioning voltage, and \( V_{1/2} \) and \( k \) are the half-maximal inactivation potential and the slope (mV) of the curve, respectively.

The clamp protocol for quantifying the time course of \( I_{\text{Ca,L}} \) inactivation was the same as that used to generate I-V curves. With Clampfit software, the time course of inactivation of \( I_{\text{Ca,L}} \) was fit with a double-exponential function as previously described (22, 45) according to

\[
I = A_1 e^{-\tau_1 t} + A_2 e^{-\tau_2 t} + C
\]

where \( I \) is the current (pA/pF) at time \( t \) (ms), \( A_1 \) (pA/pF) and \( A_2 \) (pA/pF), respectively, are the amplitudes of fast and slow components, \( \tau_1 \) and \( \tau_2 \), respectively, are the fast and slow inactivation time constants (ms), and \( C \) is the current remaining at the end of the clamp step (pA/pF).

The time course of recovery from \( I_{\text{Ca,L}} \) inactivation was determined with a double-pulse protocol at a CL of 10 s. A conditioning clamp pulse (\( P_1 \)) to \(+60 \text{ mV} \) (400-ms duration) was applied from a holding potential of \(-80 \text{ mV} \), followed at a variable interval ranging from 100 ms to 5 s by the test pulse (\( P_2 \)) of 400-ms duration to \(+60 \text{ mV} \). All experiments were performed without Cd\(^{2+}\). Sodium-free bathing solution was used to block \( I_{\text{Na}} \) in the extracellular acidosis experiments. For the intracellular acidosis experiments, \( I_{\text{Na}} \) was inactivated by application of a prepulse from \(-80 \text{ mV} \) to \(-40 \text{ mV} \) (duration 40 ms) before clamping to \(+60 \text{ mV} \). The recovery from inactivation of \( I_{\text{Ca,L}} \) was fit with a single exponential as previously described (45) according to

\[
I = Ae^{-rt} + C
\]

where \( I \) is the current (pA/pF) at time \( t \) (ms), \( A \) (pA/pF) is the amplitude, and \( C \) is the current remaining at the end of the clamp step (pA/pF).

Action Potential Voltage Clamps

AP voltage-clamp experiments (AP clamp) were performed to assess the effects of intracellular acidosis on \( I_{\text{Ca,L}} \) under more physiological conditions than with rectangular clamp pulses. The bathing and pipette solutions used for these experiments were the same as those used to generate the I-V curve, except that all of the bathing solutions also contained 2 \( \mu \text{M} \) E-4031 to block \( I_{Kr} \). AP templates (control and intracellular acidosis) were made from representative records obtained during current-clamp experiments. Each AP clamp was preceded by a prestep from \(-80 \text{ mV} \) to \(-40 \text{ mV} \) (40-ms duration) at a CL of 5 or 2 s. A train of 10 conditioning clamps was first applied in the control solution with the control AP template. They were then repeated after 2 min in the test solution with the intracellular acidosis AP template. The entire protocol was then repeated in the presence of 3 mM 4-aminopyridine to block \( I_{\text{Na}} \). The difference current was used to determine \( I_{\text{Ca,L}} \) flowing during the AP.

A very similar protocol was used to measure \( I_{\text{Ca,L}} \) in Epi and PM myocytes during AP clamps. AP templates (control and intracellular acidosis) were made from representative records obtained during current-clamp experiments. All measurements were performed in the control bathing solution. Ten conditioning AP clamp pulses were applied to the cells prior to the test clamp to ensure steady-state loading of SR Ca\(^{2+}\) when \( I_{\text{Ca,L}} \) and CaTs were recorded simultaneously (see Table 2) and when only CaTs were recorded (see Fig. 12). The control AP template served as the conditioning pulse for the control test clamp, and the intracellular acidosis AP template served as the conditioning pulse for the acidosis test clamp. The entire protocol was then repeated in the presence of 200 \( \mu \text{M} \) CdCl\(_2\) and 10 \( \mu \text{M} \) nifedipine to correct for background current.

Measurement of Intracellular pH

\( p\text{Hi} \) was measured in single resting myocytes and HEK 293T cells with carboxy-seminaphthorhodafluor-1 (carboxy-SNARF-1), as previously described (2).

Measurement of Intracellular Calcium

CaTs were detected in single myocytes with a similar epifluorescence system using the fluorescent indicator fluo-4. Cells were incubated in the normal control solution containing 10 \( \mu \text{M} \) fluo-4 AM (Molecular Probes) and 0.3 \( \text{mM} \) probenecid at 25°C for 15 min. They were then continuously bathed in the same solution containing no indicator. Probenecid (0.3 \( \text{mM} \)) was included in the bathing solutions to help retard fluo-4 loss from the cells. Fluorescence emission (535 ± 11 nm, band-pass filter) was collected with a photomultiplier tube via the \( \times 40 \) oil objective (numerical aperture 1.3) during continuous excitation at 485 nm with a 150-W xenon lamp. Fluorescence signals were background corrected and expressed as \( F/F_0 \) (the ratio of fluo-4 fluorescence during the CaT divided by diastolic fluorescence). CaT duration was measured as the time from the upstroke to 50% recovery (CaTD\(_{50}\)). CaTs were elicited with AP clamps (CL = 2 s) during superfusion with the normal bathing solution, using the appropriate AP templates for control and intracellular acidosis. A train of at least 10 conditioning clamps was applied to the cells to achieve steady-state Ca\(^{2+}\) loading of the SR. The normal pipette filling solution was used (no Cs\(^{+}\), no BAPTA) when only CaTs were measured. For experi-
ments in which CaTs and $I_{Ca,L}$ were simultaneously recorded, K$^+$ in the bathing solution was replaced with Cs$^+$ and the pipette filling solution was the normal solution used for $I_{Ca,L}$ measurements except that it did not contain BAPTA. The gain of E-C coupling was calculated as the maximum rate of rise of the CaT divided by the simultaneously recorded peak $I_{Ca,L}$, as previously described (16).

Statistics

Summarized results are expressed as means ± SE. A paired Student’s $t$-test was used to test significance between results obtained with each cell serving as its own control. An unpaired $t$-test was used to test significance between two different groups of cells. $P < 0.05$ was considered significant.

RESULTS

Changes in pH During Extra- and Intracellular Acidosis

Figure 1, A and B, illustrate the changes in pHi in rabbit ventricular myocytes resulting from 2 min of extracellular and intracellular acidosis, respectively. HCO$_3^-$-dependent transporter activity was minimized by superfusing with CO$_2$/HCO$_3^-$-free solution, while NHE activity was blocked with 30 μM cariporide. As we have previously shown in rabbit ventricular myocytes (41), 2 min of low pH$_o$ caused negligible changes in pH$_i$, reducing it by only ~0.05 units (Fig. 1, A and D). In contrast, intracellular acidosis induced by superfusion with 80.0 mM acetate (pH$_o$ 7.4) for 2 min markedly reduced pH$_i$ by 0.47 units from a mean value of 7.22 ± 0.02 to 6.75 ± 0.04 ($n = 17$; Ref. 41). We have also found that pH$_i$ is unaffected by attachment with a suction pipette filled with the normal solution (pH$_{pipette} = 7.2$; Ref. 41). The 2-min acidosis protocol was used for both types of acidosis in all subsequent myocyte experiments.

Several earlier studies used pipette acid loading to induce intracellular acidosis in cardiac myocytes, on the assumption that pH$_i$ equilibrates with the pH of the pipette filling solution (25, 29, 58, 59). However, these studies did not include measurements of pH$_i$. Figure 1C shows that 10 min of intracellular dialysis with a pipette pH of 6.6 induced a fall in the

![Fig. 1. Changes in intracellular pH (pHi) and intracellular H$^+$ concentration ([H$^+$]$_{i}$) induced by extra- and intracellular acidosis and by pipette acid loading. A: a reduction in extracellular pH (pH$_o$) from 7.4 to 6.5 for 2 min elicited only a small drop in pH$_i$. B: in contrast, exposure to 80.0 mM acetate (pH$_o$ 7.4) induced a rapid sustained fall in pH$_i$. C: example record showing the marked difference between intracellular acidosis (pH$_o$ 7.4) induced with 80 mM acetate and with pipette dialysis [pipette pH (pH$_{pipette}$) = 6.6]. D: summarized results for extracellular acidosis ($n = 7$), intracellular acidosis ($n = 17$), and pipette acid loading ($n = 4$). We previously reported the summarized results shown in D for extra- and intracellular acidosis (41). The example records in A and B have not been previously published. All results shown here were obtained from a mixture of cells from all regions of the left ventricle. **$P < 0.01$ paired, control vs. acidosis. In separate experiments, we found no significant difference between the drop in pH$_i$ induced by 80.0 mM acetate (pH$_o$ 7.4) in epicardial (Epi, $n = 4$) and papillary muscle (PM, $n = 7$) myocytes (data not shown).
measured pH$_i$ of only 0.17 units, from 7.20 ± 0.04 to 7.03 ± 0.04 (n = 4). When expressed as change in intracellular H$^+$ concentration ([H$^+$]$_i$), this represents an ~1.5× increase compared with 3.1× increase induced by the 80.0 mM acetate pulse. Thus, assuming that pH$_i$ = pH$_{pipette}$, will seriously overestimate the degree of intracellular acidosis and emphasizes the importance of measuring pH$_i$ as we have done in the present study.

Effect of Acidosis on Action Potential Repolarization in Epicardial Myocytes

Because we used myocytes from all regions of the left ventricle in our previous study of pH effects on AP repolarization, there were large cell-to-cell differences in phase 1 and the notch (41). In this series of experiments we focused on the AP response of Epi myocytes to extracellular and intracellular acidosis. As shown in Fig. 2, epicardial APs display a prominent phase 1 repolarization and notch, mediated primarily by the high density of I$_{to}$ displayed in this cell type (17).

In response to 2 min of extracellular acidosis, the plateau was depressed and APD was shortened but the notch was largely unaffected (Fig. 2, A, C, and D). The resting membrane potential (CL = 2 s) was slightly depolarized by extracellular acidosis (control = −87.1 ± 2.5 mV, acidosis = −83.8 ± 2.7 mV; n = 5, P < 0.05, paired). The effects of extracellular acidosis on cardiac AP configuration are complex, with reports of inhibition of the rapid delayed-rectifier current I$_{Kr}$ (8), I$_{Ca,L}$ (8, 41), and both peak and persistent sodium current (30). In addition, we have recently shown that the changes in rabbit ventricular repolarization induced by low pH$_o$ are largely mediated by a reduction in I$_{Ca,L}$ and are unlikely to involve I$_{to}$ (41).

In contrast, intracellular acidosis markedly slowed phase 1 repolarization, reduced the notch, elevated the plateau, and prolonged APD (Fig. 2, B–D; pacing CL = 2 s). The percent change in APD$_{90}$ induced by low pH$_i$ at CL = 2 s (+88.5 ± 9.0%, n = 11) was not significantly different from that at CL = 5 s (+86.2 ± 21.0%, n = 4). The resting membrane potential (CL = 2 s) was slightly depolarized by low pH$_i$ (control = −81.0 ± 1.0 mV, acidosis = −79.0 ± 1.5 mV; n = 11, P < 0.05, paired), but the overshoot did not change (control = 45.7 ± 1.2 mV, acidosis 45.3 ± 1.3 mV; n = 11).

We also preformed AP measurements (CL = 2s) with 10 mM BAPTA in the suction pipette to minimize Ca$^{2+}$-activated Cl$^-$ current (I$_{Cl,Ca}$). This concentration of BAPTA blocks CaTs and the rise in diastolic calcium induced by lowering pH$_i$ (41). With BAPTA dialysis the changes in AP repolarization during

Fig. 2. Effect of extra- and intracellular acidosis on epicardial action potentials (APs). A: extracellular acidosis (pHo 6.5) shortened AP duration (APD). Insert: expanded view of phase 1 repolarization. B: intracellular acidosis (80.0 mM acetate) prolonged APD, markedly slowed phase 1 repolarization, and nearly eliminated the notch. Insert: expanded view of phase 1 repolarization. C: summarized changes in APD at 90% repolarization (APD$_{90}$) in low pH$_o$ (n = 5) and low pH$_i$ (n = 11). D: changes (Δ) in APD$_{90}$ shown in C expressed as % relative to control. The normal pipette filling solution was used in these experiments (no BAPTA). Pacing cycle length (CL) for all experiments was 2 s. *P < 0.05, **P < 0.01, paired, control vs. acidosis.
both types of acidosis were qualitatively the same as those without BAPTA and included AP shortening in low pH and AP lengthening in low pHi, suggesting that changes in $I_{\text{Cl, Ca}}$ did not play a major role [intracellular acidosis: control APD$_{90}$ = 414 ± 25 ms, acidosis APD$_{90}$ = 922 ± 36 ms ($n = 2$, $P < 0.02$, paired); extracellular acidosis: control APD$_{90}$ = 585 ± 50 ms, acidosis APD$_{90}$ = 519 ± 53 ms ($n = 3$, $P < 0.05$, paired)].

Numerous studies have demonstrated that $I_{\text{to}}$ inhibition slows phase 1 repolarization, elevates the plateau, and reduces the notch (4, 17, 18, 39). Thus the results in Fig. 2 strongly suggest that the $I_{\text{to}}$ activated during APs is inhibited by intracellular acidosis but largely unaffected by extracellular acidosis.

**Effect of Acidosis and Cadmium on Steady-State Voltage Dependence of $I_{\text{to}}$ Inactivation**

In this series of experiments we examined the separate actions of extra- and intracellular acidosis on the voltage dependence of $I_{\text{to}}$ inactivation in Epi myocytes (Fig. 3). We also assessed the effect of Cd$^{2+}$ on inactivation since it was used in other protocols to block $I_{\text{Ca,L}}$. After each 500-ms-duration conditioning step, $V_m$ was clamped to +60 mV, which is near the peak of the typical rabbit ventricular AP (Fig. 1) and activates a large $I_{\text{to}}$ with minimal contamination by $I_{\text{Ca,L}}$.

Previous work with rat ventricular myocytes, measured at room temperature, reported that both extracellular acidosis (50) and simultaneous reductions in pH$_{\text{i}}$ and pHi (24) induced right shifts in the voltage dependence of $I_{\text{to}}$ inactivation. Our results, measured at 37°C without Cd$^{2+}$ or dihydropyridine Ca$^{2+}$ channel blockers, confirm that extracellular acidosis (pH$_{\text{o}}$ 6.5) has the same effect in rabbit epicardial ventricular myocytes, shifting the inactivation $V_{1/2}$ by −10 mV (Fig. 3A; Table 1, no Cd$^{2+}$). The same result was also obtained in rabbit cells bathed in sodium-free solution containing no Cd$^{2+}$ (Table 1). In accord with earlier work in rat and rabbit myocytes (1, 50, 55), we also found that Cd$^{2+}$ (200 μM) right-shifted the voltage dependence of $I_{\text{to}}$ inactivation (Fig. 3A; Table 1), compare control no Cd$^{2+}$ with control 200 μM Cd$^{2+}$). In addition, the H$^+$-induced right shift was less in the presence of Cd$^{2+}$ (Fig. 3A), as also observed in rat ventricular myocytes (50).

In striking contrast to low pH$_{\text{o}}$, intracellular acidosis had no significant effect on the voltage dependence of $I_{\text{to}}$ inactivation with or without Cd$^{2+}$ in the bathing solution (Fig. 3B; Table 1).

**Table 1. Effect of acidosis and Cd$^{2+}$ on $I_{\text{to}}$ steady-state inactivation parameters**

<table>
<thead>
<tr>
<th></th>
<th>Exocellular acidosis (n = 4)</th>
<th>Intracellular acidosis (n = 5)</th>
<th>No Cd$^{2+}$ (n = 4)</th>
<th>No Na$^{+}$, No Cd$^{2+}$ Exocellular Acidosis (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control $V_{1/2}$, mV</td>
<td>$-34.6 \pm 1.8$</td>
<td>$-34.3 \pm 1.8$</td>
<td>$-44.4 \pm 1.6$</td>
<td>$-43.5 \pm 0.4$</td>
</tr>
<tr>
<td>Acidosis $V_{1/2}$, mV</td>
<td>$-28.7 \pm 1.6^{**}$</td>
<td>$-34.4 \pm 2.4$</td>
<td>$-34.5 \pm 2.5^{**}$</td>
<td>$-38.2 \pm 1.6$</td>
</tr>
<tr>
<td>Control slope $k$</td>
<td>$5.4 \pm 0.3^{**}$</td>
<td>$5.7 \pm 0.3$</td>
<td>$5.6 \pm 0.7$</td>
<td>$4.4 \pm 0.2$</td>
</tr>
<tr>
<td>Acidosis slope $k$</td>
<td>$5.6 \pm 0.2$</td>
<td>$7.1 \pm 0.4$</td>
<td>$5.1 \pm 0.4$</td>
<td>$5.3 \pm 0.5$</td>
</tr>
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Values are mean ± SE half-maximal inactivation potential ($V_{1/2}$) and $k$ determined from best fits of Boltzmann functions. $I_{\text{to}}$, transient outward current. *$P < 0.05$, **$P < 0.01$ vs. control, paired.
This indicates that Cd$^{2+}$ can be used to block $I_{\text{Ca,L}}$ in order to study the effects of changes in pHi on the steady-state gating properties of $I_{\text{to}}$. Importantly, it also suggests that low pHi does not affect steady-state inactivation of $I_{\text{to}}$. This lack of effect has been reported for rat ventricular myocytes measured at room temperature (58, 59). However, pipette acid loading was used in that study to induce intracellular acidosis without measuring pHi, which our results demonstrate (Fig. 1, C and D) is not a reliable method for inducing quantitative changes in pHi.

**Influence of Voltage Clamp Prestep and Cd$^{2+}$ on Response of $I_{\text{to}}$ Activation to Acidosis**

It is necessary to block the contaminating effects of $I_{\text{Ca,L}}$ and $I_{\text{Na}}$ when assessing the response of $I_{\text{to}}$ activation to acidosis. $I_{\text{Ca,L}}$ can be blocked with Cd$^{2+}$, and $I_{\text{Na}}$ is readily inactivated by applying a 40-ms prestep from $-80$ mV to $-40$ mV. Here we assess the use of these two approaches for studying the effects of acidosis on $I_{\text{to}}$ activation.

**Low pHo.** The action of both low pHo and external Cd$^{2+}$ to right-shift $I_{\text{to}}$ inactivation makes it difficult to accurately assess the voltage dependence of $I_{\text{to}}$ activation during extracellular acidosis with Cd$^{2+}$ present. To avoid using Cd$^{2+}$, we examined the effect of low pHo on $I_{\text{to}}$ activation in Epi myocytes clamped to $+60$ mV, which, as noted above, is near the peak of the AP and is a voltage at which $I_{\text{Ca,L}}$ is negligible (41). To assess the effects of a prestep on $I_{\text{to}}$ activation, we used sodium-free solutions to block $I_{\text{Na}}$. Figure 4A shows that extracellular acidosis had no effect on $I_{\text{to}}$ in the absence of external sodium, external Cd$^{2+}$, and a prestep. In contrast, when the prestep was included $I_{\text{to}}$ increased during low pHo (Fig. 4B), presumably because of increased channel availability at $-40$ mV (Fig. 3A). The results are summarized in Fig. 4C. Thus inclusion of even a brief prestep will introduce errors in the measurement of $I_{\text{to}}$ activation during extracellular acidosis.

Most importantly, these results suggest that extracellular acidosis does not significantly affect $I_{\text{to}}$ in rabbit epicardial ventricular APs when they are initiated from normal resting potentials and have normal overshoots. In most subsequent experiments we focused on intracellular acidosis.

**Low pHi.** In the next series of experiments we examined the effect of Cd$^{2+}$ (200 μM) and the 40-ms prestep to $-40$ mV on $I_{\text{to}}$ activation in Epi myocytes during intracellular acidosis (Fig. 5). As in Fig. 4, the cells were clamped from a holding potential of $-80$ mV to $+60$ mV to minimize $I_{\text{Ca,L}}$. Intracellular acidosis markedly reduced $I_{\text{to}}$ in each of the four conditions: Na$^+$ plus Cd$^{2+}$ (Fig. 5Aa); Na$^+$, no Cd$^{2+}$ (Fig. 5Ab); no Na$^+$, no Cd$^{2+}$ (Fig. 5Ac); and no Na$^+$, no Cd$^{2+}$, and no prestep (Fig. 5Ad). The results are summarized in Fig. 5B and show that neither the presence of Cd$^{2+}$ nor the prestep significantly affected the action of low pHo to reduce $I_{\text{to}}$.

**Effect of Intracellular Acidosis on $I_{\text{to}}$ Activation and Kinetics of Inactivation**

On the basis of the results in Fig. 5, we further examined the voltage dependence of $I_{\text{to}}$ activation in Epi myocytes during intracellular acidosis, using a prestep with both Cd$^{2+}$ (200 μM) and normal sodium in the bathing solutions (Fig. 6). As shown in the example signals in Fig. 6A and summarized in Fig. 6B, intracellular acidosis (pHo 7.4, pHi 6.75) reduced $I_{\text{to}}$ by $\sim 50\%$ at nearly all voltages between $-40$ mV and $+60$ mV. The percent reduction in $I_{\text{to}}$ at $+40$ mV with CL = 5 s ($-56.2 \pm 3.1\%$) was not significantly different from that at CL = 1 s ($-61.1 \pm 3.8\%; n = 5$, paired).

Figure 6C summarizes the effects of intracellular acidosis on both steady-state activation and inactivation. Low pHo did not significantly change the $V_{1/2}$ of activation (11.3 ± 1.5 mV for control vs. 11.4 ± 1.9 mV for acidosis), while the slope was significantly decreased from $-19.7 \pm 0.8$ mV in control to $-15.3 \pm 0.8$ mV ($n = 9$, $P < 0.01$ paired). Steady-state inactivation was also unaffected by intracellular acidosis (with Cd$^{2+}$, same results shown Fig. 3B).

The quantitative relationship between pHo (pHo 7.4) and epicardial $I_{\text{to}}$ at $+50$ mV is summarized in Fig. 6D and shows that $I_{\text{to}}$ was inhibited by low pHo with an apparent pK of 6.92.

Figure 6E summarizes the voltage dependence of the fast ($\tau_f$) and slow ($\tau_s$) time constants of $I_{\text{to}}$ inactivation from +40 mV to +60 mV (CL = 5 s). Low pHo had no significant effect on $\tau_s$ but speeded $\tau_f$ at all voltages. Similarly, when the CL was maintained at 2 s, low pHo had no significant effect on $\tau_s$ but reduced $\tau_f$ from 10.4 ± 0.5 ms to 6.3 ± 0.6 ms at $+50$ mV ($n = 4$, $P < 0.01$, paired).

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**Fig. 4.** Effect of a voltage clamp prestep on the response of $I_{\text{to}}$ activation to extracellular acidosis (pHo, 6.5) in Epi myocytes. A: example experiment showing that, without a prestep to $-40$ mV, low pHo has no effect on $I_{\text{to}}$ activated by a voltage clamp step from $-80$ mV to $+60$ mV. B: example experiment (same cell as A) showing that low pHo increased $I_{\text{to}}$ when a 40-ms-duration prestep from $-80$ mV to $-40$ mV was included. C: summary of the results, expressed as % change in $I_{\text{to}}$ relative to control with and without the prestep ($n = 4$). All experiments in this figure were performed in sodium- and Cd$^{2+}$-free solution. Pacing CL = 5 s. **$P < 0.01$ paired, vs. without prestep.
Effect of Acidosis on Recovery from Inactivation

The time course of recovery from inactivation in Epiomyocytes was studied with a standard two-pulse protocol (Fig. 7). The low-pH₀ experiments were performed in sodium-free solution without a prestep. The recovery from inactivation of \( I_{to} \) in myocytes from control and both types of acidosis was best fit by a single-exponential function. The recovery time constant was unaffected by low pH₀ but slowed by low pHi.

Effect of Intracellular Acidosis on \( I_{to} \) During AP Clamps

To determine the effects of low pHi on Epi \( I_{to} \) under more physiological conditions than with rectangular clamp steps, we also conducted AP clamp experiments (Fig. 8). Clamps were applied to Epi myocytes at CLs of 2 and 5 s since \( I_{to} \) and phase 1 repolarization in rabbit ventricular myocytes are affected by pacing rate (17, 20). The templates used for the control and acidosis AP clamps were representative of epicardial APs recorded at both rates. Figure 8A illustrates the inhibitory effect of low pHi on \( I_{to} \) elicited by AP clamps (CL = 5 s). The peak amplitude of \( I_{to} \) was significantly reduced at both pacing rates (Fig. 8B), and there was no significant difference in the percent change relative to control between the two rates (unpaired). These results demonstrate the striking action of internal protons to inhibit \( I_{to} \) during APs.

Effect of Acidosis on Human Kv4.3 and Kv1.4 Expressed in HEK 293T Cells

On the basis of the voltage-dependent kinetics of recovery from inactivation and the time course of inactivation, transient outward current can be classified as fast (\( I_{tof} \)) and slow (\( I_{tos} \)). \( I_{tof} \) shows rapid recovery (i.e., recovery time constants on the order of 10 to hundreds of milliseconds), and \( I_{tos} \) shows slow recovery (i.e., on a timescale of seconds) (36). Evidence indicates that the pore-forming domain of the channel underlying \( I_{tof} \) is formed by a combination of Kv4.2 and/or Kv4.3 subunits, while Kv1.4 is the molecular component underlying \( I_{tos} \) (32, 34, 36). In addition, a variety of regulatory subunits have major effects on the properties of Kv1.4, Kv4.2, and Kv4.3, including channel kinetics and expression (32, 36).

Although the exact molecular basis of \( I_{to} \) in rabbit ventricle is unresolved, expression of mRNA and/or protein of Kv4.2, Kv4.3, and Kv1.4 has been reported (34, 39, 54).

To help identify which channel mediates the response of rabbit \( I_{to} \) to low pHi, we examined the effects of intracellular acidosis on hKv4.3 and hKv1.4 channels expressed in HEK 293T cells (Fig. 9). The bathing solutions contained normal sodium and no Cd²⁺, and no prestep was required to block sodium current. As shown in Fig. 9A, exposure to 40 mM acetate for 1 min (pH₀ 7.4, 30 μM cariporide) rapidly reduced...
Inactivation results same as shown in Fig. 3. (C) Inactivation kinetics. Cells were voltage clamped according to the protocol shown in Fig. 6. Effect of intracellular acidosis on ionic currents of hKv4.3 and hKv1.4 were measured by applying different concentrations of sodium acetate for 2 min while keeping extracellular pH at 7.4. The magnitude of intracellular acidosis (pHi 7.4) was varied by applying different concentrations of sodium acetate for 2 min while keeping extracellular pH at 7.4.

The kinetics of inactivation of both hKv4.3 and hKv1.4 were measured by applying different concentrations of sodium acetate for 2 min while keeping extracellular pH at 7.4. The magnitude of intracellular acidosis (pHi 7.4) was varied by applying different concentrations of sodium acetate for 2 min while keeping extracellular pH at 7.4.

Intracellular acidosis had little effect on phase 1 repolarization in PM cells, and the prolongation of APD was much less than in Epi cells. This is consistent with the finding that PM cells display a lower density of Kv4.3 channels.

**Effect of Intracellular Acidosis on AP and I_{to} in Papillary Muscle Myocytes**

Rabbit ventricular myocytes display marked regional differences in I_{to} density, with Epi cells having the highest and PM cells the lowest (17). This current gradient has large effects on AP repolarization in the intact heart. Given this spatial heterogeneity, it was of interest to also study the response of PM myocytes to intracellular acidosis.

Intracellular acidosis had little effect on phase 1 repolarization in PM cells, and the prolongation of APD was much less than in Epi cells. This is consistent with the finding that PM cells display a lower density of Kv4.3 channels.
Epi myocytes (Fig. 10, A and B). Figure 10C demonstrates that under control conditions I_{to} current density in PM myocytes was approximately half that of Epi cells, in accord with previous work (17). During intracellular acidosis I_{to} was reduced by \(50\%\) at all voltages in PM cells (Fig. 10, C and D). These results suggest that regional differences in I_{to} current density make a significant contribution to the differential response of AP repolarization to low pH in Epi and PM myocytes.

Modulation of I_{Ca,L} by pH_{i}-Induced Changes in AP Waveform

The effects of intracellular acidosis on AP repolarization in Epi myocytes are much more prominent than those in PM cells (compare Fig. 2B to Fig. 10A). Epi cells show a marked slowing of phase 1, loss of the notch, and much greater AP prolongation (Fig. 10Bb). Since phase 1, the notch, and APD have been shown to modulate I_{Ca,L}, this could have significant effects on calcium handling (42, 43). In this series of experiments we sought to determine how I_{Ca,L} is affected by pH_{i}-induced changes in AP repolarization in Epi and PM myocytes, using AP voltage clamps. Only male rabbits were used for these experiments since transmural differences in peak I_{Ca,L} density have been reported for female rabbits (37). To confirm the absence of a transmural gradient we measured I_{Ca,L} density in both cell types under control conditions (no acidosis) with a conventional rectangular voltage-clamp protocol. As shown in Fig. 11A, the I-V curves for I_{Ca,L} were virtually identical in the two cell types.

For AP voltage-clamp experiments both the control and acidosis templates were applied in the control bathing solution (K^{+} replaced with Cs^{+}) with the normal pipette filling solution for I_{Ca,L} measurements (Cs^{+}, BAPTA). This approach allowed us to determine how pH_{i}-induced changes in AP repolarization per se affect I_{Ca,L} without the complicating effects of intracellular acidosis.

As illustrated in Fig. 11, B–E, application of the acidosis template decreased the initial peak value of I_{Ca,L} and prolonged its duration, with the greatest changes occurring in Epi cells. Despite the fall in peak I_{Ca,L} (Fig. 11C), net Ca^{2+} influx via I_{Ca,L} increased in both cell types, with the greatest increase occurring in Epi cells (Fig. 11D). The changes in peak I_{Ca,L} and net Ca^{2+} influx, expressed as percentage, are summarized in Fig. 11E.

These results demonstrate that the action of low pH_{i} to slow phase 1 repolarization and prolong APD significantly alters I_{Ca,L} and net Ca^{2+} influx via I_{Ca,L}, with the greatest changes...
occurring in Epi myocytes. Thus internal H\(^+\) ions can have significant indirect effects on voltage-dependent I\(_{\text{Ca,L}}\) mediated, in part, by pHi-induced changes in I\(_{\text{to}}\) that alter the time course of repolarization.

Modulation of CaTs by pH\(_{i}\)-Induced Changes in AP Waveform

To determine whether CaTs elicited by APs are affected by pH\(_{i}\)-induced slowing of phase 1 repolarization and APD prolongation, we performed the AP clamp experiments shown in Fig. 12. All studies were performed in control bathing solution with the same AP templates as those shown in Fig. 11 and the normal pipette filling solution (no Cs\(^+\), no BAPTA). The Epi templates were applied to Epi myocytes, and the PM templates were applied to PM myocytes. Small but significant increases in the amplitude and rate of rise of the CaT occurred in Epi but not PM myocytes (Fig. 12, Ba and Bb). The duration of the CaT (CaTD\(_{50}\)) was increased by the intracellular acidosis template in both cell types (Fig. 12, A and Bc).

We also performed separate AP clamp experiments in which I\(_{\text{Ca,L}}\) and CaT were simultaneously recorded in Epi and PM myocytes. The AP templates were the same as those shown in Fig. 12A and were applied to their respective cell types. Potassium in the bathing solution was replaced with Cs\(^+\), and the pipette filling solution was the normal solution used for I\(_{\text{Ca,L}}\) measurements, except that it contained no BAPTA. The results are summarized in Table 2 and show that the intracellular acidosis template decreased peak I\(_{\text{Ca,L}}\) by \(-31.0 \pm 3.9\%\) and \(-7.8 \pm 1.7\%\) in Epi (n = 5) and PM (n = 5) myocytes, respectively. These results are very similar to those obtained with AP clamps during BAPTA dialysis (Fig. 11C). In addition, the acidosis template in Epi but not PM cells increased both the amplitude and rate of rise of the CaT. This resulted in a significant increase in E-C coupling gain in Epi but not PM myocytes, calculated as maximum rate of rise of the CaT divided by peak I\(_{\text{Ca,L}}\).

Collectively, these results demonstrate that the striking changes in AP repolarization induced by intracellular acidosis have a significant modulating influence on ventricular calcium handling that is especially evident in cells with a prominent I\(_{\text{to}}\).

DISCUSSION

Changes in myocardial pH have multiple effects on electrical activity and Ca\(^{2+}\) signaling (14, 33). This sensitivity accounts in part for the arrhythmias and depression of ventricu-
ular function that occur during myocardial ischemia, a condition associated with a fall in both pH$_i$ and pH$_o$ (3, 19). Here we examined the response of rabbit ventricular I$_{to}$ to acute extracellular and intracellular acidosis, especially intracellular, and determined the resulting effects on AP repolarization. We also studied the indirect action of these repolarization changes on I$_{Ca,L}$ and CaTs. In contrast to previous reports of pH effects on ventricular I$_{to}$ (24, 50, 58, 59), our experiments were performed at physiological temperature with a myocyte preparation with a more humanlike AP than that of rat.

We demonstrate that I$_{to}$ is rapidly and markedly reduced by intracellular acidosis (Figs. 6, 8, and 10). This occurred in cells with both high (Epi) and low (PM) I$_{to}$ densities. In both cell types low pHi reduced peak I$_{to}$ over a large voltage range (−90 to +60 mV), resulting in an ~50% fall in current density when pHi is reduced from 7.22 and 6.75. The apparent pK of this relationship in Epi cells was 6.92, demonstrating the high H$^+$ sensitivity of I$_{to}$ over the physiological range of pHi values. Interestingly, this reduction in peak current was not accompanied by shifts in either steady-state inactivation or activation curves. This contrasts with the action of external H$^+$ to induce right shifts (toward less negative potentials) in these parameters as reported here for rabbit ventricular myocytes (Fig. 3A) and previously for rat and human ventricular myocytes (50). These shifts presumably result from H$^+$ screening of and/or binding to anionic sites such as carboxylic or amine/imidazole residues on the external sarcolemma and/or the channel itself. We have recently shown that both extra- and intracellular acidosis elicit large right and left shifts, respectively, in steady-state activation and inactivation of I$_{Ca,L}$ in rabbit ventricular myocytes (41). The absence of shifts in I$_{to}$ gating parameters during intracellular acidosis cannot be explained by the present study, but perhaps it reflects a low density of intracellular anionic sites on the channel itself.

Previous studies of rat ventricular myocytes also reported that intracellular acidosis reduced peak I$_{to}$ without altering steady-state activation and inactivation (58, 59). However, those experiments were performed with pipette acid loading, without measuring pHi, and in some experiments without the same cell serving as its own control. In addition, the origin of
the cells was not specified, e.g., epicardial or endocardial. All of these issues were avoided in the present work.

The absence of voltage shifts in $I_{\text{to}}$ gating, accompanied by a large reduction in peak current during low pH, strongly suggests a direct action of internal protons to reduce channel permeability, perhaps by titrating binding sites within the channel pore. This effect has a rapid onset and thus is unlikely to reflect a decrease in channel density.

We found no significant effect of extracellular acidosis on recovery from inactivation (Fig. 7A). In contrast, we did observe a small slowing of $I_{\text{to}}$ recovery from inactivation during intracellular acidosis (Fig. 7B). However, this did not significantly affect the action of intracellular acidosis to reduce peak $I_{\text{to}}$ during AP or rectangular voltage clamps elicited at CLs of 5, 2, or 1 s. Thus acidosis-induced slowing of recovery from inactivation does not appear to contribute significantly to the inhibitory effects of low pH on $I_{\text{to}}$.

Response of Kv4.3 and 4.1 Currents to Intracellular Acidosis

Previous studies using heterologous expression systems reported that extracellular acidosis inhibited both human Kv4.3 current (49) and Kv1.4 from rat and ferret (10). In contrast, human Kv1.4 current is reported to be unaffected by extracellular acidosis but inhibited by intracellular acidosis (35). Kv1.4 and Kv4.3 are both expressed in rabbit ventricle (34, 39, 54).

To help identify the molecular basis for the inhibitory action of intracellular acidosis on rabbit $I_{\text{to}}$, we examined the effects of low pH on human Kv4.3 and Kv1.4 channels expressed in HEK 293T cells (Fig. 9). Kv1.4 current was unresponsive to a fall in pH from 7.3 to 6.7 (pHm 7.4), while Kv4.3 current magnitude was significantly reduced over the voltage range of $-110$ to $-60$ mV (Fig. 9), suggesting that Kv4.3 mediates, in part, the sensitivity of rabbit ventricular $I_{\text{to}}$ to pH. However, we cannot rule out the possibility that differences in regulatory subunits and temperature (37°C vs. 23°C) between the native channels and those expressed in HEK cells may also affect the pH sensitivity of these currents.

Relationship Between Action Potential Repolarization and $I_{\text{to}}$ Inhibition by low pH

Consistent with the higher $I_{\text{to}}$ density in Epi myocytes, the repolarization effects of intracellular acidosis to reduce $I_{\text{to}}$ were most evident in this cell type, with marked slowing of phase 1, attenuation of the notch, and elevation of the plateau (compare Fig. 2B and Fig. 10B). The role of $I_{\text{to}}$ inhibition in overall prolongation of APD in both cell types is less clear, but it seems likely that it contributed in part. Regardless of the detailed mechanism for the increased APD during low pH, the magnitude of APD prolongation in Epi cells was approximately twice that in PM cells.
Although extracellular acidosis induced a right shift in steady-state inactivation (Fig. 3A), it did not affect the magnitude of $I_{\text{to}}$ at $+60$ mV, a voltage near the peak of the AP, when activated from $V_m$ near the normal diastolic value (Fig. 4A). In addition, both extra- and intracellular acidosis had only small effects on diastolic $V_m$ (Fig. 2). Thus it seems unlikely that $I_{\text{to}}$ is involved in the pHo-induced shortening of APD we observed in this preparation, a conclusion supported by our previous computer simulations (41).

**Table 2. Effect of AP repolarization on simultaneously measured $I_{\text{Ca,L}}$ and CaTs**

<table>
<thead>
<tr>
<th></th>
<th>Epi Template ($n = 5$)</th>
<th>PM Template ($n = 5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Intracellular acidosis</td>
</tr>
<tr>
<td>Initial peak $I_{\text{Ca,L}}$, pA/pF</td>
<td>$-7.64 \pm 0.59$</td>
<td>$-5.35 \pm 0.70^{**}$</td>
</tr>
<tr>
<td>Net Ca$^{2+}$ influx via $I_{\text{Ca,L}}$, pC/pF</td>
<td>$0.31 \pm 0.03$</td>
<td>$0.40 \pm 0.05^{*}$</td>
</tr>
<tr>
<td>CaT amplitude (F/F₀)</td>
<td>$1.95 \pm 0.23$</td>
<td>$2.09 \pm 0.23^{**}$</td>
</tr>
<tr>
<td>Rate of rise, F/F₀/s</td>
<td>$51.8 \pm 16.4$</td>
<td>$65.2 \pm 14.4^{*}$</td>
</tr>
<tr>
<td>E-C coupling gain, (F/F₀/s)/(pA/pF)</td>
<td>$7.36 \pm 2.66$</td>
<td>$13.8 \pm 4.22^{*}$</td>
</tr>
</tbody>
</table>

All values are means ± SE. All experiments were performed in the absence of acidosis with appropriate action potential (AP) templates for control and intracellular acidosis. $I_{\text{Ca,L}}$, L-type calcium current; CaT, calcium transient; Epi, epicardial; PM, papillary muscle; F/F₀, ratio of fluorescence during CaT divided by diastolic fluorescence; E-C, excitation-contraction. *$P < 0.05$, **$P < 0.01$ vs. control, paired; ***$P < 0.01$ vs. control with Epi template, unpaired.
Modulation of Calcium Handling by Intracellular Acidosis During Action Potentials

Our AP clamp experiments, performed with normal pHo and pHi, revealed that the changes in AP configuration induced by intracellular acidosis have significant effects on peak $I_{\text{Ca,L}}$, net $\text{Ca}^{2+}$ influx via $I_{\text{Ca,L}}$, and the CaT (Fig. 11, Fig. 12, Table 2). These effects appear to be mediated, in part, by pH-induced changes in $I_{\text{Ca,L}}$ and are more prominent in Epi than PM myocytes. Several earlier studies reported that changes in phase 1 repolarization significantly affect $I_{\text{Ca,L}}$ and CaTs during APs (6, 11, 43, 44). However, to our knowledge, this is the first demonstration that changes in ventricular repolarization induced by low pHi have significant indirect effects on calcium handling.

Our finding of a reduction in peak $I_{\text{Ca,L}}$, resulting from $I_{\text{to}}$ inhibition (Fig. 11), is consistent with canine myocyte simulations (21) and rat myocyte experiments (11, 43, 44) and is likely mediated by the reduced voltage driving force acting on $\text{Ca}^{2+}$ current. Epi myocytes displayed the greatest change in peak $I_{\text{Ca,L}}$, reflecting their higher $I_{\text{o}}$ density and thus more pronounced slowing of phase 1. Despite the drop in peak $I_{\text{Ca,L}}$, net $\text{Ca}^{2+}$ influx via $I_{\text{Ca,L}}$ increased in both Epi and PM cells, presumably because of the prolonged APs. The largest increase in $\text{Ca}^{2+}$ influx occurred in Epi cells, reflecting the greater AP lengthening (Fig. 11D).

The prolongation of the AP also caused CaT duration to increase in both cell types (Fig. 12Bc). However, the amplitude and rate of rise of the CaT as well as EC coupling gain increased significantly only in Epi myocytes (Fig. 12, Ba and Bb, Table 2). These increases occurred despite a drop in the peak amplitude of Epi $I_{\text{Ca,L}}$. Under normal conditions, for a given magnitude of $I_{\text{Ca,L}}$, the amplitude of the CaT is steeply dependent on SR calcium load (46). It seems likely that the much larger increase in net $\text{Ca}^{2+}$ influx in Epi myocytes promoted increased SR $\text{Ca}^{2+}$ loading, leading to increased gain and CaT amplitude. Supporting this hypothesis is the finding in rat ventricular myocytes of increased SR $\text{Ca}^{2+}$ load and CaT amplitude when APD is increased (42). Similarly, AP prolongation increases CaT amplitude in rabbit ventricular myocytes (38).

In contrast to our results, both rabbit and rat ventricular myocytes displayed reduced CaT amplitude when subjected to AP clamps using human AP templates of heart failure in which the notch was reduced and APD increased (11). The authors proposed that the smaller CaT amplitude resulted from a reduction in peak $I_{\text{Ca,L}}$ that decreased synchrony of SR $\text{Ca}^{2+}$ release. We cannot rule out dysynchronous $\text{Ca}^{2+}$ release in our experiments. However, it seems possible that its effect on CaT amplitude was mitigated by enhanced SR $\text{Ca}^{2+}$ content mediated by the much greater AP prolongation during low pHi (~88%; Fig. 2D) compared with that of the heart failure AP templates (~25%) used in the Cooper et al. study (11).

In summary, we have shown that, in contrast to low pHo, intracellular acidosis has a marked inhibitory effect on rabbit ventricular $I_{\text{to}}$ perhaps mediated by Kv4.3. The repolarization effects of this inhibition were much greater in Epi than PM myocytes and included slowing of phase 1, attenuation of the notch, and elevation of the plateau. In addition, the low pHi-induced prolongation of APD was greatest in Epi myocytes. In the intact heart this may have the undesirable effect of promoting transmural heterogeneity of repolarization under pathological conditions in which pHi falls. Increased dispersion of repolarization has been shown to facilitate the occurrence of reentrant arrhythmias (26). By altering the trajectory of the AP waveform, low pHi modulates calcium handling as reflected in a reduction in initial peak $I_{\text{Ca,L}}$, increased net $\text{Ca}^{2+}$ influx via $I_{\text{Ca,L}}$, and increased CaT amplitude and duration. Thus, in addition to the direct action of [H+]i, on $I_{\text{Ca,L}}$ (41), SR $\text{Ca}^{2+}$ release and uptake (28), NCX (15), and diastolic Ca2+ (41), low pHi also exerts a significant indirect effect on E-C coupling. This illustrates the striking diversity of mechanisms that contribute to the overall response of ventricular myocytes to acute increases in [H+]i.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.S., V.G., and K.W.S. conception and design of research; N.S., V.G., and K.W.S. performed experiments; N.S., V.G., and K.W.S. analyzed data; N.S., V.G., and K.W.S. interpreted results of experiments; N.S. and K.W.S. prepared figures; N.S. and K.W.S. drafted manuscript; N.S., V.G., and K.W.S. revised manuscript; N.S., V.G., and K.W.S. approved final version of manuscript.

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