Beat-to-beat dynamic regulation of intracellular pH in cardiomyocytes

Highlights

- Cardiomyocytes exhibit beat-to-beat cellular acidification, termed “pHi transient.”
- pHi transients are coupled to cardiomyocyte contractions and are tightly regulated.
- Inhibitions of the mitochondrial electron transport chain attenuate pHi transients.
- pHi transients may reflect a rhythmic metabolic status in cardiomyocytes.
Beat-to-beat dynamic regulation of intracellular pH in cardiomyocytes

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SUMMARY

The mammalian heart beats incessantly with rhythmic mechanical activities generating acids that need to be buffered to maintain a stable intracellular pH (pHi) for normal cardiac function. Even though spatial pHi non-uniformity in cardiomyocytes has been documented, it remains unknown how pHi is regulated to match the dynamic cardiac contractions. Here, we demonstrated beat-to-beat intracellular acidification, termed pHi transients, in synchrony with cardiomyocyte contractions. The pHi transients are regulated by pacing rate, Cl⁻/HCO₃⁻ transporters, pHi buffering capacity, and β-adrenergic signaling. Mitochondrial electron-transport chain inhibition attenuates the pHi transients, implicating mitochondrial activity in sculpting the pHi regulation. The pHi transients provide dynamic alterations of H⁺ transport required for ATP synthesis, and a decrease in pHi may serve as a negative feedback to cardiac contractions. Current findings dovetail with the prevailing three known dynamic systems, namely electrical, Ca²⁺, and mechanical systems, and may reveal broader features of pHi handling in excitable cells.

INTRODUCTION

The heart has the most extraordinary metabolic demands per gram of tissue among excitable cells because of the constant mechanical work (Doenst et al., 2013; Wang et al., 2010). ATP powers cardiac contraction. The production of ATP generates H⁺ and CO₂ in aerobic reactions and lactic acid in anaerobic metabolism, yielding acidic products that alter intracellular pH (pHᵢ). pHi is a crucial regulator of cellular structure and function (Casey et al., 2010; Cordat and Casey, 2009). Cells employ mobile HCO₃⁻, less mobile protein buffers and H⁺-equivalent transporters to maintain a stable pH, (Casey et al., 2010; Roos and Boron, 1981). In the absence of H⁺ regulatory mechanisms, large pH swings ensue (Vaughan-Jones et al., 2009; Wang et al., 2014), resulting in untoward accumulation of intracellular H⁺ (Casey et al., 2010; Vaughan-Jones et al., 2009). An unregulated decline of pH in the heart leads to reduced contractility, deranged Ca²⁺ signaling, and arrhythmias (Vaughan-Jones et al., 2009). Thus, the precise homeostatic control of cardiac pH is critical for normal cardiac function.

Studies have shown that acid-extruders, such as Na⁺/H⁺ exchanger (NHE) and Na⁺-HCO₃⁻ cotransporter (NBC), as well as acid-loaders, like Cl⁻/HCO₃⁻ (CBE) and Cl⁻/OH⁻ (CHE) exchangers, mediate cardiac pH regulation (Vaughan-Jones et al., 2009). Methods for cardiac pH measurement have continued to evolve since the early 1960s ranging from the use of open-tipped glass micropipette electrodes, 5,5 dimethyl-2,4-oxazolidinedione (DMO), isotopes, pH-sensitive microelectrodes, nuclear magnetic resonance, and fluorescent dyes (Bers and Ellis, 1982; Blank et al., 1992; Ellis and Thomas, 1976a; 1976b; Hunjan et al., 1998; Kirschenlohr et al., 1988; Lavallee, 1964; Schroeder et al., 2010; Valkovic et al., 2019; Wad- dell and Bates, 1969). Cardiac pH was documented to range between 7 and 7.3 when HCO₃⁻ was used as the buffer. Due to the relatively large size of cardiomyocytes, compartmentalization of cellular activities causes spatial pH non-uniformity, and a pH gradient of up to ~0.1 unit was previously reported (Swie-tach and Vaughan-Jones, 2005b; Vaughan-Jones et al., 2006), suggesting that there exists a relatively weak coupling of pHi and H⁺-equivalent transporters with local regulatory mechanisms of pHi. A recent study using confocal line-scan mode recorded ~3.6 nM proton concentration changes in the cytosol, which resulted from acid-yielding ATP hydrolysis during cardiomyocyte contraction (Hulikova and...
Swietach, 2016). However, it remains unknown how the pH is regulated to match the beat-to-beat contraction of the heart.

We hypothesize that cardiac pH may experience beat-to-beat changes resulting from the burst of myofilament ATPase activity and rhythmic metabolic events, dampened by endogenous pH buffering. Results from simultaneous measurement of pH and sarcomere lengths demonstrate unequivocally that cardiac pH, experiences beat-to-beat dynamic changes, termed “pHi transients.” The pH transients are coupled to cardiomyocyte contraction and regulated by pacing rate, sarcolemmal Cl⁻/HCO₃⁻ transporters, pH buffering capacity, and β-adrenergic signaling. Inhibiting the mitochondrial electron transport chain (ETC) activities attenuates the pH transients suggesting the possible contributions of mitochondria in generating pH transients. Indeed, the beat-to-beat dynamic changes in pH during cardiac contraction reflect a rhythmic metabolic status in cardiomyocytes. We propose that temporal pH acidification may provide a transient increase in the driving force for H⁺ transport to enhance mitochondrial ATP synthesis during cardiac contraction to meet the increased energy demand. Moreover, a decrease in pH may serve as a negative feedback to control the cardiac contraction due to the inhibitory effects on the contractile machinery by low pH. Our findings reveal a dynamic pH regulatory system in the heart that dovetails with the prevailing dynamic electrical, Ca²⁺, and mechanical regulatory systems and have critical physiological and disease ramifications.

RESULTS

Intracellular pH in cardiomyocytes experiences dynamic beat-to-beat changes

Simultaneous pH, and sarcomere lengths were measured and quantified in single ventricular cardiomyocytes during the contraction–relaxation cycle. To ensure that the changes in fluorescence signals were not due to motion artifacts, we positioned the relaxed cardiomyocyte within the imaging frame to capture the fluorescence signals from the whole cells. Moreover, two different fluorescent indicators including pHrodo green, an intensity-based pH dye, and SNARF-1, a ratiometric pH dye, were used to verify the results. Indeed, the pH, in cardiomyocytes experiences dynamic beat-to-beat changes coupled with the contraction, termed pH transients (Figures 1A, and S2). Mouse and rabbit left ventricular myocytes were loaded with pHrodo green dye, bathed in HCO₃⁻-buffered Tyrode’s solution and paced at 0.5 Hz (Figure 1A. Left: rabbit; Right: mouse). A significant acidification of pH was observed in response to each sarcomere contraction. For comparisons of the time courses of the pH and sarcomere contraction, ten cycle average traces of simultaneous pH, and sarcomere length were overlayed and shown in the lower panel.

To further ensure that the pH transient observed was not due to motion artifacts or dependent on a specific pH dye, SNARF-1, a ratiometric dye was used to verify the findings. The major advantage of the ratiometric dye is the fact that it is less prone to motion artifacts from cell contractions. Similar dynamic beat-to-beat pH changes were observed using SNARF-1 (Figure 1B. Left: rabbit; Right: mouse; and Figure S2). The baseline pH is summarized in Figure 1C, together with the absolute pH transient amplitude (ΔpHi) in Figure 1D. The baseline pH, measured in mouse and rabbit cardiomyocytes using pHrodo green and SNARF-1 was similar; however, the pH transient amplitude in the mouse was significantly smaller than that in the rabbit cardiomyocytes (Figure 1D). Thus, we choose rabbit cardiomyocytes and use pHrodo green for subsequent studies.

We further measured pH, and sarcomere length at physiological temperature (36°C) and pacing rate (1 Hz) in rabbit ventricular myocytes shown in Figure 1E. The baseline pH is significantly lower compared to that at RT (Figure 1F), and the absolute amplitude of pH transient at physiological temperature is significantly larger than that at RT (Figure 1G).

We analyzed the kinetics of the beat-to-beat pH changes in rabbit cardiomyocytes recorded at RT. The time-to-peak of the pH transient was 489 ± 28 ms (N = 7, n = 21. N represents animal numbers, while n represents cell numbers), while the time to the peak velocity was 112 ± 16 ms (n = 21). The recovering phase of the pH transient was fitted with a single exponential function with a time constant of 293 ± 24 ms (N = 7, n = 21). Additionally, the time-to-peak of the contraction (time to the maximum contraction or the shortest sarcomere length) was 524 ± 24 ms (N = 7, n = 21), which is not significantly different from that of the pH transient (489 ± 28 ms). In contrast, the time-to-peak contraction velocity (time to the maximum sarcomere contraction speed) was 45 ± 3 ms (N = 7, n = 21), which was significantly shorter than that of the pH transient (p = 0.00034). The relaxation phase of the contraction was well fitted by a single exponential function with a time constant of 203 ± 19 ms (N = 7, n = 21), which was significantly smaller than that of the pH transient (293 ± 24 ms) (p = 0.0057). The kinetic analyses support the coupling of the
pHi transient to the sarcomere contraction with a similar time-to-peak. However, the nearly 50% slower decay for the pHi transient versus relaxation supports the notion that the signal is not likely a motion artifact. Indeed, if the pHi transients reflect rapid ATP consumption, it is probable that the pHi recovery may take longer than relaxation. In mouse cardiomyocytes, the time-to-peak of the pHi transient was 170 ± 16 ms (N = 6, n = 15), which is significantly smaller than that of rabbit (p = 1.7 × 10⁻⁹); the time constant of mouse pHi transient recovering phase was 143 ± 15 ms (N = 6, n = 15), which is also significantly smaller.
than that of rabbit ($p = 0.000052$). The differences in the kinetics may be related to differential myosin subtypes in adult rabbit ($\beta$-myosin) and mouse ($\alpha$-myosin) cardiomyocytes.

We did not find significant differences in the pH transients between the male and female mice (Figure S3A). In both mouse and rabbit atrial myocytes, the baseline pH is significantly lower than that in left ventricular myocytes, and the absolute amplitude of pH transients is larger than that in left ventricular myocytes (Figure S3B). We further examined the rabbit left and right ventricular myocytes, and we did not find significant differences in baseline pH, and pH transients between rabbit left and right ventricular myocytes (Figure S3C).

**Inhibition of cardiac contraction reduces the intracellular pH transient**

To generate a cardiac contraction, coupled electrical and mechanical activities, as well as Ca$^{2+}$ signaling and myofilament ATP hydrolysis, are required. ATP hydrolysis results in the acidification of pHi. Moreover, mechanical activities alter the metabolic states of cardiomyocytes and activate mechanosensitive ion channels and transporters. These events are also expected to change the pH.

To decipher the underlying mechanisms for the observed beat-to-beat pH transients, we separate myofilament ATP consumption from ionic fluxes and other cellular events by taking advantage of a myosin ATPase inhibitor blebbistatin (BLEB, 25 $\mu$M) (Kovacs et al., 2004). Figure 2A shows that BLEB reduces the sarcomere contractions and causes a significant reduction of pHi transient accompanied by an acidification of baseline pH. Example traces before (control), during (BLEB), and after (washout) of BLEB are shown in the right three panels in Figure 2A. Summary of the changes in baseline pH, and the absolute amplitude of pH transients are shown in Figure 2C. Upon washout, the baseline pH, and pH transients recovered slowly accompanied by partial recovery of the sarcomere contraction, suggesting partially reversible inhibitory effects of BLEB.

To further investigate the contraction-coupled pH transients, we applied another excitation–contraction (E–C) uncoupler, 2,3-butanedione 2-monoxime (BDM, 10 mM), to inhibit the contraction of cardiomyocytes (Backx et al., 1994). BDM significantly inhibited the contraction of cardiomyocytes as well as the pH transients (Figure 2B), but did not significantly affect the baseline pH (Figure 2D). Example traces before (control), during (BDM), and after (washout) BDM application are shown in the right three panels. The effect of BDM was reversible upon washout. We further tested the inhibitory effect of BDM in physiological conditions, and the results are similar to those obtained at RT as shown in Figure S4.

The observed significant reduction of pH transients suggests that the pH transient is coupled to the contraction of cardiomyocytes, and may result, in part, from the myofilament ATP hydrolysis.

**Regulation of intracellular pH transients by intracellular pH buffering capacity**

pHi in cardiomyocytes is tightly regulated in normal cardiac function. However, in the pathological condition such as ischemia, the pH falls significantly in the infarcted myocardium (Vaughan-Jones et al., 2009). Moreover, respiratory or metabolic acidosis or alkalosis results in significant alterations in systemic pH as well as cardiac pH (Valley et al., 1990). To test the cellular mechanisms underlying the pH transients, we kept extracellular pH (pHe) constant, but altered the pHi by including sodium acetate or NH$_4$Cl in the bath solutions as previously described (Roos and Boron, 1981; Sirish et al., 2017; Thomas, 1984). We recorded pHi transients and sarcomere lengths during the application of NH$_4$Cl and acetate as shown in Figure 3. Perfusion by NH$_4$Cl containing solutions significantly increased the baseline pH, and enhanced the sarcomere contraction, but reduced the pHi transients (Figures 3A and 3C). In contrast, inclusion of acetate significantly decreased the baseline pH, reduced the contraction but increased the pHi transients (Figure 3B and 3D). These findings demonstrate that amplitudes of pHi transients are decoupled from the sarcomere contraction when the baseline pHi was altered, likely as a result of endogenous pHi buffering capacity changes. Indeed, pHi is critical in modulating the pHi transients, providing an important regulatory mechanism of pHi transients in physiological and pathological conditions.

**Regulation of intracellular pH transients by extracellular HCO$_3^-$ and Cl$^-$**

The pHi in cardiomyocytes is tightly regulated by a group of ionic transporters, including acid-extruders and acid-loaders. NHE and NBC mediate acid extrusion, while CBE and CHE mediate acid loading.
(Vaughan-Jones et al., 2009). Because of the acidification of pHi transients during cardiac contractions, we hypothesize that acid loaders might play important roles in the regulation of pHi transients. We, therefore, tested the effects of Cl−/HCO3− on cardiac pHi and contractions. We compared the effects of HEPES-buffered with that of HCO3−-buffered Tyrode’s solutions as shown in Figure 4A. After switching the perfusate from HCO3− to HEPES-buffered solution, the baseline pHi increased significantly, accompanied by an enhancement of the contraction. However, the amplitude of the pHi transient was significantly reduced (Figure 4A). The exposure to the HEPES-buffered solution gradually reduced the contraction and pHi transients (Figure 4D). This observation supports the significant role of HCO3− and HCO3− transporters in regulating the baseline pHi, pHi transients, and contractions. The acute alkalinization induced by HEPES-buffered solution is likely due to intracellular HCO3− combining with a proton and leaving the cell as CO2. The baseline alkalinization decreases the intrinsic buffering capacity in HEPES-buffered solution (Leem et al., 1999); however, the pHi transients were reduced likely as a result of the smaller contraction.

As CBE functions as an acid-loader, we predict that the reduction of extracellular Cl− will reverse the direction of Cl−/HCO3− exchange and increase the baseline pHi. Thus, we tested the effects of extracellular Cl− on the pHi transient and contractions (Figure 4B). To reduce the Cl− concentration, NaCl was replaced by an equal concentration of Na-Glutamate in HCO3−-buffered solution. After switching the perfusate from
high Cl\(^{-}(130 \text{ mM})\) to low Cl\(^{-}(10 \text{ mM})\) solution, the baseline pH increased significantly, accompanied by a slight reduction in contractions. Additionally, the pH transient was significantly reduced (Figures 4B and 4E). The findings support the roles of Cl\(^{-}/\text{HCO}_3^{-}\) exchangers in the regulation of baseline pH and pH transients, consistent with the results shown in Figures 3A and 4A. We further tested the role of NHE, an acid extruder, on pH transients using 10 \(\mu\text{M}\) EIPA, a specific inhibitor of NHE (Masereel et al., 2003). EIPA did not alter the baseline pH or pH transients significantly, suggesting negligible contributions from NHE at physiological pH (Figures 4C and 4F).

**Regulation of intracellular pH transients by the beating rate of cardiomyocytes**

The mechanical activities of cardiomyocytes are fueled by cardiac metabolism, which may contribute to the pH transients. Heart rates significantly alter cardiac metabolic demand and affect cardiac contractions. As pH transients are linked to the contraction, we reason that the beating rate of cardiomyocytes may alter the myofilament ATPase activity as well as the pH transients. We recorded pH transients from cardiomyocytes paced at 0.1, 0.5, 1.2, and 4 Hz (Figure 5A). As the beating rate was increased, the sarcomere length in diastole shortened. The amplitude of contractions was reduced and the corresponding pH transient was decreased with increasing pacing rates (Figure 5B). The relationship between the time-averaged sarcomere length and the time-averaged pH at different pacing rates is shown in Figure 5C. The time-averaged sarcomere lengths represent the shortening of the myocytes during contraction as well as the diastronic
sarcomere lengths as the relaxation is incomplete at higher rates (Figures 5A and 5C). Hence, the time-averaged sarcomere lengths reflect the averaged force, an index of ATP consumption rate (Brandes and Bers, 1996, 1997). With higher pacing rates, the time-averaged sarcomere length became shorter, consistent with higher averaged ATP consumption (and protons produced) and the lower averaged pHi (Figure 5C). Figure 5D quantifies the amplitude of the steady-state sarcomere shortening ($\Delta$Sarcomere length) including the residual diastolic shortening against the amplitude of the pHi transients ($\Delta$pHi). Similar to Figure 5C, the plot in Figure 5D also accounts for the progressive diastolic shortening at higher frequency. However, $\Delta$pHi was reduced with increased $\Delta$Sarcomere length (including the residual diastolic shortening) at higher pacing frequencies with increased ATP consumption. This suggests that the higher averaged ATP consumption may not be the sole mechanism underlying the generation of pHi transients. To support the physiological relevance and significance, we performed the pacing experiment at 36°C, and similar results were obtained shown in Figure S5. To exclude the time-dependent effects, cells were returned to the lower

Figure 4. Effects of sarcolemma H+-equivalent transporter activities on baseline pHi and pHi transients
(A) The time course of pHi measurement when the cell was perfused with Tyrode’s solutions either using HCO₃⁻ or HEPES as pH buffer (left).
(B) The time course of pHi measurement when the cell was perfused with Tyrode’s solutions containing higher (130 mM) and lower (10 mM) Cl⁻ (left).
(C) The time course of pHi measurement when the cell was perfused by Tyrode’s containing 10 μM EIPA (left). The right three panels in A, B, and C showed three fragments of the time course at three conditions as shown on the top of the panel.
(D) Comparisons of baseline pHi, and $\Delta$pHi, of A (*p = 0.0039, n = 8; **p = 0.0039, n = 9; paired Wilcoxon signed-rank test).
(E) Comparisons of baseline pHi, and $\Delta$pHi, of B (*p = 0.022, n = 6; **p = 0.0046, n = 9; paired sample t test).
(F) Comparisons of baseline pHi, and $\Delta$pHi, of C (n = 5). N and n represent animal numbers and cell numbers, respectively. Data are represented as mean ± SEM.
pacing rate after pacing at high rates. There was a significant recovery of the baseline pHi and pHi transients 
upon returning to the lower pacing rate (Figure S6).

Activation of β-adrenergic signaling increases the intracellular pH transient
Acute activation of β-adrenergic signaling induces positive inotropic effects on cardiac myocyte contraction. 
Submicromolar isoproterenol was previously documented to be effective to increase intracellular 
Ca$^{2+}$ levels (Zou et al., 2001). Figure 6A shows the effects of 50 nM isoproterenol on the contraction and 
pHi transients. Isoproterenol stimulated a brief transient alkalinization of baseline pH, and a more gradual 
rise in the amplitude of contraction and pHi transients. Figure 6C shows significant increases in both myo-
cyte contractions and pHi transients after isoproterenol stimulations. The findings reflect the increase in averaged force and thus, an index of energy consumption rate and pHi transients. In contrast, Ca$^{2+}$ channel 
blocker, verapamil, has negative inotropic effects on cardiac contractions (Bell and McDermott, 1995; Ka-
naya et al., 2006). We further tested the effects of verapamil on cardiac contractions and pHi transients. Fig-
ures 6B and 6D demonstrate the inhibitory effects of 2.5 μM verapamil on the contraction, with reduced pHi, 
transients and elevated baseline pHi in rabbit cardiomyocytes.

Inhibiting mitochondrial electron transport chain reduces the intracellular pH transients
Mitochondria are the powerhouse for cardiomyocytes, and the generation of an H$^{+}$ gradient across the 
inner mitochondrial membrane is essential for ATP synthesis. We hypothesize that in addition to beat-to-beat ATP hydrolysis and energy consumption, the pH, transients may also be coupled to the mitochondrial H$^{+}$ transfer driving the mitochondrial ATP synthesis. Therefore, we applied different 
inhibitors to probe the functional impacts of the mitochondrial ETC on the baseline pH, and pH, 
transients.

Figure 5. Increasing the pacing rate reduced the pHi transients
(A) The time course of pHi measurement was shown on the left, and the right three panels showed three fragments of the time course at three pacing rates. 
(B) Comparisons of ΔpHi at different pacing rates (The p-values for a: 0.018; b: 0.0087; c: 0.01. n = 6, one-way repeated measures ANOVA combined with 
Tukey’s post hoc analyses). Animal numbers (N) are shown in the bar graphs.
(C) The relationship between the time-averaged sarcomere length and the time-averaged pH, during pacing at different pacing rates (n = 6).
(D) The relationship between ΔpHi and ΔSarcomere length (including the residual diastolic shortening) at different pacing rates (n = 6). N and n represent 
animal numbers and cell numbers, respectively. Data are represented as mean ± SEM. 
See also Figures S5 and S6.
Rotenone and antimycin A (Wiersma et al., 2019), which inhibit complex I and III, respectively, reduce proton pumping out of the matrix, which is expected to cause cytosolic alkalinization. Indeed, either inhibitor acutely alkalinized the baseline pHi, reduced the pHi transient, and increased contractions (Figures 7A, 7B, 7D, and 7E). The increased contractility likely results from pHi alkalization, and as the alkalinization subsided contractility declined. Finally, we examined the effect of 2-[[2-[4-(trifluoromethoxy) phenyl]hydrazinylidene]propanedinitrile (FCCP) (Bassani et al., 1992; Neary et al., 2014; Thai et al., 2018), a protonophore and an uncoupler of the ETC on pHi. Although FCCP initially allows mitochondrial H+ entry (transiently alkalinizing the cytosol), the uncoupling results in ATP synthase reversal as a major futile ATPase that generates acid decreasing the pHi. Indeed, FCCP acidified baseline pHi, abolished contractions, and hence abolished the pHi transient associated with the contraction (Figures 7C and 7F). A summary diagram in Figure 7G illustrates the beat-to-beat pHi regulatory system that dovetails with the prevailing dynamic electrical, Ca2+, and mechanical regulatory systems.

DISCUSSION
Cardiac function is shaped by three tightly coupled dynamic systems, including electrical excitation, Ca2+ signaling, and mechanical contraction. Our study identifies a dynamic beat-to-beat pHi system in the heart, that is tightly coupled with the other three dynamic systems due to the exquisite effects of pH on cardiac excitability, Ca2+ signaling, and contractions (Vaughan-Jones et al., 2009).
Figure 7. Inhibition of mitochondrial ETC reduced the pH\textsubscript{i} transients
(A–C) Time courses of pH\textsubscript{i} measurement when the cell was perfused by Tyrode’s containing 2 \textmu M rotenone (A, left), or
containing 4 \textmu M antimycin A (B, left), or containing 4 \textmu M FCCP (C, left). The right three panels showed three fragments of
the time course at three conditions as shown on the top.
(D) Comparisons of baseline pH\textsubscript{i} (**p = 0.000083, n = 11, paired sample t test) and \Delta\text{pH}\textsubscript{i} of A (\textasteriskcentered p = 0.0078, n = 9; \Delta\text{pH}\textsubscript{i}: 0.046 ± 0.008 in control vs 0.038 ± 0.006 with rotenone, paired Wilcoxon signed-rank test). After washout, \Delta\text{pH}\textsubscript{i} = 0.045 ± 0.007, and is not statistically different compared to control (p = 0.86).
(E) Comparisons of baseline pH\textsubscript{i} (**p = 0.00056, n = 17, paired sample t test) and \Delta\text{pH}\textsubscript{i} of B (*p = 0.00031, n = 15. \Delta\text{pH}\textsubscript{i}: 0.046 ± 0.007 in control vs 0.037 ± 0.006 with antimycin A, paired Wilcoxon signed-rank test). After washout, \Delta\text{pH}\textsubscript{i} = 0.045 ± 0.005, and is not statistically different compared to control (p = 0.67).
(F) Comparisons of baseline pH\textsubscript{i} (**p = 0.0002, n = 7, paired sample t test) and \Delta\text{pH}\textsubscript{i} of C (*p = 0.016, n = 7, paired
Wilcoxon signed-rank test. \Delta\text{pH}\textsubscript{i}: 0.057 ± 0.006 in control vs 0.003 ± 0.002 with FCCP). After washout, \Delta\text{pH}\textsubscript{i} = 0.055 ±
Diomyocytes suggests local regulatory machinery and spatial control of pHi. In depressed cardiac contractility, abnormal intracellular Ca²⁺ signaling, and arrhythmias (Garlick et al., 1995). Previous studies demonstrate that there is a significant spatial non-uniformity in pHi of cardiomyocytes, cardiac workload and heart rate (Bountra et al., 1988; Elliott et al., 1994; Vaughan-Jones et al., 2009). Pre-

Cardiomyocytes are vulnerable to pHi changes as the molecules participating in the regulation of cardiac excitability and contractility such as ion channels, transporters, and contractile proteins are exquisitely sensitive to pHi (Sirish et al., 2017; Thorsen et al., 2017; Vaughan-Jones et al., 2009; Wang et al., 2014). In pathological conditions such as myocardial ischemia, a large fall of pHi (~6.5) occurs, resulting in depressed cardiac contractility, abnormal intracellular Ca²⁺ signaling, and arrhythmias (Garlick et al., 1979; Steenbergen et al., 1977; Vaughan-Jones et al., 2009). The mammalian heart beats incessantly with rhythmic and robust electrical and mechanical activities consuming ATP and generating acids. Even though the intrinsic and HCO₃⁻ mobile buffering capacity in cardiomyocytes is strong, there remain pH fluctuations and non-uniformity in cardiomyocytes. Fluctuations of pH may occur during changes in cardiac workload and heart rate (Bountra et al., 1988; Elliott et al., 1994; Vaughan-Jones et al., 2009). Previous studies demonstrate that there is a significant spatial non-uniformity in pHi of cardiomyocytes, attributed to the low intracellular H⁺ mobility (Swietach et al., 2005; Swietach and Vaughan-Jones, 2005a; 2005b; Vaughan-Jones et al., 2002; Vaughan-Jones et al., 2006). The heterogeneity of pHi in cardiomyocytes suggests local regulatory machinery and spatial control of pH.

Cardiac pH, measurement and regulation have been extensively investigated over the past several decades since the 1960s (Bers and Ellis, 1982; Blank et al., 1992; Ellis and Thomas, 1976a; 1976b; Hunjan et al., 1998; Kirschensohl et al., 1988; Lavalle, 1964; Schroeder et al., 2010; Valkovic et al., 2019; Waddell and Bates, 1969). However, to eliminate the motion-induced instability for recordings by microelectrodes or reduce the motion distortion in confocal microscopic imaging, previous studies were performed mostly using quiescent cardiac tissues and cardiomyocytes. Furthermore, low temporal resolution limited the capture of the fast pH changes. A recent study explored the nuclear proton dynamics in rat cardiomyocytes and showed a ~3.6 nM increase in proton concentration (from 95.3 ± 0.002 to 99.0 ± 0.01 nM) in the cytosol along the long axis of the cell during contraction, and the index of contraction was shown by the width changes of the fluorescence signals. This measurement was performed using confocal line scan mode and the contraction of the cardiomyocytes was not directly quantified. The authors concluded that the small proton concentration change may result from acid-yielding ATP hydrolysis during cardiomyocyte contractions (Hulikova and Swietach, 2016).

Our study directly quantifies simultaneous changes of sarcomere length and pH, during the cardiac cycle and documents a significantly larger increase in free H⁺ concentration at RT and physiological conditions (36°C with 1 or 2 Hz pacing). The average value of pH, transient amplitudes in rabbit cardiomyocytes is ~0.055 unit at RT or ~0.12 at 36°C, which corresponds to a free H⁺ concentration increase of ~10 nM at RT or ~30 nM at 36°C from baseline pH, of 7.7–7.4. The proton buffering power in cardiomyocytes is in the order of 20–90 mM per pH unit (Crampin et al., 2006). If we use a median value of 50 mM per pH unit for the calculation of the total H⁺ release using the method previously reported (Hulikova and Swietach, 2016), 10 and 30 nM changes in free H⁺ concentrations represent 2 and 6 mM changes in total H⁺ concentrations, respectively. A previous study has shown that cooling from 37 to 27°C decreases both the intrinsic buffer power and acid extrusion from the cell, and the resting pH is ~0.1 pH units lower at 37°C (Ch’en et al., 2003). Our resting pH values measured at 36°C (6.97 ± 0.05, n = 13) and RT (7.17 ± 0.02, n = 14) are consistent with this previous finding (Ch’en et al., 2003). However, the absolute amplitude of pH, transients is significantly larger at 36°C (0.12 ± 0.01, n = 13) than that at RT (0.055 ± 0.005, n = 9), suggesting that the buffering power is not the only regulatory factor for the pH, transients.
To ensure that the recorded pH transients were not due to motion artifacts, we agonize over several important features in our recordings. First is to position the cells completely within the imaging frame during the recordings to capture fluorescence signals from the entire cell. Next, we use two different fluorescence indicators including pHrodo green and SNARF-1, a ratiometric dye, to directly verify the results. Additionally, we analyzed the time course of pH, transients and sarcomere contraction to compare the kinetic parameters and coupling. The time to peak contraction velocity for sarcomere contraction was used to compare to the time to peak velocity of pH transients, revealing a significantly slower kinetics in pH, transients than that of sarcomere contraction. Furthermore, data shown in Figure 3 provide clear evidence that pH, transients can be decoupled with the sarcomere contraction. Specifically, the decreased pH, transients were accompanied by enhanced contractions (Figure 3A) or vice versa (Figure 3B). Moreover, the immediate effect of the inhibition of ETC is the enhancement of contractions. However, this is accompanied by reduced pH, transients (Figure 7A and 7B), illustrating the decoupled changes of contractions and pH, transients, and providing further evidence that the pH, transient does not result from the motion artifacts.

The differences of the pH, transient amplitude between rabbit and mouse cardiomyocytes may result from the differential fractional shortening (FS) with significantly higher FS in rabbit cardiomyocytes (rabbit: 17 ± 1%, n = 19; mouse: 8 ± 2%, n = 12. p = 0.000022), as well as differences in the metabolic demands for cardiomyocyte contractions in rabbits and mice. The faster kinetics of pH, transients in mouse cardiomyocytes may be due in part to the higher velocities of mouse α-myosin compared to rabbit β-myosin (Malmqvist et al., 2004).

The differences in baseline pH, and pH, transients between atrial and ventricular myocytes may result from distinct expression levels of H⁺-equivalent transporters, metabolic profiles, and pH, buffering capacity, which need to be further investigated.

Actions of 2,3-butanedione 2-monoxime and blebbistatin on intracellular pH transients
Both BDM and BLEB inhibit myofilament force production by binding to myosin. BLEB is a more specific inhibitor and binds to the motor domain of myosin II, while BDM is less specific with low affinity due to its inhibition of other myosins and its interactions with other proteins (Bond et al., 2013). Our results show that both inhibitors significantly reduce pH, transients with simultaneous inhibition of contractions. BDM effect is reversible, while the BLEB effect is only partially reversible. However, the baseline pH, decreases during the application of BLEB while the baseline pH, remains the same upon the application of BDM, suggesting different mechanisms of BLEB and BDM in altering the ATP hydrolysis and metabolic status of cardiomyocytes. The effects of BLEB and BDM on pH, demonstrated in our study suggest that alterations in pH, need to be carefully considered in the result interpretations when these reagents are used in studies of E–C coupling.

Regulation of intracellular pH transients
The proton buffering capacity of the cell is governed by the protonated states of the buffers. In turn, the pH, alters the protonated states of the buffers depending on the pH, , while the CO₂/bicarbonate buffering capacity increases monotonically with pH, . As a result, the total buffering capacity increases with a rise of pH, in CO₂/HCO₃⁻-buffered conditions (Leem et al., 1999). In our studies, we used CO₂/bicarbonate-buffered solutions during pH recordings. We demonstrate that the amplitudes of pH, transients are significantly regulated by pH, , with decreased pH, transients by increased pH, and vice versa (Figure 3). This highlights the effect of pH, buffering capacity on pH, transients. Lower extracellular Cl⁻ also causes intracellular alkalization resulting in reduced pH, transient, but the contraction is slightly inhibited, which needs to be further investigated. The alkalization caused by lower extracellular Cl⁻ in our experiment is faster than that in sheep Purkinje fibers (Vaughan-Jones, 1979), which may result from the relatively slower solution penetration of Purkinje fibers and different recording methods. These results support the critical roles of membrane Cl⁻/HCO₃⁻ exchangers in the regulation of pH, transients. Cl⁻/HCO₃⁻ exchangers may regulate pH, transients by altering the baseline pH, and the buffering capacity. On the other hand, inhibition of NHE did not significantly affect the pH, , suggesting that NHE is not activated with neutral pH, and, therefore, has no effects on pH, transients.
A previous study using ion-selective microelectrodes reported that the increase in pacing rate causes the fall of baseline pH in sheep Purkinje fibers suggesting frequency-dependent intracellular acidosis (Bountra et al., 1988). Our data show cellular acidosis with higher pacing rates in rabbit ventricular myocytes consistent with the previous findings (Figures 5 and S5). However, the increase in pacing rates reduces the contraction amplitude and in turn, the pH transients. The increase in intracellular Ca^{2+} and Na^{+} concentrations with higher pacing rates may affect the cell contraction. In addition, higher pacing rates reduce the time-averaged sarcomere length suggesting increased force and more ATP hydrolysis. The time-averaged sarcomere length represents cardiomyocyte shortening during a contraction that includes the diastolic sarcomere lengths, which reflects the averaged force and is an index of ATP consumption rate. We further evaluated the relationship between the amplitude of pH transients with the delta sarcomere length including the residual diastolic shortening (delta sarcomere length plus diastolic sarcomere length changes) (Figure 5D). Specifically, even though the delta sarcomere length is shorter at higher pacing frequency, the residual diastolic shortening is actually larger which indicated the higher ATP consumption rate. In this case, we observed an inverse relationship, that is, the amplitude of pH, transients is reduced at higher pacing frequency, suggesting that ATP hydrolysis may not be the sole mechanism underlying the generation of pH, transients. A possible factor that may affect the pH is the breakdown of phosphocreatine to restore ATP at higher pacing rate with increased workload (Zervou et al., 2016). The effect of increased pacing rates on pH, transients is opposite to the effect of β-adrenergic signaling stimulation. The underlying mechanisms may involve different posttranslational modifications in the cellular calcium handling, sarcomeric proteins, rate-limiting steps in the SERCA transport cycle, and altered pH, and mitochondrial function. Additionally, the biological function of isoproterenol on cardiomyocytes may not be limited to increased contractility. Isoproterenol also alters intracellular signaling by activating different protein kinases which may contribute to pH, regulation. These will be further investigated in our future studies.

Role of mitochondria in generating intracellular pH transients
Mitochondria govern the metabolic and energetic status of cardiomyocytes. ETC in the inner membrane of mitochondria plays critical roles in ATP synthesis through oxidative phosphorylation. The central role of ETC is to establish the proton-motive force by generating an H^{+} gradient across the inner membrane (Santo-Domingo and Demaurex, 2012). As the mitochondrial outer membrane is freely permeable to H^{+}, we expect a rapid equilibrium of H^{+} with the mitochondrial intermembrane space. We demonstrate that the inhibition or uncoupling of ETC reduces or abolishes pH, transients suggesting the possible contributions of mitochondria in the generation of pH, transients. The inhibition of H^{+} pumping into the intramembranous space causes cytosolic alkalinization and reduces the pH, transients. The partial recovery phase from the rapid alkalinization caused by rotenone and antimycin A may stem from the compensatory mechanisms in the mitochondrial ETC. The mitochondrial uncoupler FCCP causes an increase of the respiration rates and cytosolic acidification with thermodynamically unrestrained H^{+} pumping and possibly with the reverse-mode of ATP synthase which hydrolyzes ATP to generate acid, resulting in the cessation of pH, transients and contractions. The elevation of mitochondrial Ca^{2+} is essential for controlling the ATP production to match cardiac demands during contractions (Bell et al., 2006; Dedkova and Blatter, 2013; Lu et al., 2013; Robert et al., 2001). Thus, pH, transients in cardiomyocytes may report the dynamic beat-to-beat oscillations of mitochondrial H^{+} transfer and bioenergetics that couple with the mitochondrial Ca^{2+} and mechanical signaling. The pH, transients may provide a transiently increased H^{+} driving force to enhance the ATP synthesis during cardiomyocyte contractions to meet the increased energy demand for contractions, suggesting a rhythmic metabolic status in cardiomyocytes. Taken together, H^{+} transport across the mitochondrial inner membrane may play critical roles in the generation of pH, transients in cardiomyocytes.

Physiological implications and future studies
Our study has important physiological implications. The pH, transients in cardiomyocytes suggest the beat-to-beat dynamic regulation of mitochondrial function for ATP synthesis and myofilament ATP hydrolysis synchronize the energy production and consumption for contractions. Additionally, the acidification caused by pH, transients during contractions may provide a negative feedback to the contractile elements, and facilitate the relaxation of cardiomyocytes due to the reduced cardiac contractility by low pH, (Harrison et al., 1992; Orchard and Kentish, 1990; Vaughan-Jones et al., 2009). The effects of mitochondrial inhibitors are statistically significant; however, the biological relevance needs to be further investigated. In addition, many aspects of the cell function may affect the pH. Therefore, future experimental and computational
studies are required to further test the mechanisms of mitochondria and other cellular processes in the generation of pH transients, including real-time measurement of ATP production and consumption in cardiomyocytes as well as mathematical pH modeling.

**Limitations of the study**

ATP hydrolysis is accompanied by the release of $\text{H}^+$ with a stoichiometry coefficient less than 1 depending on pH, and intracellular cation concentrations (Kushmerick, 1997); ATP synthesis also needs the proton transfer by mitochondrial ETC, and each ATP generated requires the transport of an estimated 4 protons (Turina et al., 2003). Therefore, ATP consumption and production will affect the total $\text{H}^+$ concentrations in cytosol, and they are one of the important mechanisms for dynamic pH regulation. Due to the complexity of the pH regulation system in the cells involving $\text{H}^+$ generation and buffering, $\text{H}^+$ transport, $\text{Ca}^{2+}$ buffering and signaling, and other metabolic activities, it is difficult to quantitatively correlate pH transients with the ATP consumption and production in this study.

Additionally, although we optimized the experimental condition to load the dye to the cytosol, due to the dyes' intrinsic properties, we could not rule out the effects of the dye loading into intracellular organelles which may affect our results. To this end, saponin treatment was performed to quantify the dye loading, demonstrating specific cytosol loading in our experiments. Secondly, the fluorescent dye may be subjected to photobleaching and leakage, which may cause the baseline pH drift. To circumvent this effect, we have conducted control experiments to record the baseline signals which have been used for the correction of the pH measurement (Figure S1).

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103624.

**ACKNOWLEDGMENTS**

We thank Dr. Joe Soughayer from IonOptix for his support in the configuration and troubleshooting of the measurement system. This work was supported by NIH R56 HL138392 to X.D.Z., NIH R01 HL085727, HL085844, HL137228, and VA Merit Review Grant 101 BX000576 and CX001490 (N.C.), and additional support provided by NIH R01 HL123526 (Y.C.I.), NIH R01 HL081562 (D.M.B., J.B.), NIH R01 DC015135, AG051443, DC015252, DC016099, AG060504 (E.N.Y), and Postdoctoral Fellowship from NIH/NHLBI Institutional Training Grant in Basic and Translational Cardiovascular Science T32 HL086350, and NIH F32 HL149288 (P.N.T.), and American Heart Association (AHA) Predoctoral Fellowship Award 18PRE34030199 (L.R.). NC is the holder of the Roger Tatarian Endowed Professorship in Cardiovascular Medicine and a part-time staff physician at VA Northern California Health Care System, Mather, CA.

**AUTHOR CONTRIBUTIONS**

X.D.Z. proposed and developed the concept, designed the study, initially tested the concept by experiments, analyzed the data, made figures, and wrote the manuscript. N.C. helped with experiment design, data analysis and interpretation, and revised the manuscript; Y.L. performed the intracellular pH and contraction measurement, data analysis, and organization; P.N.T. and L.R. tested dye loading conditions, conducted part of the imaging experiment, and contributed to figure making; V.T. isolated the mouse heart.
cardiomyocytes and contributed to the writing of STAR Methods; Z.J. isolated the rabbit cardiomyocytes; S.P. helped on the initial pH measurement experiments; K.S.G. and J.B. supervised the rabbit cell isolation, and K.S.G. contributed to the writing of STAR Methods and J.B. revised the manuscript; D.M.B. revised the manuscript and helped on the data analysis and interpretation; J.O. prepared and distributed the cardiomyocytes; Y.C.I. sponsored the rabbit cardiomyocyte isolation and revised the manuscript; E.N.Y. revised the manuscript and helped on the data acquisition and analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. One or more of the authors of this paper self-identifies as living with a disability.

Received: August 9, 2021
Revised: November 10, 2021
Accepted: December 10, 2021
Published: January 21, 2022

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| pHrodo™ Green AM | ThermoFisher Scientific | P35373 |
| Carboxy SNARF®-1 AM | ThermoFisher Scientific | C1272 |
| (+)-Blebbistatin | Abcam | Ab120425 |
| 2,3-Butanediol monoxime | Sigma-Aldrich, USA | B0753 |
| (+)-Isoproterenol hydrochloride | Sigma-Aldrich | I6504 |
| 5-(N-Ethyl-N-isopropyl) amiloride | Sigma-Aldrich | A3085 |
| Rotenone | Sigma-Aldrich | R8875 |
| Antimycin A | Sigma-Aldrich | A8674 |
| 2-[2-[(4-trifluoromethoxy) phenyl] hydrazinylidene]-propanedinitrile (FCCP) | Sigma-Aldrich | C2920 |
| Verapamil hydrochloride | Sigma-Aldrich | V4629 |
| Collagenase type II | Worthington, USA | LS004176 |
| Protease type XIV | Sigma-Aldrich | P5147 |
| Ketamine hydrochloride injection (100mg/ml) | Akorn Inc., USA | NDC 50989-161-06 |
| Xylazine sterile solution (20mg/ml) | Akorn Inc., USA | NDC59399-110-20 |
| Heparin (1000 USP units/ml) | Fresenius Kabi USA, LLC | NDC63323-540-57 |
| Fetal bovine serum | ThermoFisher Scientific | 10437028 |
| Penicillin-Streptomycin | ThermoFisher Scientific | 15-140-122 |
| Novolin R human insulin | Novo Nordisk USA | NDC 0169-1833-11 |
| Bovine serum albumin | Sigma-Aldrich | A9418 |
| Other salt and chemicals | https://www.sigmaaldrich.com | |
| **Experimental models: organisms/strains** | | |
| New Zealand white rabbits | Western Oregon Rabbit Company | 2495 Rosecrest Dr, Philomath, OR 97370, USA |
| C57BL/6J mice | The Jackson Laboratory | https://www.jax.org |
| **Software and algorithms** | | |
| IonWizard (64-bit) 7.2.7.138 | IonOptix | https://www.ionoptix.com |
| GraphPad Prism 9 | GraphPad Software | https://www.graphpad.com |
| Origin Pro 2021 | OriginLab Corp., Northampton, MA | https://www.originlab.com |
| BioRender | BioRender | https://www.biorender.com |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiao-Dong Zhang (xdzhang@ucdavis.edu).

Materials availability
All unique reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability
The data supporting the current study have not been deposited in a public repository because they are only used for the publication of this manuscript. This paper does not report original code. Any additional
information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

All animal care and procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of California, Davis, and per National Institutes of Health guidelines. Male and female animals were used. All experiments described in the study were conducted in a blinded fashion with different investigators for animal handlings, cardiomyocyte isolations, data collection and analysis. The cardiomyocytes used for each experiment were isolated from ~68 rabbits (New Zealand White rabbits, male, 3-6 months of old with 2-5 kg of body weight, Western Oregon Rabbit Company, Philomath, OR, USA) and ~21 mice (C57 BL/6J, male and female, 10-16 weeks old, Jackson Laboratory, Bar Harbor, ME, USA) depending on the sample size, and randomly assigned to the experimenters.

**METHOD DETAILS**

**Cell isolation and chemicals**

The chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specifically indicated. Blebbistatin was purchased from Abcam (Abcam, Cambridge, UK). Rabbit and mouse cardiomyocytes were isolated using the method we reported (Bartos et al., 2017; Hegyi et al., 2015; Jian et al., 2016; Kim et al., 2013; Sirish et al., 2017). Rabbit cardiomyocytes were isolated from hearts excised from New Zealand White rabbits. Briefly, rabbits were administered heparin at 400 USP units per kg subcutaneously 15–30 min before surgery. The animal was anesthetized with isoflurane inhalation (3-5%). The heart was excised and washed in a cold perfusion solution containing (in mM): 140 NaCl, 5.4 KCl, 1 MgCl₂, 5 Glucose, 5 HEPEs (free acid), 5 HEPEs (sodium salt), and pH 7.4, supplemented with 40 units of Novolin R human insulin, 50,000 units penicillin, and 50,000 micrograms of streptomycin per litter, and was then cannulated and mounted on a Langendorff apparatus. The heart was initially perfused with perfusion solution with added 2800 USP heparin per litter for 5 min to clear the blood. The heart was then perfused with perfusion solution with added collagenase type II (1 mg/ml Worthington Biochemical Corporation, Lakewood, NJ, USA), protease Type XIV (0.04 mg/ml, Sigma Aldrich, St. Louis, MO, USA), 5 mM taurine, and 0.02 mM CaCl₂ under constant flow (3-6 ml/min/gram heart weight) until it loses most of its integrity (typically 25-30 min). The temperature of the perfusion solutions was kept at 37°C. The heart was then removed from the perfusion system, and the digestion was stopped by incubation of the heart in perfusion solution with added 10% fetal bovine serum (FBS) and 0.02 mM CaCl₂. The ventricles and atria were minced and pipetted in the solution to liberate individual ventricular and atrial myocytes as needed. Cells were filtered through nylon mesh, and stored in perfusion solution with added 0.125 mM CaCl₂ at room temperature for experimental use. Isolation of mouse cardiomyocytes followed the conventional enzymatic dissociation methods we reported (Jian et al., 2016; Sirish et al., 2017). Mice were anesthetized by intraperitoneal injection of 80 mg/kg of ketamine and 5 mg/kg of xylazine. Intraperitoneal administration of 300 USP units of heparin was performed 10-15 minutes before the surgery. Mice were exsanguinated by rapid heart excision. The excised heart was quickly immersed in the perfusion solution containing (in mM): 113 NaCl, 4.7 KCl, 1.2 MgSO₄, 0.6 Na₂HPO₄, 0.6 KH₂PO₄, 12 NaHCO₃, 10 KHCO₃, 30 Taurine, 10 Hepes, and 10 glucose, and pH = 7.4. After trimming off the associated tissues, the heart was cannulated on a Langendorff apparatus and first perfused with perfusion solution. After 3 min, the heart was then perfused with perfusion solution with added collagenase (1 mg/mL, type II, Worthington Biochemical Corporation, Lakewood, NJ, USA). The perfusion pressure was monitored, and the flow rate was adjusted to maintain the initial perfusion pressure at ≈ 60 mmHg. The temperature of the perfusion solutions was kept at 37°C. After 15 to 20 min of collagenase digestion, the heart was then removed from the perfusion apparatus and transferred to petri dish containing perfusion solution with the addition of 10% FBS. The ventricles and atria were minced and pipetted to obtain ventricular and atrial myocytes, respectively. Cells were harvested by filtering through nylon mesh, and the Ca²⁺ concentration in the cell storage solution was restored stepwise using solutions containing 0.2, 0.5, or 1 mM CaCl₂ (in mM): 133.5 NaCl, 4 KCl, 1.2 Na₂HPO₄, 10 HEPES, 1.2 MgSO₄, 10 glucose, 20 taurine, 0.2 (or 0.5, or 1) CaCl₂, pH = 7.4, supplemented with bovine serum albumin (1 mg/ml). Cardiomyocytes were maintained in the cell storage solution containing 1 mM CaCl₂ at room temperature until ready for experiments.

**Measurement of pHi and sarcomere length**

We used IonOptix contractility (IonOptix LLC., Westwood, MA, USA) system (Jian et al., 2014; Sirish et al., 2017) to measure single cardiomyocyte contraction and pHi. Cardiomyocytes were loaded with SNARF-1
Cardiomyocytes were seeded in a chamber with two field stimulation electrodes connected to a Myopacer (IonOptix LLC., Westwood, MA, USA). The chamber was mounted on the stage of the Zeiss Axio Observer A1 inverted fluorescent microscope imaged by using a 63x or 40x oil lens. The chamber was perfused continuously during recordings. Cardiomyocytes were paced with a square bipolar pulse with 2 ms width and 10 V amplitude at different frequencies ranging from 0.1 Hz to 4 Hz. The measurement was performed at room temperature (RT, 20–22°C) and 36°C. The temperature control was achieved by using Warner TC-344C temperature controller (Warner Instruments, Holliston, MA, USA). The signals were acquired by IonWizard 7 software.

For the measurement of pHi, a xenon lamp was used as an excitation light source. For SNARF-1 measurement, the dye loaded cardiomyocytes were excited by a selected wavelength of 550 ± 10 nm as previously reported (Buckler and Vaughan-Jones, 1990), and the emitted fluorescent light was split by a 605 nm long-pass dichroic mirror with the shorter wavelengths through a band-pass filter of 585 ± 10 nm, and the longer wavelengths through a band-pass filter of 630 ± 15 nm to two photomultipliers, respectively. For pHrodo green measurement, the dye loaded cardiomyocytes were excited by a selected wavelength of 500 ± 10 nm, and the emitted fluorescent light was filtered using a band-pass filter of 535 ± 15 nm and collected by a photomultiplier. The contraction was measured using a high-speed camera (MyoCam-S, 240 to 1000 frames/s) to record the sarcomere movement. The sarcomere pattern was used to calculate the sarcomere length using an FFT algorithm.

The solutions for pH measurement include Tyrode’s solution buffered by HEPES containing (in mM): 120 NaCl, 24 Na-Glutamate, 4 KCl, 1 MgCl₂, 10 Glucose, 0.33 NaH₂PO₄, 10 HEPES, and pH 7.4, with added either 5 μM SNARF-1 AM or 5 μM pHrodo green for dye loading. After removing the loading solution, cardiomyocytes were washed twice using Tyrode’s solution and resuspended with Tyrode’s solution for pH measurement. To reduce the effects of photobleaching and dye leakage of pHrodo green on the measurement with longer duration, we recorded on pHrodo green loaded cardiomyocytes without any interventions for ∼10 mins with pacing and without pacing, and the photobleaching process of the fluorescent signal was well fitted by a linear equation (Figure S1). The slope resulting from the linear fitting was used to correct the pH measurement to minimize the effects of photobleaching and dye leakage by subtracting the slope factor.

The SNARF-1 emission ratio (F₅₈₀/F₆₄₀) was converted to a pHi value using standard calibration (Blank et al., 1992; Buckler and Vaughan-Jones, 1990; Niederer et al., 2008; Sirish et al., 2017). The pHrodo green intensity was converted to a pH value using the calibration method provided by the manufacturer. The calibration was performed by measuring the SNARF-1 emission ratios and pHrodo green intensities when the cells were perfused by calibration solutions with four different pH values of 5.5, 6.5, 7.5, and 8.5 (Figure S2). For pHrodo green calibration, the fluorescence intensity signals were normalized by the peak intensity at pH 5.5, and pH recordings performed on each cardiomyocyte were completed by perfusion using pH 5.5 calibration solution to acquire the peak fluorescence intensity value for normalization and proper calibration for each cardiomyocyte. The normalized intensities at calibration solutions were fitted by a sigmoidal function to obtain the standard calibration curve for pHrodo green (Figure S2B). The representative pHrodo green and SNARF-1 fluorescence signals recorded in rabbit cardiomyocytes are shown in Figures S2A and S2C. The representative calibration trace and calibration curve for SNARF-1 are shown in Figure S2D.
Ten cycles of the pH transients and sarcomere shortening traces were averaged and used for analyses. The representative traces shown in the figures were chosen to represent the mean values of the experimental group if applicable.

To ensure that the changes in fluorescence signals were not due to motion artifacts introduced by cell contractions, we positioned the relaxed cell completely within the imaging frame using either 63× or 40× objectives depending on the cell size. After each measurement, the cardiomyocyte was moved away from the imaging frame for recording background fluorescence signals, and background fluorescence signals were subtracted for each channel during the data analysis. For pacing experiment, we did not count the cardiomyocytes that have less than 50% of averaged normal fractional shortening at lowest pacing rate.

To quantify the cytosolic dye loading, the loaded cells were perfused by Tyrode’s solution containing 50 μg/ml saponin. Only (10.5 ± 1.4)%(pHrodo green, n = 7), (7.5 ± 1.5)% (SNARF-1/F580, n = 8), (8.1 ± 1.6)% (SNARF-1/F640, n = 8) fluorescence signals remained after saponin treatment, suggesting predominant cytosolic dye loading. The intrinsic fluorescence signals of cardiomyocytes were measured before the loading of the dye, and the ratios of the intrinsic signals to the dye signals were quantified and are less than one percent (pHrodo green: (0.78 ± 0.35)%; SNARF-1 F580: (0.92 ± 0.27)%, F640: (0.62 ± 0.27)%). The intrinsic fluorescence signal is subtracted from the dye signals.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

We estimate a sample size of 5 per experiment to detect at least 15% difference before and after the change of the conditions with alpha=0.05 for a two-tailed test to give the power of the study >0.95, assuming the standard deviation of the differences to be 5% (SigmaStat, Systat Software Inc.). No data were excluded. Data are presented as mean ± S.E.M. Shapiro-Wilk test was used for normality test. Statistical comparisons were achieved by one-way ANOVA combined with Tukey’s post hoc analyses among three or more groups, or paired sample t-test (two-tailed) or two-sample t-test when the data follow a normal distribution, if comparing the effects before and after the application of drugs on the same cell. If the data did not follow a normal distribution or with small sample number, non-parametric paired Wilcoxon signed-rank test was used. Statistical significance was set at p < 0.05. The statistical analyses were performed using Origin Pro 2021 (OriginLab, Northampton MA, USA) and GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).