Na\textsuperscript{+} ions as spatial intracellular messengers for co-ordinating Ca\textsuperscript{2+} signals during pH heterogeneity in cardiomyocytes

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Aims

Contraction of the heart is regulated by electrically evoked Ca\textsuperscript{2+} transients (CaTs). H\textsuperscript{+} ions, the end products of metabolism, modulate CaTs through direct interactions with Ca\textsuperscript{2+}-handling proteins and via Na\textsuperscript{+}-mediated coupling between acid-extruding proteins (e.g. Na\textsuperscript{+}/H\textsuperscript{+} exchange, NHE1) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. Restricted H\textsuperscript{+} diffusivity in cytoplasm predisposes pH-sensitive Ca\textsuperscript{2+} signalling to becoming non-uniform, but the involvement of readily diffusible intracellular Na\textsuperscript{+} ions may provide a means for combating this.

Methods and results

CaTs were imaged in fluo3-loaded rat ventricular myocytes paced at 2 Hz. Cytoplasmic [Na\textsuperscript{+}] ([Na\textsuperscript{+}]) was imaged using SBFI. Intracellular acidification by acetate exposure raised diastolic and systolic [Ca\textsuperscript{2+}] (also observed with acid-loading by ammonium prepulse or CO\textsubscript{2} exposure). The systolic [Ca\textsuperscript{2+}] response correlated with a rise in [Na\textsuperscript{+}] and sarcoplasmic reticulum Ca\textsuperscript{2+}-load, and was blocked by the NHE1 inhibitor cariporide (CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}-free media). Exposure of one half of a myocyte to acetate using dual microperfusion (CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}-free media) raised diastolic [Ca\textsuperscript{2+}] locally in the acidified region. Systolic [Ca\textsuperscript{2+}] and CaT amplitude increased more uniformly along the length of the cell, but only when NHE1 was functional. Cytoplasmic Na\textsuperscript{+} diffusivity (D\textsubscript{Na}) was measured in quiescent cells, with strophanthidin present to inhibit the Na\textsuperscript{+}/K\textsuperscript{+} pump. With regional acetate exposure to activate a local NHE-driven Na\textsuperscript{+}-influx, D\textsubscript{Na} was found to be sufficiently fast (680 μm\textsuperscript{2}/s) for transmitting the pH–systolic Ca\textsuperscript{2+} interaction over long distances.

Conclusions

Na\textsuperscript{+} ions are readily diffusible messengers that expand the spatial scale of cytoplasmic pH–CaT interactions, helping to co-ordinate global Ca\textsuperscript{2+} signalling during conditions of intracellular pH non-uniformity.

Keywords

Acidosis • Calcium • Diffusion • E–C coupling • Na\textsuperscript{+}–H\textsuperscript{+} exchange

1. Introduction

A brief rise of cytoplasmic [Ca\textsuperscript{2+}] in cardiac myocytes (the Ca\textsuperscript{2+} transient, CaT) couples the action potential to cellular contraction.\textsuperscript{1} This CaT displays considerable plasticity. An important example is during changes of intracellular pH (pHi), where the CaT can increase or decrease in amplitude, depending on conditions.\textsuperscript{2} In healthy isolated myocytes, pHi is typically close to 7.2 (equivalent to ~60 nM [H\textsuperscript{+}]). This value represents a balance among metabolic acid/base production within the cell and H\textsuperscript{+}-equivalent transport across the sarcolemma. pHi decreases by 0.1–0.2 units with increasing cardiac work-load and can vary during neurotransmitter and hormonal stimulation.\textsuperscript{2} The pHi also decreases dramatically (up to ~0.8 units) during clinical conditions such as myocardial ischaemia, where it contributes to acute contractile failure and electrical arrhythmia.\textsuperscript{2–4} In all these examples, a coupling between pHi and intracellular Ca\textsuperscript{2+} signalling plays a role in shaping the myocardial response.

Cardiomyocytes have a propensity to develop pHi non-uniformity. Intracellular gradients of 0.1–0.5 units have been measured in ventricular myocytes under various experimental conditions.\textsuperscript{5–7} Key factors that predispose to non-uniformity are the low myoplasmic H\textsuperscript{+} diffusivity\textsuperscript{8} (~100 μm\textsuperscript{2}/s), high sarcolemmal H\textsuperscript{+}-equivalent flux during acid/base

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disturbances,9 the expression of H+-equivalent transporters in spatially segregated sarcolemmal domains,6 and regional heterogeneity in the extracellular concentration of membrane-permeant weak acids, such as lactic acid and CO2 (e.g. at the borders of regionally ischaemic zones).10–12 While the effects of whole-cell pH change on intracellular Ca2+ signaling have been well characterized, far less is known about the effects of spatial pHi non-uniformity.

There are multiple routes that couple intracellular [Ca2+2+]/[Ca2+2+] ([(Ca2+2+)]i) with pH, in the mammalian cardiomyocyte. Resting [Ca2+2+]i increases during acidosis, because of Ca2+-unloading from pH-sensitive intracellular buffers, such as troponin C, histidyl dipeptides (HDPs), and ATP,13 an effect reinforced by H+-induced slowing of Ca2+ extrusion on sarcolemmal Na+/Ca2+ exchange (NCX).13 Modulation of the CaT by low pH also relies on the effect of H+-ions on L-type Ca2+ current.14 On Ca2+ re-uptake into the sarcoplasmic reticulum (SR) by the SR Ca ATPase (SERCA),15,16 and on SR Ca2+ release channels (ryanodine receptor channels, RyRs).17,18 The integrated influence of all these effects during a global acidosis is a reduction in SR Ca2+ release, and hence a decrease in the amplitude of the CaT (for convenience, defined here collectively as a direct inhibitory H+-effect on the CaT). Low pH, however, exerts an additional influence on the CaT. This occurs through stimulation of sarcolemmal Na+/H+ exchange (NHHE1) and Na+-HCO3- co-transport (NBC). These membrane transporters extrude intracellular acid from cardiomyocytes in exchange for the entry of extracellular Na+. As a result, cytoplasmic [Na+]i can rise rapidly by several mM during a global fall of pH. By acting on sarcolemmal NCX, the [Na+]i rise then causes retention of intracellular Ca2+, which facilitates greater SR Ca2+ loading via SERCA, leading ultimately to enhanced SR Ca2+ release, and hence an increase in CaT amplitude15 (for convenience, defined here as an indirect stimulatory H+-effect on the CaT). The modulation of CaT amplitude by global acidosis therefore reflects a balance between the direct inhibitory and indirect stimulatory effect of H+-ions on Ca2+-signalling. Depending on which effect is the greater, reducing pH can either increase or decrease CaT amplitude. The increase, when it occurs, can be physiologically advantageous as it helps to maintain myocardial contraction during acidosis, which would otherwise be depressed owing to decreased Ca2+ binding to the regulatory subunit, troponin C.2

Although a global acidosis can powerfully modulate the CaT, it is not known whether localized pH changes, when they occur, produce local or more global effects within the cell. We have recently shown5 that a localized acidic microdomain, when induced experimentally in the bulk cytoplasmic compartment can, within seconds, instigate a localized and stable microdomain of elevated resting [Ca2+2+]. This phenomenon is mediated by cytoplasmic HDPs and ATP molecules acting as diffusible Ca2+/H+-exchangers. It ensures that any spatial non-uniformity of [H+]i, is matched by a comparable non-uniformity in resting [Ca2+2+]. It is not known, however, whether the characteristics of the dynamic CaT signal can similarly be localized by pH. If so, this would lead to spatial dysynchrony in the CaT, during local acid/base disturbances, potentially compromising the contractile efficiency of the cell.

In the present work, we have investigated the effects of local pH changes on the spatial characteristics of the CaT in the isolated rat ventricular myocyte. We have induced a local intracellular acid-load by using a dual microperfusion apparatus to expose one end of the cell to a permeant weak acid. Our results show that although the direct inhibitory response of the CaT to H+-ions occurs locally within the cell, the indirect stimulatory response, which is driven by sarcolemmal NHHE1/NBC activity, is dominant and manifested globally. We have investigated the possible reason for this latter effect. Global stimulation of the CaT by a localized intracellular acidosis appears to be dependent on a high myoplasmic diffusivity of intracellular Na+-ions, contrary to an earlier suggestion for a low value.20 High myoplasmic Na+-diffusivity permits a locally activated influx of Na+ into an acidic region of the cell to spread rapidly downstream, and facilitate the stimulation of SR Ca2+ loading and subsequent release in non-acidic regions, thus promoting a more global CaT response. We have used confocal fluorescence imaging to track the spatial movement of intracellular Na+ under these conditions, to confirm its high myoplasmic mobility. Thus, in addition to emphasizing the importance of intracellular Na+ for enhancing the CaT during acidosis, our work identifies an unexpected role for the Na+-ion as a spatial intracellular messenger, involved in the global regulation of Ca2+-signalling.

2. Methods

2.1 Isolation of ventricular myocytes

Enzymatic isolation of myocytes from rat heart ventricles was performed using a published method.7 Animals were sacrificed by Schedule 1 killing (cervical dislocation) approved by the UK Home Office.

2.2 Solutions

Normal Tyrode (NT; in mM): 135 NaCl, 4.5 KCl, 1 CaCl2 (or as indicated otherwise), 1 MgCl2, 11 glucose, 20 HEPES, and pH 7.4 (37°C). Acetate-containing solutions had equivalently reduced [Cl−]. Ammonium-containing solutions had equivalently reduced [Na+]. Where indicated, Ca2+ in acetate-containing solutions was raised 23% to compensate for Ca2+-acetate binding.14 CO2/HCO3−-buffered solutions contained 22 mM NaHCO3 in place of HEPES and were bubbled with 5% CO2 (for pH 7.4) or 20% CO2 (for pH 6.8). ONa+/OCa2+ solutions contained N-methyl-β-glycine (NMDG) in place of Na+ and EGTA in place of Ca2+. All experiments were performed at 37°C.

2.3 Dual microperfusion

Dual microperfusion was performed in a Perspex superfusion (37°C) chamber mounted on a Leica IRBE microscope.7 One of the two microstreams contained 15 mM sucrose to visualize the interstream boundary. The position of the boundary was adjusted to change between dually micro-perfusing a myocyte with two microstreams (i.e. boundary across the middle of the cell) and uniformly superfusing with either microstream. Two platinum wires delivered a 2 ms pulse of field stimulation at 2 Hz to evoke contractions.

2.4 Ionic fluorescence imaging

Loading of acetoxymethyl esters of dyes was performed at room temperature (10 min for cSNARF1, 10 min for fluo3, and 2 h for SBFI). Fluorescence was measured using a Leica TCS NT confocal system7 with the following settings: cSNARF1 excitation 514 nm, emission 580 ± 20 and 640 ± 20 nm (ratiometrically); fluo3 excitation 488 nm and emission 520 ± 50 nm; SBFI excitation 361 nm and emission 440 ± 40 nm and >550 nm (ratiometrically). cSNARF and SBFI images were taken in a X-Y mode (2 s/frame). Fluo3 images were taken in a linescan mode (2 ms/frame). Photobleaching was minimized by exciting fluo3 along the raster line only with low laser power (yet sufficient for a good signal/noise ratio). Compared with UV-excitable (ratiometric) dyes, fluo3 offers superior spatio-temporal resolution of Ca2+-dynamics. Images were analysed with ImageJ to determine fluorescence time courses in regions of interest (ROIs) along length of myocyte. Myocyte movement and contraction were corrected by edge detection in the
direction of the long axis (cell outline defined as threshold of 5% mean intracellular fluorescence).

2.5 Calibration of fluorescent dyes
cSNARF1 was calibrated using nigericin (10 μM). Fluorescence $K$ was measured in situ at $pH_1 = 7.25$ to be 0.84 μM by pipette-loading cells, with 10 μM $Ca^{2+}$ to measure maximal fluorescence ($F_{\text{max}}$) relative to $F_0$ ($F_{\text{max}}/F_0 = 9.3 \pm 0.3$). The mild $pH$ sensitivity of fluo3 was characterized previously and was accounted for in the calibration equation by adjusting $K$ with cariporide ($0.4048 \times [H^+] + 813.3$ [nM]). For validation in situ, see Swietach et al. Fluo3 ratio was calibrated using monensin (40 μM) and gramicidin D (2 μM).

2.6 Statistical analysis
Paired t-tests performed at 5% significance level. Where multiple comparisons are being made for statistical testing (e.g. data from adjacent ROIs), the critical $P$-value was corrected by the Holm–Bonferroni method. The number of observations is reported as ‘X/Y rats’ (X cells from Y hearts).

3. Results

3.1 Global intracellular acidification affects diastolic $Ca^{2+}$ and CaT amplitude
Figure 1A shows specimen CaTs (2 Hz field stimulation) from a cell bathed in NTHCO/HCO3-free, HEPES-buffered. Figure 1Aa displays the CaT averaged, for several cells, in 14 serial ROIs (mean cell length 137.6 ± 3.0 μm and mean ROI length 9.8 μm), confirming that the $Ca^{2+}$ signal displays good spatial (longitudinal) uniformity (CaT time courses re-plotted with error bars in Supplementary material online, Figure S1).

To manipulate $pH_1$, myocytes were subjected to the NH4 per se technique. Global superfusion with 20 mM NH4 transiently raised $pH_1$ and the return to NT produced a transient acidification (Figure 1Bb). $pH_1$ recovery from acidosis was blocked by 30 μM cariporide (a high affinity NHE1 inhibitor; Figure 1B), confirming that it was mediated by sarcolemmal acid extrusion on NHE1 (lack of CO2/HCO3+ the CaT averaged, for several cells, in 14 serial ROIs (mean cell length averaged, for several cells, in 14 serial ROIs (mean cell length 137.6 ± 3.0 μm and mean ROI length 9.8 μm), confirming that the $Ca^{2+}$ signal displays good spatial (longitudinal) uniformity (CaT time courses re-plotted with error bars in Supplementary material online, Figure S1).

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3.2 $Ca^{2+}$ amplitude, but not $[Ca^{2+}]_{\text{dia}}$, tracks $[Na^+]_{\text{dia}}$
Figure 2Ai quantifies the rapid intracellular acidification produced by 80 mM acetate superfusion. This was followed by a slow $pH_1$ recovery and accompanied by a rise of $[Na^+]_{\text{dia}}$ (reported by intracellular SBF fluorescence; Figure 2Bi), both of which were inhibited by cariporide (Figure 2Aii and Bii), confirming the activity of NHE1 (NB: the small acetate-induced decrease in SBF ratio in the presence of cariporide reflects the $pH$-sensitivity of the dye). $[Ca^{2+}]_{\text{dia}}$ again, increased during the acidosis, both in the presence and absence of cariporide (cf. Figure 2Gi and ii), indicating that the effect was independent of NHE1 activity, and thus of the rise in $[Na^+]_{\text{dia}}$. In contrast, $[Ca^{2+}]_{\text{sys}}$ and hence CaT amplitude, increased more slowly during the acidosis, in parallel with $[Na^+]_{\text{dia}}$ (Figure 2Gi). Notably, this rise of $[Ca^{2+}]_{\text{sys}}$ failed to occur when the $[Na^+]_{\text{dia}}$ elevation was inhibited by cariporide (Figure 2Gi). Thus, the enhancement of CaT amplitude during acidosis tracked the NHE1-dependent rise of $[Na^+]_{\text{dia}}$, whereas the rise of $[Ca^{2+}]_{\text{sys}}$ was independent of $[Na^+]_{\text{dia}}$. Furthermore, when NHE1 had been inhibited, the fall of $pH_1$ during acetate superfusion decreased CaT amplitude (see calibrated and averaged time courses of individual CaTs shown as insets in Figure 2C). These observations match those seen earlier with a NH4+ rebound acidosis (Figure 1B). Results shown in Figures 1 and 2 thus indicate that intracellular acidosis raises $[Ca^{2+}]_{\text{sys}}$ and CaT amplitude via different mechanisms, only the latter correlating with the rise of $[Na^+]_{\text{dia}}$, driven by NHE1 activity.

The spatial effect of globally reducing $pH_1$ on $Ca^{2+}$ signalling, visualized along the length of the cell, is shown in Figure 2D (time courses re-plotted with error bars in Supplementary material online, Figures 2E and S3). After 90 s of the acid-load, $[Ca^{2+}]_{\text{dia}}$ and $[Ca^{2+}]_{\text{sys}}$ had increased uniformly. Thus, the response of $Ca^{2+}$ signalling to a global acidosis is spatially the same throughout the bulk cytoplasmic compartment. The slower relaxation of the CaT during acidosis is consistent with the known inhibitory effects of $H^+$ ions on SERCA and NCX.

3.3 Intracellular acidification raises SR $Ca^{2+}$ content
Under conditions of active NHE1, the rise in $[Ca^{2+}]_{\text{sys}}$ at low $pH_1$ is believed to reflect greater SR $Ca^{2+}$ loading. To confirm this, the SR load was probed by rapid application of 10 mM caffeine, following a 10 s suspension of pacing. Caffeine superfusion was performed either under control conditions or after 2 min of acetate exposure. The SR load, assessed from the peak fluo3 fluorescence response, was increased at low $pH_1$ when NHE1 was active (Figure 2Ei), but not when NHE1 was inhibited (Figure 2Eii). Note also that the subsequent relaxation of this caffeine response (an approximate indicator of NCX activity in rat ventricular myocytes) was slowed at reduced $pH_1$ (Figure 2Eii), as expected from the inhibitory effect of $H^+$ ions on NCX.
3.4 Local acidosis regulates $[\text{Ca}^{2+}]_{\text{dia}}$ and $[\text{Ca}^{2+}]_{\text{sys}}$ over different spatial domains

To induce a local microdomain of acidity, acetate exposure was restricted to one end of the myocyte, using a dual microperfusion device [7] (see Supplementary material online, Methods for justification of using acetate to induce acidosis locally). This releases two parallel microstreams (Figure 3A i), one containing 80 mM acetate, separated by a sharp inter-stream boundary of $< 10 \mu$m. Within $\sim 20$ s, subjecting half the cell length ($\sim 60 \pm 7 \mu$m) to acetate produced a smooth longitudinal gradient of $pH_i$, with the majority of acidosis confined to the acetate-exposed end [7].

The $pH_i$ gradient was stable over time (for several minutes [5]) and unaffected by adding cariporide ($\sim 0.45$ pH units end-to-end; Figure 3A ii and iii), confirming that the technique can be used to clamp a spatial $pH_i$ gradient, while probing effects of NHE1 activity on $\text{Ca}^{2+}$. The effect of the $pH_i$ gradient on $\text{Ca}^{2+}$ signalling was assessed in 12 adjoining ROIs along the cell length.

Figure 3B i (HEPES-buffered superfusates) shows the time course of $[\text{Ca}^{2+}]_{\text{dia}}$ and $[\text{Ca}^{2+}]_{\text{sys}}$ changes, averaged in the three outermost ROIs in the acetate-exposed end where NHE1 is strongly activated by low concentration of bicarbonate buffer ($5 \%$ CO$_2$-HCO$_3^{-}$). Binding of $\text{Ca}^{2+}$ to acetate reduces $[\text{Ca}^{2+}]_o$ by 23%.

Figure 1 Intracellular acid-loading on CaTs. (Ai) Time course of two CaTs reported by fluo3 fluorescence ($F/F_0$) in rat ventricular myocyte paced at 2 Hz superfused in NT. Arrow indicates 2 ms field stimulation voltage pulse. (ii) Visualization of CaT time courses ($n = 20$ myocytes from four rats) plotted in 14 equally spaced ROIs of the myocyte (corrected for movement and contraction). (Bi) Time courses of $pH_i$ (cSNARF1; $n = 3$/2 rats) and diastolic and systolic $[\text{Ca}^{2+}]$ (fluo3; $n = 7$/3 rats) in response to $20$ mM NH$_4^+$ pre-pulse solution manoeuvre (HEPES-buffered solutions). Removal of NH$_4^+$ imposes an acid load that is then extruded by NHE1. (ii) Experiment repeated in the presence of $30 \mu$M cariporide to inhibit NHE1 ($n = 7$/3 rats). Dotted lines show time courses in the absence of cariporide to highlight the effect of NHE activity on $pH_i$ and CaTs. (C) Effect of raising superfusate CO$_2$ from 5\% (pH 7.4) to 20\% (pH 6.8) on CaTs ($n = 8$/3 rats). Media contain 22 mM HCO$_3^-$, (D) Effect of applying 80 mM acetate (HEPES-buffered media; CO$_2$/HCO$_3^-$-free) on CaT (fluo3; $n = 6$/2 rats) when total extracellular $[\text{Ca}^{2+}]$ was 1 mM. Binding of $\text{Ca}^{2+}$ to acetate reduces $[\text{Ca}^{2+}]_o$ by 23%.
Figure 2  Acid-evoked rise in systolic \([\text{Ca}^{2+}]\) is \([\text{Na}^+]\)-dependent. (A) Time course of pH, (cSNARF1) in response to superfusion with 80 mM acetate (HEPES-buffered; \(\text{CO}_2/\text{HCO}_3^{-}\)-free) under conditions of constant free extracellular \([\text{Ca}^{2+}]\). (i) Control conditions, under which NHE is activated by fall in pH, \((n = 12/4 \text{ rats})\). (ii) In the presence of 30 \(\mu\text{M} \) cariporide to block NHE \((n = 12/4 \text{ rats})\). (B) Time course of \([\text{Na}^+]\), (SBFI) in response to intracellular acidification by acetate under (i) control conditions \((n = 6/2 \text{ rats})\) and (ii) in the presence of cariporide \((n = 7/2 \text{ rats})\). (C) Time course of diastolic and systolic \([\text{Ca}^{2+}]\) in response to intracellular acidification by acetate under (i) control conditions \((n = 20/5 \text{ rats})\) and (ii) in the presence of cariporide \((n = 17/5 \text{ rats})\). Insets show calibrated CaT amplitude before and during acetate exposure. (D) Visualization of CaT time courses in 12 ROIs along the length of cell, measured before exposure to acetate (labelled as ‘NT’ in C) or after 90 s of exposure to 80 mM acetate (labelled as ‘Ac’ in C) under (i) control conditions and (ii) in the presence of cariporide. (E) Response to rapid application of 10 mM caffeine (evokes release of SR \[\text{Ca}^{2+}\]), quantified in terms of fluo3 \(F/F_0\) amplitude and half-time of recovery from peak \(F/F_0\). Experiments under (i) control conditions \((n = 9/3 \text{ rats})\) or (ii) in the presence of cariporide \((n = 10/3 \text{ rats})\). *\(p < 0.05\) (vs. NT).
pH, and in the three outermost ROIs at the opposite (alkaline) end where NHE1 activity is at a low level. The insets show calibrated time courses of individual CaTs (offset to baseline). Within 90 s of dual microperfusion, [Ca\(^{2+}\)]\(_{\text{dia}}\) increased in the more acidic end, whereas in the more alkaline end it was unchanged (Figure 3Bi). Strikingly, [Ca\(^{2+}\)]\(_{\text{sys}}\) and CaT amplitude increased significantly (P < 10\(^{-3}\), paired t-test) in both regions. Thus, although the acidic microdomain caused an increase in [Ca\(^{2+}\)]\(_{\text{sys}}\) locally, it increased the CaT amplitude globally over a distance of ~130 \(\mu\)m.

The spatial effects of regional acidosis on CaTs are visualized in Figure 3Bii, where control CaTs (non-shaded transients) are compared with measurements made at 90 s of dual microperfusion (shaded transients; time courses re-plotted with error bars in Supplementary material online Figures S4 and S5). Whereas the [Ca\(^{2+}\)]\(_{\text{dia}}\)-rise co-localized with the acidic microdomain, the enhancement of CaT amplitude was more uniform along the whole-cell length. Furthermore, elevation of [Ca\(^{2+}\)]\(_{\text{sys}}\), but not [Ca\(^{2+}\)]\(_{\text{dia}}\), was blocked by 30 \(\mu\)M cariporide (Figure 3Ci). In the presence of the inhibitor, the local acidic microdomain now induced a local reduction in CaT amplitude without significantly affecting the CaT at the far, non-acidic end of the cell. Figure 4 quantifies these effects of local and global acidosis on Ca\(^{2+}\) signalling. The global

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**Figure 3** Acidic microdomain of cytoplasm can influence CaTs remotely. (A) Generating pH\(_i\) gradient along the length of myocyte. (i) Cartoon of dual microperfusion device releasing NT microstream (lower) and acetate-containing microstream (upper). (ii) pH\(_i\) gradient along cell length after 90 s of dual microperfusion. (iii) Experiments repeated in the presence of 30 \(\mu\)M cariporide to block NHE. (B) Time course of diastolic and systolic [Ca\(^{2+}\)] recorded in three adjacent ROIs at either end of the cell (proximal denotes the acetate-exposed end) (n = 24/6 rats). Note the transmission of the systolic [Ca\(^{2+}\)] response to the distal end of the cell (P < 0.01; paired t-test). Insets show calibrated CaT amplitude measured before dual microperfusion (dashed line) and after 90 s of dual microperfusion (continuous line). (ii) Visualization of CaT time courses in 12 equally spaced ROIs along cell length at times labelled I and II. (C) Experiments repeated in the presence of 30 \(\mu\)M cariporide to block NHE (n = 14/5 rats). Note the absence of systolic [Ca\(^{2+}\)] response distally.

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response of CaT amplitude to both whole-cell and local acidosis (Figure 4A) required functional NHE1 activity. With NHE1 inhibition (Figure 4B), the CaT response became local (i.e. CaT amplitude declined only in acidic zones). In contrast, [Ca\(^{2+}\)]\(_{\text{dia}}\) elevation during acidosis was always local, and was independent of NHE1 activity. Diastolic [Ca\(^{2+}\)] and the CaT amplitude therefore respond very differently to acidosis.

### 3.5 Local acidosis globally enhances CaT via cytoplasmic Na\(^{+}\) diffusion rather than intra-SR Ca\(^{2+}\) diffusion

CaT amplitude is determined largely by Ca\(^{2+}\) release from the SR. During a global acidosis, this amplitude rises because of enhanced SR Ca\(^{2+}\) loading. When acidification is restricted regionally, the global increase in CaT amplitude may arise from a rapid re-distribution of the SR Ca\(^{2+}\) load from acidic to non-acidic regions, via lumenal Ca\(^{2+}\) diffusion. Alternatively, it may arise from a rapid transmission of Na\(^{+}\) ions in cytoplasm. Since the delay in CaT response between acidic and non-acidic ends of myocytes is of the order of seconds (e.g. Figure 3B), the underlying mechanism would need to be adequately fast, characterized by diffusivity of the order of \(\approx 10^3 \text{ mm}/\text{s}\).

The feasibility of the first postulated mechanism (rapid redistribution of SR Ca\(^{2+}\)) was tested by radically changing the SR release pattern locally, and observing whether this had a rapid effect on release in remote regions of the cell. Using dual microperfusion, one half of a myocyte (distal half) was exposed to NT solution containing normal (1 mM) Ca\(^{2+}\), and the other half (proximal half) to a microstream containing 80 mM acetate with low (100 \(\mu\text{M}\)) Ca\(^{2+}\). Both microstreams contained 30 \(\mu\text{M}\) cariporide to prevent NHE1 activation. Figure 5A shows the time course of [Ca\(^{2+}\)]\(_{\text{dia}}\) and [Ca\(^{2+}\)]\(_{\text{sys}}\), changes averaged in three outermost ROIs at the proximal and distal ends of the cells. At the proximal (acidic/low-[Ca\(^{2+}\)]) exposed end of the cell, [Ca\(^{2+}\)]\(_{\text{dia}}\) was raised (due to the acidification), whereas [Ca\(^{2+}\)]\(_{\text{sys}}\) was reduced (low [Ca\(^{2+}\)]\(_{\text{sys}}\)) as well as low pH, greatly reduces SR Ca\(^{2+}\) release when NHE1 is inhibited. Upon return to uniform superfusion with NT, [Ca\(^{2+}\)]\(_{\text{sys}}\) at the proximal end greatly overshot control levels for a

![Figure 4](image-url) **Summary of the effects of global and local acidosis on diastolic and systolic Ca\(^{2+}\) in the presence and absence of acid-evoked Na\(^{+}\) entry.** (A) Response of (i and iii) diastolic [Ca\(^{2+}\)] (triangles) and systolic [Ca\(^{2+}\)] (circles) and (ii and iv) CaT amplitude to regional (i and ii) or whole-cell (iii and iv) exposure to acetate. Filled symbols denote control conditions (uniform exposure to NT). Open symbols denote response to regional or whole-cell exposure to 80 mM acetate. \(\ast\) denotes significance \(P < 0.05\) (paired t-test), corrected for multiple comparisons by the Holm–Bonferroni method. (B) Analysis of experiments performed in the presence of cariporide to block NHE. Key shows the direction of significant change in diastolic [Ca\(^{2+}\)] (dia-[Ca\(^{2+}\)]) and systolic [Ca\(^{2+}\)] (sys-[Ca\(^{2+}\)]) and the CaT amplitude ([CaT]). Light symbols: effects in acetate-exposed regions; black symbols: effects in acetate-free regions.
period of ~20 s, which reflects a time-dependent resetting of SR and surface membrane Ca\textsuperscript{2+} fluxes. At the distal end (where NT had been present throughout the experiment), [Ca\textsuperscript{2+}] \textsubscript{sys} remained at control levels, despite the major CaT changes < 100 \mu m away. Figure 5B plots the shape of individual CaTs measured longitudinally in 12 ROIs along the length of the myocyte, during the dual microperfusion (unshaded CaTs), and then after 10 s of returning to uniform NT exposure (shaded CaTs). Proximal CaT amplitude increased by nearly 10-fold while at the distal end the CaT was unaffected (time courses re-plotted with error bars in Supplementary material online, Figure S6). Results indicate that the myocyte behaves as a series of adjoining compartments that do not rapidly communicate changes in SR Ca\textsuperscript{2+}. This is consistent with low global intra-SR Ca\textsuperscript{2+} diffusivity (~10 \mu m\textsuperscript{2}/s).\textsuperscript{24}

Results shown in Figure 5 demonstrate that intra-SR Ca\textsuperscript{2+} diffusion is too slow to explain fast global systolic Ca\textsuperscript{2+} responses to local acidosis. Thus, the alternative hypothesis of rapid Na\textsuperscript{+} transmission was tested. Cytoplasmic Na\textsuperscript{+} diffusivity (D\textsubscript{Na}) was measured in myocytes equilibrated initially in Na\textsuperscript{+}-free/Ca\textsuperscript{2+}-free solution to deplete intracellular Na\textsuperscript{+} without causing Ca\textsuperscript{2+} overload via reverse mode NCX. A local influx of Na\textsuperscript{+} was then triggered, by exposing one end of the cell (proximal end, ~30% of cell length; Figure 6Ai) to solution containing 140 mM Na\textsuperscript{+}, with 80 mM acetate (to stimulate NHE1), and 100 \mu M strophanthidin (to block Na\textsuperscript{+}-extrusion by the Na\textsuperscript{+}/K\textsuperscript{+} pump). The remaining 70% (distal) length of the cell was exposed to a Na\textsuperscript{+}-free/Ca\textsuperscript{2+}-free solution, containing 100 \mu M strophanthidin to block the Na\textsuperscript{+}/K\textsuperscript{+} pump, and 30 \mu M cariporide to block NHE1. This protocol ensured that any observed [Na\textsuperscript{+}]-rise in distal regions would necessarily be due to cytoplasmic Na\textsuperscript{+} diffusion from the proximal end, [Na\textsuperscript{+}]. This was imaged by using intracellular SBFI. As shown in Figure 6Aii, SBFI reported a large and rapid proximal rise of [Na\textsuperscript{+}] upon locally evoking Na\textsuperscript{+} influx. A similar rise was observed distally, but after a short time-delay of ~6 s. Best-fitting these data to a diffusion model\textsuperscript{25} suggested a D\textsubscript{Na} of 682 \pm 184 \mu m\textsuperscript{2}/s (n = 8). This is 40 \pm 10% of the value for D\textsubscript{Na} in pure water\textsuperscript{26} at 37 \degree C. The rapid rate of cytoplasmic Na\textsuperscript{+} diffusion is thus consistent with short longitudinal Na\textsuperscript{+} diffusion delays, of the order of seconds. To test whether intracellular Na\textsuperscript{+} diffusion was also fast under more physiological conditions (i.e. starting from a more normal [Na\textsuperscript{+}]), and with a functional sarcolemmal Na\textsuperscript{+}/K\textsuperscript{+} pump), electrically paced myocytes were exposed proximally to a microstream containing 140 mM Na\textsuperscript{+}, with 80 mM acetate (to reduce pHi locally), but with no strophanthidin in any solution (Figure 6B). The proximal activation of NHE1 by acetate increased [Na\textsuperscript{+}], near-uniformly along the whole length of the cell, consistent with a high value for D\textsubscript{Na} (Figure 6Bi). The [Na\textsuperscript{+}]-rise was blocked completely by cariporide (Figure 6Bii), showing that it was due to NHE1 stimulation. Results suggest that bulk Na\textsuperscript{+} ion diffusivity in cytoplasm is fast enough to produce a near-uniform rise in releasable Ca\textsuperscript{2+} (despite slow intra-SR Ca\textsuperscript{2+} ion diffusion), and therefore a more coordinated global rise in CaT amplitude, even when the source of Na\textsuperscript{+} influx is highly localized.

4. Discussion

4.1 Cytoplasmic H\textsuperscript{+} ions spatially modulate [Ca\textsuperscript{2+}]\textsubscript{dia}

It is well established that pH modulates Ca\textsuperscript{2+} signalling in ventricular myocytes, and strongly influences cardiac function, by regulating CaTs, and even triggering aberrant forms of signalling, such as Ca\textsuperscript{2+} waves. Most experimental studies have considered the effect of global (uniform) changes in pH, on Ca\textsuperscript{2+} signalling. With the discovery that cardiac pH need not always be uniform (see Introduction), effects of pH heterogeneity should also be considered. In a previous study, we demonstrated that a pH gradient in quiescent rat ventricular myocytes produces an overlying spatial gradient of cytoplasmic [Ca\textsuperscript{2+}]. H\textsuperscript{+} ions displace Ca\textsuperscript{2+} ions from cytoplasmic buffers, such as histidyl residues on proteins, resulting in a rise in baseline [Ca\textsuperscript{2+}], that is independent of NHE activity and hence [Na\textsuperscript{+}]. Importantly, when a pH gradient evokes a baseline [Ca\textsuperscript{2+}] gradient, the latter can be stable because...
Ca\textsuperscript{2+} ions are recruited uphill into acidic regions via a spatial cytoplasmic Ca\textsuperscript{2+}/H\textsuperscript{+} exchange process, mediated by small diffusible buffer molecules, such as HDPs.\textsuperscript{5} This process offsets diffusive dissipation of the Ca\textsuperscript{2+} microdomain. The present study confirms that [Ca\textsuperscript{2+}]\text{dia} in electrically paced myocytes (Figure 4Ai and Bi) behaves in the same way as resting [Ca\textsuperscript{2+}]\text{dia} in quiescent cells, in response to an imposed pHi non-uniformity. The process of cytoplasmic Ca\textsuperscript{2+}/H\textsuperscript{+} exchange, which accumulates Ca\textsuperscript{2+} in acidic microdomains, is functional only when pHi is non-uniform. For this reason, it had not been observed in experiments where myocytes were acid-loaded uniformly. Since cytoplasmic H\textsuperscript{+} ion diffusivity is low\textsuperscript{2} and the factors that affect pHi (metabolism, blood perfusion, and acid/base membrane transport) can be highly heterogeneous and compartmentalized,\textsuperscript{27} the effects of non-uniform pHi on Ca\textsuperscript{2+} signalling should be considered as physiologically relevant. The spatial coupling between pHi and [Ca\textsuperscript{2+}]\text{dia} may be important for overcoming the inhibitory effects of H\textsuperscript{+} ions on Ca\textsuperscript{2+}-sensitive proteins regulated by basal [Ca\textsuperscript{2+}]\text{dia}, for example, forms of calmodulin/calcineurin-dependent signalling.\textsuperscript{28}

### 4.2 Local acidosis modulates CaT amplitude both locally and globally

To furnish a more complete understanding of pH–Ca\textsuperscript{2+} coupling, the present study has explored the spatial effects of pHi on the CaT. In agreement with previous findings,\textsuperscript{23,29,30} we observe that the influence of low pHi on CaT amplitude can be both inhibitory (when sarcolemmal NHE1 and NBC are not active) and excitatory (when these transporters are active, leading to a rise of [Na\textsuperscript{+}]), which ultimately, via NCX, enhances SR Ca\textsuperscript{2+} loading). Interestingly, the inhibitory effect, which is largely attributable to attenuation of SERCA, RyR, and L-type Ca\textsuperscript{2+} channel activity, is closely localized to acidic cytoplasmic regions, rather like the local effects of pHi on [Ca\textsuperscript{2+}]\text{dia}. The surprising finding, however, is that this local inhibitory CaT response is transformed into a global excitatory response, when Na\textsuperscript{+}-dependent acid extrusion is functional. In the present experiments using HEPES-buffered NT, such extrusion was via NHE1, although NBC will also have contributed in CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{−}-buffered conditions (e.g. Figure 1C). We have shown that an acidic cytoplasmic microdomain, which activates NHE1 locally, raises CaT amplitude both in the acidified domain and more remotely in non-acidic regions (Figure 4Ai and Bi). Thus, local acidosis is capable of increasing CaT amplitude over a much greater volume of the cell, provided an H\textsuperscript{+}-activated Na\textsuperscript{+}-entry pathway is present (Figure 7). This remote coupling between intracellular H\textsuperscript{+} and Ca\textsuperscript{2+} signals is unprecedented, given that high intracellular buffering greatly restricts their effective ionic diffusivity. The functional significance of expanding the spatial scale over which H\textsuperscript{+} ions affect systolic [Ca\textsuperscript{2+}] may be to even out CaT heterogeneity under conditions of non-uniform pHi. This may help to decrease the incidence of aberrant forms of Ca\textsuperscript{2+} signalling.\textsuperscript{31,32}

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**Figure 6** Na\textsuperscript{+} ions are rapidly diffusible messengers. (A) Measuring cytoplasmic Na\textsuperscript{+} diffusivity using dual microperfusion to produce local Na\textsuperscript{+}-influx. (i) Cartoon of dual microperfusion device. Myocytes were pre-treated in Na\textsuperscript{+}/Ca\textsuperscript{2+}-free media to deplete intracellular Na\textsuperscript{+}. One end (30%) of a quiescent myocyte was exposed to Na\textsuperscript{+}/Ca\textsuperscript{2+}-free solution containing 140 mM Na\textsuperscript{+} (source of Na\textsuperscript{+} influx) and 80 mM acetate (to stimulate NHE); the remainder of the myocyte was exposed to Na\textsuperscript{+}/Ca\textsuperscript{2+}-free media containing 100 μM streptophanthen and 30 μM cariporide. (ii) Rise in cytoplasmic [Na\textsuperscript{+}] (SBFI) during dual microperfusion (n = 7/2 rats). (iii) Averaged data shown with superimposed best-fit for diffusivity 680 μm\textsuperscript{2}/s. (Bi) Cytoplasmic [Na\textsuperscript{+}] response during dual microperfusion with 80 mM acetate (along mid-point of the cell) in the presence of normal extracellular [Na\textsuperscript{+}], [Ca\textsuperscript{2+}], and Na\textsuperscript{+}/K\textsuperscript{+} pump activity. (ii) Experiments repeated in the presence of 30 μM cariporide. Result also demonstrates that the SBFI ratio is not substantially pH-sensitive.
A key factor affecting Ca\(^{2+}\) signalling uniformity is the distribution of Ca\(^{2+}\) in the SR. Luminal Ca\(^{2+}\) ions are buffered, but the extent to which their diffusivity is restricted is controversial,\(^{13}\) with reports of fast,\(^{34}\) slow,\(^{34}\) and spatially heterogeneous Ca\(^{2+}\) mobility.\(^{15}\) This study provides additional evidence that the SR content is weakly coupled by Ca\(^{2+}\) diffusion over distances of multiple sarcomeres. In the absence of an H\(^+\)-activated Na\(^+\) influx, manipulating the CaTs at one end of a myocyte does not affect Ca\(^{2+}\) signalling at the other (Figure 5), arguing that global apparent intra-SR Ca\(^{2+}\) diffusivity is low. Thus, Ca\(^{2+}\) signalling is predisposed to becoming non-uniform, in part because of slow Ca\(^{2+}\) diffusion in both the cytoplasm and SR. A means of coordinating SR Ca\(^{2+}\) content, in order to unify Ca\(^{2+}\) signalling, would thus be helpful for maintaining contractile synchrony within the myocyte.

4.3 Na\(^+\) ions are rapidly diffusible messengers for Ca\(^{2+}\) signalling

Among the three ionic species of the H\(^+\)–Na\(^+\)–Ca\(^{2+}\) signalling triumvirate,\(^{19}\) Na\(^+\) ions are the least buffered and therefore the most likely to diffuse rapidly in cytoplasm. The present work measured cytoplasmic Na\(^+\) diffusivity by restricting Na\(^+\) influx to one region of the cell under conditions of sarcolemmal Na\(^+\)/K\(^+\) pump inhibition (i.e. limiting the transmembrane ‘loss’ of Na\(^+\)) that may otherwise lead to an erroneous estimate of Na\(^+\) diffusivity). Our measured value for \(D_{Na}\) is 40% of that in pure water, i.e. near its physical limit for a tortuous cytoplasmic compartment, considering that macromolecular crowding increases diffusion paths by approximately two-fold.\(^{25,26}\) The present estimate of \(D_{Na}\) is in agreement with measurements made on skeletal myoplasm\(^{26}\) and with the fast mobility of a similar-sized monovalent cation, NH\(_4\)^+ estimated in cardiac myocytes.\(^{7}\) The magnitude of \(D_{Na}\) is sufficient to produce a near-uniform rise in [Na\(^+\)], when evoked locally by transporters such as NHE1. In contrast, an earlier estimate\(^{26}\) of \(D_{Na}\), which was 60-fold lower, would not be compatible with our observations of [Na\(^+\)], near-uniformity during regional NHE1 stimulation. Since intra-SR Ca\(^{2+}\) mobility is substantially lower than \(D_{Na}\), remote pH\(_i\)–Ca\(^{2+}\) coupling must be dependent on fast cytoplasmic Na\(^+\) transport. The rapid spread of Na\(^+\) ions, even when influx is localized, will therefore assist in coordinating the SR Ca\(^{2+}\) load, helping to unify the electrically evoked CaTs.

4.4 Potential for Na\(^+\) diffusion in multicellular myocardium

In the present work, the principle of remote pH\(_i\)–Ca\(^{2+}\) coupling was demonstrated by experimentally imposing a large pH\(_i\) gradient (\(\sim 0.5\) units) along an isolated cell (\(\sim 100\) \(\mu\)m). Although physiological pH\(_i\) gradients are typically smaller than this (e.g. up to 0.2 units over \(\sim 30\) \(\mu\)m during stimulated NHE1 activity\(^{3}\)), the functional importance of Na\(^+\) diffusion will remain the same, helping to even out local variations of CaT amplitude caused by the pH\(_i\) non-uniformity. This spatial role of Na\(^+\) may become particularly prominent in the intact myocardium. Here, the spatial range over which Na\(^+\) diffuses is likely to exceed the dimensions of a single myocyte, because Na\(^+\) ions readily permeate gap-junctional channels at intercalated discs\(^{36}\) (rather than requiring a permeant carrier molecule, as is the case for H\(^+\) ions\(^{37}\)). Thus, Na\(^+\) diffusion may regulate CaTs in cells adjacent to acidified myocytes. Within the myocardium, local acidosis, evoked for example by compromised capillary perfusion, can greatly exceed 0.2 pH\(_i\) units\(^{3}\) resulting in considerable spatial pH\(_i\) non-uniformity. Since Na\(^+\) ions diffuse \(\sim 7\) times faster than H\(^+\) ions in the coupled syncytium,\(^{3,37}\) a large acid-induced rise of [Na\(^+\)] may extend beyond the acidified domain, provided that gap junctions remain open during acidosis,\(^{37}\) and Na\(^+\)-coupled acid-extruders remain active. In this case, local myocardial acidity may promote significant pH\(_i\)–Ca\(^{2+}\) coupling remotely. Under conditions of excessive Na\(^+\) influx into a restricted region, cell-to-cell transmission of Na\(^+\) may also predispose neighbouring cells to SR Ca\(^{2+}\) overload, Ca\(^{2+}\) waves, and delayed after-depolarizations. This behaviour would be the spatial equivalent of events occurring during cardiac ischaemia–reperfusion, where a substantial Ca\(^{2+}\) overload, driven by [Na\(^+\)], accumulation, and persisting at normal (recovered) pH\(_i\), is pro-arrhythmogenic and...
is notable that 23Na NMR imaging studies of regional myocardial ischaemia have demonstrated [Na+] gradients of sufficient magnitude to drive a lateral diffusive Na+ flux and plausibly modulate Ca2+ handling in remote regions, whereas Na+ overloading of one myocyte of a coupled pair, using a patch-pipette, has been shown to drive hypercontracture in the adjacent cell. 

4.5 Conclusions

We have shown that, within the ventricular myocyte, rapid diffusion of Na+ ions in cytoplasm can couple Ca2+ signaling to spatially remote H+ signals. The different spatial domains for the pH sensitivity of diastolic and systolic [Ca2+] add a novel and important degree of complexity to the regulation of Ca2+ signaling in the heart. During pH disturbances, high Na+ ion mobility will assist in the unification of Ca2+ signaling, by coordinating the degree of SR Ca2+ load, and overcoming the potential for heterogeneity caused by low intra-SR Ca2+ diffusivity. This novel role for Na+ ions need not be limited to the acid response, as any local Na+ influx should have a comparable effect.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References

1. Bers DM. Cardiac excitation-contraction coupling. Nature 2002;415:198–205.
2. Vaughan-Jones RD, Spitzer KW, Swietach P. Intracellular pH regulation in heart. J Mol Cell Cardiol 2009;46:318–331.
3. Orchard CH, Cringley HE. Acidosis and arrhythmias in cardiac muscle. Cardiovasc Res 1994;28:1312–1319.
4. Steenberg C, Deleev G, Rich T, Williamson JR. Effects of acidosis and ischemia on contractility and intracellular pH of rat heart. Circ Res 1977;41:845–858.
5. Swietach P, Youm JB, Saegusa N, Leem CH, Spitzer KW, Vaughan-Jones RD. Coupled Ca2+/H+ transport by cytoplasmic buffers regulates local Ca2+ and H+ signaling. Proc Natl Acad Sci USA 2013;110:E2064–E2073.
6. GarciaCedro, MA, YuL, Swietach P, Hulu L, Vaughan-Jones RD. Sarcolemmal localisation of Na+/H+ exchange and Na+/HCO3− co-transport influences the spatial regulation of intracellular pH in rat ventricular myocytes. J Physiol 2013;591:2287–2306.
7. Swietach P, Leem CH, Spitzer KW, Vaughan-Jones RD. Experimental generation and computational modeling of intracellular pH gradients in cardiac myocytes. Biophys J 2005;88:3018–3037.
8. Swietach P, Spitzer KW, Vaughan-Jones RD. pH-Dependence of extrinsic and intrinsic H+ ion mobility in the rat ventricular myocyte, investigated using flash photolysis of a caged-H+ compound. Biophys J 2007;92:641–653.
9. Leem CH, Lagadic-Gossmann D, Vaughan-Jones RD. Characterization of intracellular pH regulation in the guinea-pig ventricular myocyte. J Physiol 1999;517(Pt 1):159–180.
10. Bauer WR, Hiller KH, Galuppo P, Neubauer S, Kopke J, Haase A, Waller C, Erti G. Fast high-resolution magnetic resonance imaging demonstrates fractality of myocardial per- fusion in microscopic dimensions. Circ Res 2001;88:340–346.
