J Physiol 592.15 (2014) pp 3179–3188

The Journal of Physiology

SYMPOSIUM REVIEW

Pumping Ca^{2+} up H^+ gradients: a Ca^{2+}–H^+ exchanger without a membrane

Pawel Swietach[1], Chae-Hun Leem[2], Kenneth W. Spitzer[3] and Richard D. Vaughan-Jones[1]

[1] *Burdon Sanderson Cardiac Science Centre, Department of Physiology, Anatomy and Genetics, Oxford, UK*
[2] *Department of Physiology, University of Ulsan College of Medicine, Seoul, Republic of Korea*
[3] *Nora Eccles Harrison Cardiovascular Research and Training Institute, Salt Lake City, UT, USA*

Abstract Cellular processes are exquisitely sensitive to H^+ and Ca^{2+} ions because of powerful ionic interactions with proteins. By regulating the spatial and temporal distribution of intracellular [Ca^{2+}] and [H^+], cells such as cardiac myocytes can exercise control over their biological function. A well-established paradigm in cellular physiology is that ion concentrations are regulated by specialized, membrane-embedded transporter proteins. Many of these couple the movement of two or more ionic species per transport cycle, thereby linking ion concentrations among neighbouring compartments. Here, we compare and contrast canonical membrane transport with a novel type of Ca^{2+}–H^+ coupling within cytoplasm, which produces uphill Ca^{2+} transport energized by spatial H^+ ion gradients, and can result in the cytoplasmic compartmentalization of Ca^{2+} without requiring a partitioning membrane. The mechanism, demonstrated in mammalian myocytes, relies on diffusible cytoplasmic buffers, such as carnosine, homocarnosine and ATP, to which Ca^{2+} and H^+ ions bind in an apparently competitive manner. These buffer molecules can actively recruit Ca^{2+} to acidic microdomains, in exchange for the movement of H^+ ions. The resulting Ca^{2+} microdomains thus have the potential to regulate function locally. Spatial cytoplasmic Ca^{2+}–H^+ exchange (cCHX) acts like a 'pump' without a membrane and may be operational in many cell types.

(Received 4 November 2013; accepted after revision 2 February 2014; first published online 10 February 2014)
Corresponding author P. Swietach: Oxford University, Physiology, Anatomy and Genetics, Sherrington Building, Parks Road, Oxford OX1 3PT, UK. Email: pawel.swietach@dpag.ox.ac.uk

Abbreviations cCHX, cytoplasmic Ca^{2+}–H^+ exchange; HDP, histidyl dipeptide; NCX, Na^+–Ca^{2+} exchanger; PMCA, Ca^{2+}–H^+-ATPase pump; SERCA, sarco–endoplasmic reticulum Ca^{2+}-ATPase pump; SR, sarcoplasmic reticulum.

The signalling ion calcium is highly compartmentalized in cardiac cells

Calcium (Ca^{2+}) ions regulate a wide variety of intracellular signalling cascades (Clapham, 2007), which, in the heart, include electrical excitation, contraction, growth and development (Bers, 2002). The chemical basis for these interactions is the ability of Ca^{2+} ions to bind to proteins and modulate their function. Cells, however, cannot exercise control over Ca^{2+} signals by means of

Pawel Swietach is a Royal Society University Research Fellow and University Lecturer at the Department of Physiology, Anatomy and Genetics, Oxford University, UK. From Poland, he completed his BA in Physiological Sciences at Oxford and later a doctorate on pH regulation in the heart in the lab of Richard Vaughan-Jones. Pawel obtained further research training in electrophysiology and Ca^{2+} signalling with Kenneth Spitzer (Salt Lake City, USA), and in cancer biology with Adrian Harris (Oxford). **Richard Vaughan-Jones** is Professor of Cellular Physiology at Oxford, Joint Director of the Burdon Sanderson Cardiac Centre, and Tutorial Fellow at Exeter College. His first degree (Physiology) and PhD were from Bristol University. He was a Medical Research Council Senior Fellow at Oxford, before appointment to the Department of Physiology. Collaborative work with Kenneth Spitzer (University of Utah, USA) dates from 1997. He is currently Deputy President of the Physiological Society.

This review was presented at the symposium *Intracellular signalling microdomains in heart*, which took place at IUPS 2013, Birmingham, UK on 25 July 2013.

DOI: 10.1113/jphysiol.2013.265959

synthesis or break-down, as is the case for many other signalling agents such as cyclic nucleotides (Hardman *et al.* 1971). Instead, Ca^{2+} signalling operates on the principle of sub-cellular compartmentalization by ion transport (Berridge *et al.* 2000). Microdomains of elevated [Ca^{2+}] may modulate biological function locally, or could be the source of Ca^{2+} for release into adjacent regions for dynamic signalling (Cheng *et al.* 1993).

The size and stability of [Ca^{2+}] non-uniformity is limited by Ca^{2+} dissipation down electrochemical gradients. The lipid matrix of membranes is a barrier that reduces the magnitude of dissipative Ca^{2+} fluxes and allows large [Ca^{2+}] gradients to be formed across plasmalemmal, sarcoplasmic reticulum (SR) and lysosomal membranes (Fig. 1). Within aqueous compartments, the magnitude and longevity of [Ca^{2+}] gradients depend on the ion's diffusivity. Cytoplasmic Ca^{2+} diffusivity is reduced by volume exclusion due to macromolecules (which increase diffusion path length) and by reversible binding to buffer molecules (e.g. troponin, calmodulin or ATP) (Kushmerick & Podolsky, 1969; Baylor & Hollingworth, 1998). Typically, only one in a hundred Ca^{2+} ions is free to diffuse, and the diffusivity of the remaining calcium depends on the mobility of the Ca^{2+}–buffer complex (Berlin *et al.* 1994; Baylor & Hollingworth, 1998). These phenomena reduce cytoplasmic Ca^{2+} diffusivity to a level that supports, albeit transiently, spatially confined signalling modalities such as Ca^{2+} sparks (Cheng *et al.* 1993).

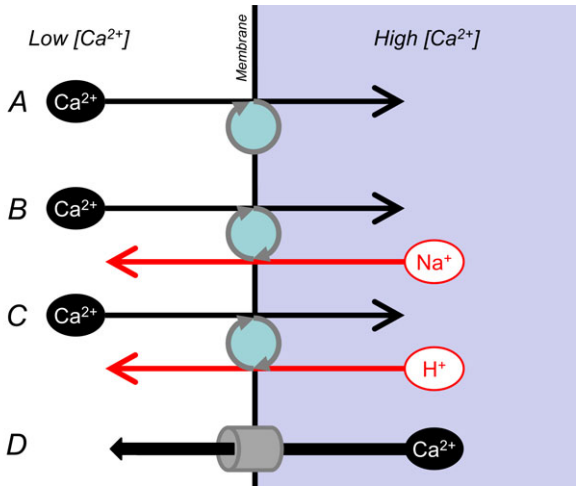

Figure 1. Membrane transport can compartmentalize calcium ions within cardiac ventricular myocytes
Calcium ions cannot be synthesized or degraded, but transport can produce local elevations in their concentration. Spatial non-uniformity of [Ca^{2+}] within cells can lead to local modulation of biological function and provide the driving force for Ca^{2+} transfer between adjacent regions for dynamic signalling. It is generally accepted that stable compartmentalization of Ca^{2+} is achieved by transporters that pump Ca^{2+} across membranes (e.g. SERCA, A). In some cases, pumping of Ca^{2+} is coupled to the movement of other ionic species (e.g. Na^+–Ca^{2+} exchange, B, or plasmalemmal Ca^{2+}–H^+-ATPase, C). Compartments of high [Ca^{2+}] can be discharged by increasing the 'leak' across membranes, e.g. by activating Ca^{2+} channels (such as ryanodine receptors, IP_3 receptors or voltage-gated Ca^{2+} channels, D).

Restricted Ca^{2+} diffusivity in aqueous compartments, and low Ca^{2+} permeability across lipid bilayers are not absolute barriers to Ca^{2+} back-flux. Nonetheless, steep [Ca^{2+}] gradients can be maintained across membranes by active transport, which compensates for Ca^{2+} leakage. Active transport involves membrane-embedded proteins that couple uphill translocation of Ca^{2+} with hydrolysis of ATP (primary active transport) or downhill movement of an ion other than Ca^{2+} (secondary active transport). In cardiac cells, the primary active Ca^{2+} transporters are plasmalemmal Ca^{2+}–H^+-ATPase pumps (PMCA) and sarco–endoplasmic reticulum Ca^{2+}-ATPase pumps (SERCA), while secondary active transport is by Na^+–Ca^{2+} exchangers (NCX) (Bassani *et al.* 1994; Bers, 2002). SERCA sequesters Ca^{2+} into the SR and produces a steep (up to 10^4-fold) [Ca^{2+}] gradient that remains stable during diastole. The activity of NCX and PMCA keeps cytoplasmic [Ca^{2+}] considerably lower (up to 10^4-fold) than extracellular [Ca^{2+}] (Bassani *et al.* 1994; Bers, 2002). These transporter proteins are able to produce a net flux because they are anchored in a specific orientation in the lipid matrix of membranes. Since cytoplasm cannot similarly restrict the orientation of soluble proteins, it has commonly been accepted that the aqueous compartments cannot support stable [Ca^{2+}] gradients. Our recent findings challenge this notion by demonstrating that a class of soluble molecules can mediate uphill Ca^{2+} transport driven by a cytoplasmic [H^+] ([H^+]$_i$) gradient but without a membrane (Swietach *et al.* 2013). The basis for this novel paradigm in ion exchange is the mechanism of H^+ ion transport in cytoplasm.

Cytoplasmic H^+ transport involves the exchange of protonated and unprotonated molecules

H^+ ions, like Ca^{2+} ions, bind avidly to intracellular buffer molecules. However, there are many more cytoplasmic binding sites for H^+ compared to Ca^{2+}, such that only a negligible fraction (~1:500,000 in cardiac myoplasm) of H^+ ions is free to diffuse (Vaughan-Jones *et al.* 2009). These buffer sites include titratable residues on large (essentially immobile) proteins. If buffering capacity were attributable exclusively to these immobile sites, H^+ diffusivity in cytoplasm would be reduced ~500,000-fold (Junge & McLaughlin, 1987; Irving *et al.* 1990). To test this, the apparent H^+ diffusion coefficient can be measured experimentally by injecting H^+ ions into a small region of cytoplasm (either via acid-filled patch pipette or by local photolytic H^+ uncaging) and recording the pH response

in downstream regions of cytoplasm (Vaughan-Jones *et al.* 2002; Zaniboni *et al.* 2003; Swietach *et al.* 2007*b*). In myocyte cytoplasm, H^+ ion diffusivity is considerably slower than in pure water, yet much faster than expected from immobile buffering alone (Fig. 2*Aa*). These findings suggest that a sub-population of H^+-binding buffers is mobile, i.e. of low molecular weight. The apparent H^+ diffusion coefficient was also found to increase with intracellular pH (pH_i), which is consistent with an increase in the ratio of mobile-to-fixed buffering capacity (Fig. 2*Ab*) and argues for chemically distinct populations of mobile and fixed buffers (Swietach *et al.* 2007*b*).

Carbonic (CO_2–HCO_3^-) buffer is an important and highly mobile contributor to cytoplasmic H^+ buffering, particularly at alkaline pH_i. Excluding CO_2–HCO_3^- from experimental solutions removes this component of buffering from the cytoplasm (Leem *et al.* 1999), but only reduces H^+ diffusivity by a third (Swietach *et al.* 2007*b*). These findings indicate that *intrinsic* mobile buffers must be present in myocyte cytoplasm. Biochemical assays have identified a family of histidyl dipeptides (HDPs), including carnosine (histidine coupled with β-alanine), homocarnosine (histidine with γ-amino butyric acid, GABA) anserine, (methylhistidine with β-alanine) and their acetylated derivatives, present collectively in cardiac myoplasm at 10–20 mM (O'Dowd *et al.* 1988; House *et al.* 1989). The small size (~240 Da) and the presence of a titratable imidazole group (pK_a ~7), make HDPs suitable for mediating the transport of H^+ ions in cytoplasm (Vaughan-Jones *et al.* 2002). Interestingly, neonatal heart cells have lower HDP levels and slower cytoplasmic H^+ diffusion (Swietach *et al.* 2010). Other small molecules present in cytoplasm, such as inorganic phosphate, ATP and phosphocreatine, provide an additional degree of mobile buffering, but this only amounts to about half of that accounted for by HDPs (Vaughan-Jones *et al.* 2002).

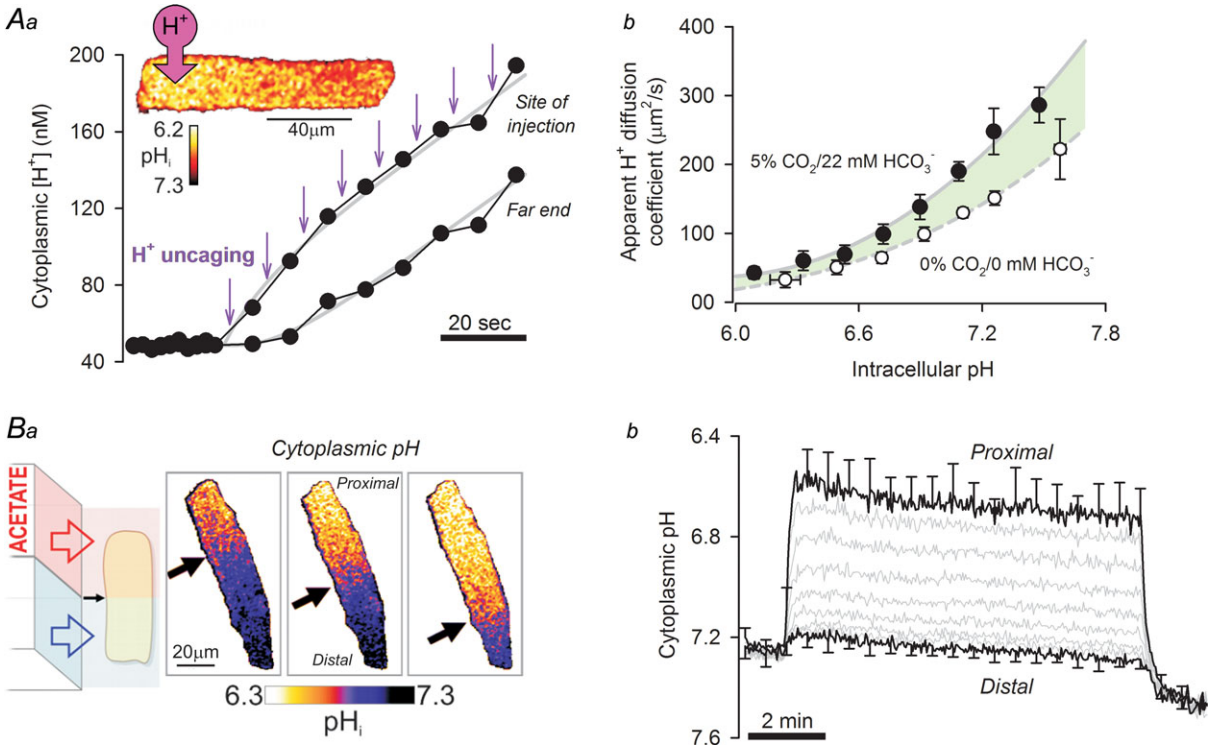

Figure 2. Cytoplasmic transport of H^+ ions requires mobile H^+ buffers
A, H^+ ions are heavily buffered in cytoplasm, to the extent that free H^+ ion diffusion is negligible. Cytoplasmic H^+ ion mobility is therefore determined by the diffusivity of H^+-binding buffers. *Aa*, photolytic uncaging of H^+ ions from the caged H^+-donor 2-nitrobenzaldehyde in a small region of a rat ventricular myocyte produces an acidic microdomain that dissipates with relatively slow diffusivity (~120 $\mu m^2\ s^{-1}$), as reported by the pH-sensitive dye cSNARF1. *Ab*, cytoplasmic H^+ diffusivity increases with cytoplasmic pH and is greater in the presence of physiological CO_2–HCO_3^- buffer (shaded area). *B*, rat ventricular myocyte superfused with Na^+-free, Ca^{2+}-free solutions to block major transmembrane Ca^{2+} and H^+ fluxes. *Ba*, dual microperfusion device exposes the rat myocyte to two sharply separated microstreams of solution (black arrow indicates boundary position). Regional exposure to 80 mM acetate (proximal end) produces a local influx of acid (acetic acid entry) that is dissipated (acetic acid exit) in the unexposed (distal) region. H^+-equivalent fluxes across membranes and along the cytoplasm achieve, at steady state, a large and stable pH gradient (measured with cSNARF1). *Bb*, one-half of myocyte exposed to acetate. Cytoplasmic pH gradient remains stable for the duration of dual microperfusion because of the constant exchange between protonated and deprotonated mobile buffer molecules.

The passive shuttling of H^+ ions aboard mobile buffers, from regions of low pH to high pH, necessitates the return of deprotonated buffer molecules to the source of H^+ ions. Diffusive exchange of these protonated and deprotonated molecules can be studied at steady-state by introducing constant H^+ influx and H^+ efflux at opposite ends of a myocyte's cytoplasmic compartment. This is attainable experimentally by exposing one-half of a myocyte to the salt of a membrane-permeant weak acid, such as acetate, using a dual microperfusion system (Spitzer *et al.* 2000; Swietach *et al.* 2005; Fig. 2*Ba*). Transmembrane acetic acid entry at the acetate-exposed end of the myocyte and exit from the other end of the cell result in compartmentalized H^+ influx and H^+ efflux, which are coupled by cytoplasmic H^+ diffusion. Since intracellular H^+ diffusivity is low, a large cytoplasmic pH gradient is necessary to drive an H^+ flux that matches the substantial transmembrane H^+ fluxes at steady state (Fig. 2*Bb*). In the case of buffers with rapid protonation and deprotonation kinetics, such as imidazoles, the chemical reactions at the H^+ ion source and sink are not rate-limiting relative to buffer-mediated H^+ diffusion (Swietach *et al.* 2005). CO_2–HCO_3^- is unusual because of its slow spontaneous chemical reactions (time constant ~5 s). The degree to which this is accelerated by carbonic anhydrases is only modest in myocyte cytoplasm (~3-fold; Leem & Vaughan-Jones, 1998; Schroeder *et al.* 2013). Thus, the ability of CO_2–HCO_3^- to shuttle H^+ ions spatially can be rate-limited by chemical reactions. This explains the observation that a relatively small fraction of diffusive H^+ traffic is carried by CO_2–HCO_3^-, despite this buffer being a major contributor to the total buffering capacity at equilibrium in myocytes (Swietach *et al.* 2005).

Aside from differences between buffer types with respect to size or kinetics, there are also important chemical differences between the protonated and deprotonated forms of each buffer. As explained below, the difference in electric charge gives rise to some surprising ionic interactions of physiological importance.

Preferential binding of Ca^{2+} to deprotonated mobile buffer can result in Ca^{2+} transport

According to the ping-pong model of membrane-embedded transporters, transfer of ions across a membrane arises from the translocation of an ion-bound state from one side of the membrane to the other. If the returning state were able to bind selectively to a different type of ion, the transport cycle would result in ion exchange, as is the case for NCX. Exchange of protonated and deprotonated buffer-molecules resembles the transport cycle of membrane-embedded proteins, except that it occurs in cytoplasm rather than across a membrane. Following this analogy, coupled ion exchange in cytoplasm would be possible if ions other than H^+ were able to bind preferentially to the deprotonated buffer molecule.

The principal cytoplasmic monovalent ions (Na^+, K^+, Cl^-) bind only very weakly, if at all, to most organic molecules. In contrast, the higher charge density of divalent ions (e.g. Ca^{2+}, Mg^{2+}) allows for stronger interactions. The mobile H^+ buffer ATP binds Mg^{2+} and Ca^{2+} ions with micromolar affinity (Kushmerick, 1997; Baylor & Hollingworth, 1998). Interestingly, HDPs were originally described as biochemical anti-oxidants on the basis of their ability to chelate divalent cations such as copper(II) (Boldyrev, 1993; Pavlov *et al.* 1993; Baran, 2000). Carnosine, a representative HDP, also binds Ca^{2+} and Mg^{2+} with affinity ~1 mM. Over the physiological concentration range of intracellular Ca^{2+} and Mg^{2+} (0.1–1.0 μM and 0.5–1.0 mM, respectively), Mg^{2+} occupancy exceeds Ca^{2+} occupancy by several orders of magnitude. Nonetheless, the amount of Ca^{2+} carried by HDPs (1–10 μM, over typical $[Ca^{2+}]$ range) is biologically meaningful because the total concentration of these buffers in cells is very high (10–20 mM).

Divalent ions are expected to bind more stably to deprotonated molecules, i.e. at more alkaline pH (Baran, 2000). This was tested *in vitro* by measuring the stability of Ca^{2+}-bound buffers in response to acidification (Swietach *et al.* 2013). Buffer-containing solutions (in equilibrium with a fixed $[Ca^{2+}]$) were set in agarose and subjected to photolytic H^+ uncaging. In response to acidification, a substantial rise in free $[Ca^{2+}]$ was observed in the presence of carnosine and ATP (Fig. 3*Aa*), as expected for an apparent competition between Ca^{2+} and H^+ ions.

A global decrease of cytoplasmic pH will release divalent cations from mobile buffers, such as carnosine and ATP, because of a shift towards the protonated form. In the case of a *local* fall of cytoplasmic pH, the depletion of the deprotonated form would, additionally, drive a diffusive influx from adjacent cytoplasmic regions, in exchange for the protonated form. Indeed, this is the mechanism by which H^+ ions diffuse out of the acidic microdomain. Since divalents bind preferentially to the deprotonated form, the exchange of protonated for deprotonated buffer results in net transport of divalents towards acid regions (Fig. 3*Ac*). This process resembles the transport cycle for canonical Ca^{2+}–H^+ exchangers, except that it does not require a partitioning membrane.

Acidic microdomains in cytoplasm recruit Ca^{2+} and generate stable Ca^{2+} gradients

H^+-linked transport of divalents may produce microdomains of elevated $[Ca^{2+}]$ and $[Mg^{2+}]$, unless this is short-circuited by passive back-flux. The magnitude of divalent back-flux depends on cytoplasmic diffusivity, which is slow in the case of Ca^{2+} because of extensive buffering. The ability of a cytoplasmic pH gradient to

produce an uphill Ca^{2+} flux in excess of dissipative back-flux was tested experimentally by imposing a standing gradient of pH in the cytoplasm of isolated ventricular myocytes, using dual microperfusion. The Ca^{2+}-sensitive fluorophore Fluo3 reported a ~80 nM rise of $[Ca^{2+}]$ at the acidic end of the cell (Fig. 3*Ba*). Importantly, the $[Ca^{2+}]$ gradient did not collapse over time.

These findings demonstrate that spatial gradients of Ca^{2+} can, in principle, be formed in cytoplasm over regions of pH non-uniformity. Paradoxically, a partitioning membrane is not required for compartmentalizing Ca^{2+} ions because Ca^{2+} back-flux (set by cytoplasmic Ca^{2+} diffusivity) is low and surmountable by an evoked uphill Ca^{2+} flux. However, it is important to note that adequate diffusivity of the H^+-sensitive Ca^{2+} carrier is required to ensure that the uphill Ca^{2+} transport overcomes the dissipative back-flux. For this reason, immobile buffers, alone, cannot support this form of interaction, even if they exhibit competitive Ca^{2+}–H^+ binding. Similarly, organelles that exchange H^+ ions for Ca^{2+} ions by means of a membrane-embedded transporter, such as Letm1 in mitochondria (Tsai *et al.* 2014), cannot, alone, support the observed standing $[Ca^{2+}]$ gradient. However, these sources of Ca^{2+} can provide the cargo for mobile buffer exchange.

For the experiment shown in Fig. 3*B*, the ratio of the rise in free $[Ca^{2+}]$ per rise in free $[H^+]$ is 1:2. This, is merely an apparent stoichiometry because the buffering capacities for H^+ and Ca^{2+} ions are not equal. The apparent exchange ratio is also sensitive to the size of the underlying pH gradient because of the pH-dependence of buffering. Computational models predict that a 0.1 pH unit gradient would produce a 30 nM $[Ca^{2+}]$ gradient under resting conditions, i.e. an exchange ratio approaching ~$2Ca^{2+}$:$1H^+$ (Swietach *et al.* 2013).

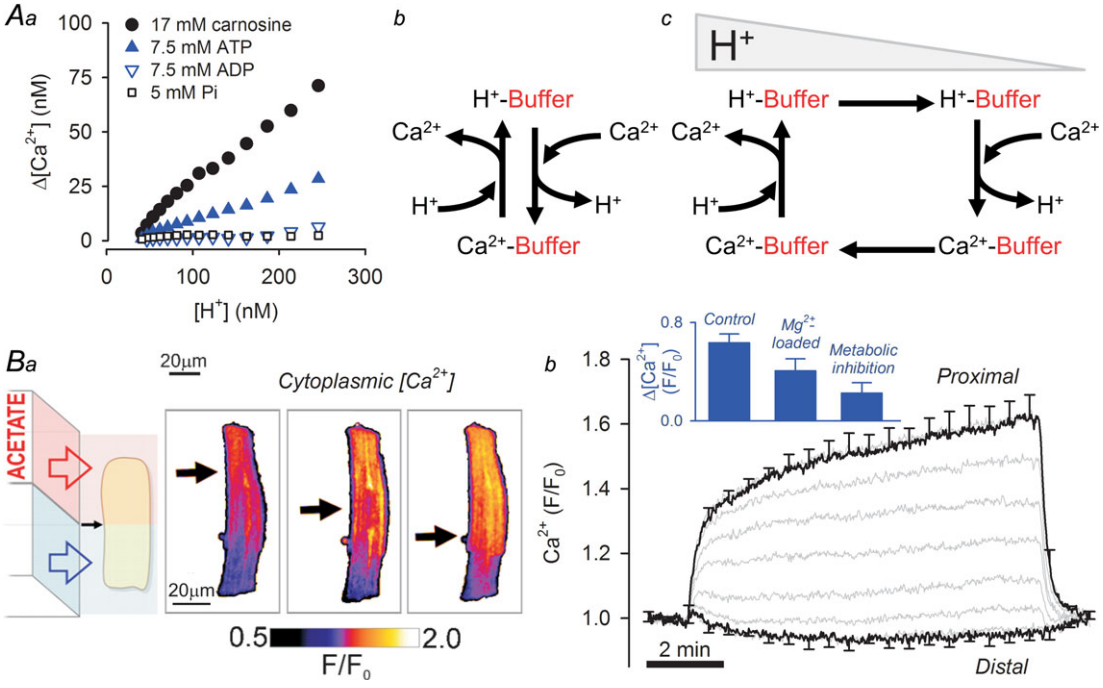

Figure 3. pH-sensitive binding of Ca^{2+} to mobile H^+ buffers results in cytoplasmic Ca^{2+}–H^+ exchange
A, Ca^{2+} binds to many small molecules, including mobile buffers. *Aa*, stability of Ca^{2+} complexes of carnosine (a histidyl dipeptide mobile buffer), ATP, ADP and inorganic phosphate (P_i) during acidification (photolytic H^+ uncaging), determined *in vitro* from rise in free $[Ca^{2+}]$ in agarose-set solutions. *Ab*, binding of Ca^{2+} and H^+ ions to carnosine and ATP shows a degree of apparent competitiveness. *Ac*, a locally imposed $[H^+]$ gradient produces a spatial gradient of Ca^{2+}-loaded mobile buffers. H^+- and Ca^{2+}-bound mobile buffers diffuse down their respective gradients, producing Ca^{2+}–H^+ exchange. *B*, rat ventricular myocyte superfused with Na^+-free, Ca^{2+}-free solutions. *Ba*, response of resting $[Ca^{2+}]$ (measured with Ca^{2+} dye Fluo3) during regional exposure to 80 mM acetate (arrow indicates boundary between acetate-containing and acetate-free microstreams). A gradient of $[Ca^{2+}]$ maps spatially onto the imposed gradient of $[H^+]$. *Bb*, one-half of myocyte exposed to acetate. Rise of $[Ca^{2+}]$ at the proximal (acidic) end of myocyte does not dissipate towards distal end, indicating that an uphill Ca^{2+} flux is balancing the diffusive back-flux. Inset shows size of $[Ca^{2+}]$ gradient measured at 8 min of dual microperfusion under control conditions, following a protocol that raises cytoplasmic $[Mg^{2+}]$ fourfold (superfusion with 30 mM Mg^{2+}-containing, Na^+-free solution) and following metabolic inhibition with rotenone (10 μM), antimycin (10 μM) and deoxyglucose (5 mM). Raised $[Mg^{2+}]$ reduces the Ca^{2+}-carrying capacity of histidyl dipeptides. Metabolic inhibition depletes ATP, a key mobile buffer, and raises cytoplasmic $[Mg^{2+}]$.

Uphill H^+ transport by reverse-mode cytoplasmic Ca^{2+}–H^+ exchange requires large Ca^{2+} gradients

Cytoplasmic Ca^{2+}–H^+ exchange (cCHX) could, in principle, be reversed to produce uphill H^+ transport powered by a $[Ca^{2+}]$ gradient. However, the apparent H^+/Ca^{2+} stoichiometry of this process is very high and not a simple inverse of H^+-driven Ca^{2+} transport. To explain this, it is important to consider the traffic of ions carried by buffers that are capable of binding H^+ and Ca^{2+} ions competitively. An imposed pH_i gradient drives the diffusive flux of buffers that, by and large, exhibit Ca^{2+}/H^+ competition. Due to the overwhelming extent of H^+-buffering, no significant flux of free H^+ ions occurs. In contrast, an imposed Ca^{2+} gradient evokes a sizeable flux of free Ca^{2+} ions (which, by definition, cannot be coupled to H^+ ions) plus movement of Ca^{2+} aboard buffers, many of which are not significant H^+ buffers. Consequently, a steep $[Ca^{2+}]$ gradient is necessary to drive a sufficient flux of H^+-carrying buffers, capable of establishing a meaningful pH gradient.

Ca^{2+}-driven uphill transport of H^+ ions has been demonstrated experimentally in the cytoplasm of ventricular myocytes by activating NCX transport in opposite directions on either end of a cell using dual micro-perfusion (Swietach *et al.* 2013). However, the underlying cause of this $[Ca^{2+}]$ gradient is not physiological. A more plausible source of $[Ca^{2+}]$ non-uniformity is the SR Ca^{2+} release event. At the peak of a Ca^{2+} transient, bulk cytoplasmic $[Ca^{2+}]$ can approach 1 μM, whereas near the SR release sites at dyadic spaces, it may reach 70 μM (Cannell & Kong, 2012). This magnitude of $[Ca^{2+}]$ non-uniformity is predicted to be sufficient for evoking uphill H^+ transport by reverse-mode cCHX. Although the Ca^{2+} release event is transient, its cyclical and regular pattern will result in a time-averaged $[Ca^{2+}]$ gradient that may be sufficient to generate an acidic nanodomain at the dyadic space. A substantial accumulation of dyadic H^+ ions may feed-back negatively on the release process through the pH_i sensitivity of ryanodine receptor (RyR) channels (Xu *et al.* 1996; Balnave & Vaughan-Jones, 2000). Furthermore, this interaction may show rate dependence, as the time-averaged Ca^{2+} gradient is likely to become steeper at faster heart rates.

The cell's energetic status can affect cytoplasmic Ca^{2+}–H^+ exchange

Mathematical simulations predict that HDPs are the major contributors to cCHX because of their high concentration, an acid-dissociation constant that is near resting cytoplasmic pH (pK ~6.8, ensuring comparable concentrations of protonated and deprotonated forms) and adequate Ca^{2+} binding (Swietach *et al.* 2013). In contrast, the contribution of ATP to cCHX is small, despite a high affinity for Ca^{2+} ($10^{-4.6}$ M) and abundance (~7.5 mM in cardiac myocytes), because the low acid-dissociation constant (pK_a 6.5) makes it a weaker H^+ buffer than HDPs. Nonetheless, the unique biological properties of ATP, stemming from its role as an energy source, result in important effects on cCHX.

Compromised ATP production or uncompensated raised demand will reduce the magnitude of cCHX and result in smaller cytoplasmic Ca^{2+} gradients (i.e. a lower Ca^{2+}–H^+ exchange ratio). This has been observed experimentally by measuring the size of $[Ca^{2+}]$ gradients evoked by an acidic microdomain in metabolically inhibited ventricular myocytes. The degree of inhibition of cCHX, however, was larger than expected from the depletion of intracellular ATP alone (Fig. 3*Bb*). The additional decrease in the Ca^{2+}–H^+ exchange ratio can be explained in terms of the rise in $[Mg^{2+}]$ caused by ATP hydrolysis to ADP, a nucleotide with much lower divalent affinity (Kushmerick, 1997). The additional Mg^{2+} released into the cytoplasm from net ATP hydrolysis is buffered by HDPs, at the expense of reducing their Ca^{2+}-carrying capacity (i.e. lower Ca^{2+}–H^+ exchange ratio). This effect was confirmed experimentally in metabolically normal myocytes, after cytoplasmic $[Mg^{2+}]$ had been raised globally by driving whole-cell transmembrane Na^+–Mg^{2+} exchange in the outward current mode. The observed end-to-end $[Ca^{2+}]$ gradient in response to a longitudinal standing pH_i gradient was now reduced (Fig. 3*Bb*). Thus, the inhibitory effect of raising intracellular $[Mg^{2+}]$ on cCHX is comparable to the effect of competitive antagonists on membrane-embedded transporter-proteins. cCHX is therefore exquisitely sensitive to ATP levels (i.e. the cell's metabolic status) but in a unique manner compared to primary active transporters.

The fact that ATP is a highly labile chemical component of cells means that gradients of cytoplasmic [ATP] can be produced rapidly as a result of regional differences in energy demand and supply. The diffusive exchange of ATP and ADP between such regions can evoke uphill Ca^{2+} transport – even without an underlying pH gradient – because of preferential divalent binding to ATP (Swietach *et al.* 2013). Since net ATP hydrolysis yields H^+ ions, non-uniformity in the ATP demand/supply ratio will generate pH and Ca^{2+} gradients concurrently.

Comparing cytoplasmic and transmembrane uphill transport of Ca^{2+}

Until recently, transporter proteins embedded in membranes have been recognized as the only means of producing uphill transport of ions, leading to ionic compartmentalization. Although a transient and localized increase in ionic concentration in cytoplasm, such as during a Ca^{2+} spark, may be considered a form of

compartmentalization, the cytoplasm has not generally been believed to have the intrinsic means to 'pump' ions. The cytoplasmic Ca^{2+}–H^+ exchanger is thus a novel paradigm in ionic transport (Figure 4). Like secondary active transporters (e.g. NCX), cCHX is driven by an underlying solute gradient (in this case, an H^+ gradient). Also, in common with membrane-embedded transporters, the cytoplasmic Ca^{2+}–H^+ exchanger can be 'antagonized' (by Mg^{2+}) and described kinetically using the Michaelis–Menten formalism (affinity and maximal velocity).

Despite a number of qualitative similarities with canonical membrane-embedded transporters, major differences are apparent. Firstly, the back-flux that short-circuits cCHX is comparably much higher than membrane 'leakage' in the case of transmembrane transport. This limits the size of $[Ca^{2+}]$ gradients and the distance over which they spread. Considering that cytoplasmic pH gradients are unlikely to exceed one unit in magnitude, cCHX could, at most, generate $[Ca^{2+}]$ in the sub-micromolar range, spanning distances in the micrometre range. In contrast, membrane-embedded transporters are capable of generating much larger $[Ca^{2+}]$ gradients of up to 10^4-fold across a 10 nm-wide membrane. Whereas membrane-embedded transporters can support sharply demarcated boundaries between regions of dramatically different degrees of Ca^{2+} activation, cytoplasmic transport produces a more graded distribution of Ca^{2+} activation. The latter may be optimal for fine-tuning cellular physiology spatially across several sarcomeres. In contrast, only membrane transport would have the capacity to create Ca^{2+} stores necessary for dynamic signalling, such as Ca^{2+} transients.

A second difference is the scope for regulating the transport process. Membrane-transporters can be regulated at multiple levels, from gene expression through to allosteric modulation, and very often in a highly targeted manner. In contrast, cCHX operates on the basis of small molecules that are not coded directly by genes and are not subject to the same degree of regulation as transporter proteins. The key buffers responsible for cCHX serve additional roles, and this restricts the scope of targeting the Ca^{2+}–H^+ exchange process selectively for regulation. ATP is the cell's energy store and, unsurprisingly, ATP levels are controlled principally by energetic cues. HDPs are anti-oxidants, major pH buffers, and possibly important metabolic regulators. Proteins involved in the synthesis (carnosine synthase CARNS1; Drozak *et al.* 2010), break-down (cytosolic carnosinase CN2; Teufel *et al.* 2003) and uptake (peptide transport PepT1 and 2; Yamashita *et al.* 1997; Vistoli *et al.* 2012) of carnosine have been described (Boldyrev *et al.* 2013), but the precise mechanisms by which these are co-ordinated to meet the cell's demand for HDPs remains unclear.

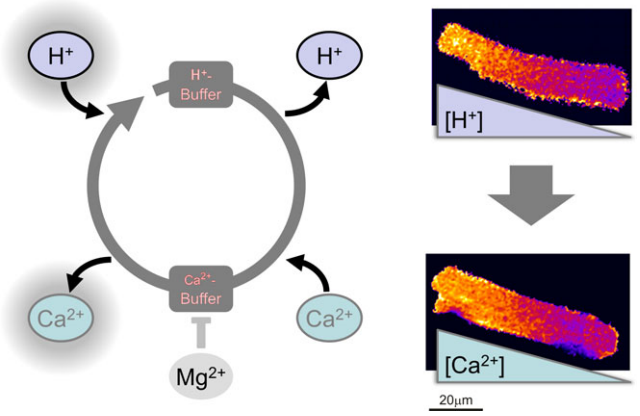

Figure 4. Cytoplasmic Ca^{2+}–H^+ exchange by the mobile buffer shuttle is a new paradigm in spatial Ca^{2+}–H^+ interactions
Diffusive exchange of H^+-bound mobile buffer (down a spatial $[H^+]$ gradient) for Ca^{2+}-bound mobile buffer, produces uphill Ca^{2+} transport that can result in the establishment of a stable spatial $[Ca^{2+}]$ gradient. Non-uniformity of pH (resulting from gradients in metabolic output, regional exposure to membrane-permeant weak acids/bases or compartmentalized acid–base membrane transport) will automatically produce a microdomain of Ca^{2+} that can locally regulate function.

Cytoplasmic Ca^{2+}–H^+ exchange is physiologically important

Cytoplasmic Ca^{2+}–H^+ exchange is driven automatically between regions with different concentrations of protonated and unprotonated forms of buffers like HDPs and ATP pH. Gradients of pH_i occur physiologically in the heart myoplasm as a result of the extensive system of acid–base transporters at membranes, regional differences in blood perfusion and metabolic rate.

In ventricular myocytes, membrane acid extrusion on Na^+/H^+ exchanger 1 (NHE1) produces spatial gradients of pH_i of up to 0.1 units (with subsarcolemmal regions more alkaline than bulk cytoplasm), which can last for several tens of seconds (Swietach & Vaughan-Jones, 2005; Garciarena *et al.* 2013). By evoking cCHX, such H^+ microdomains will drive uphill Ca^{2+} transport towards the more acidic regions of the cell. A prompt buffer-mediated rise of resting $[Ca^{2+}]_i$ during acidosis is likely to provide physiological compensation for H^+ interference with Ca^{2+}-activated processes that share common Ca^{2+}/H^+ binding sites. By diverting Ca^{2+} towards acidic regions, cytoplasmic Ca^{2+}–H^+ exchange may help spatially to unify Ca^{2+} responses during periods of local pH_i non-uniformity.

Intracellular acidity has a direct inhibitory effect on the contractile apparatus, but an up-regulation in Ca^{2+} signalling can correct for this, and even produce positive inotropy (Bountra & Vaughan-Jones, 1989; Choi *et al.* 2000). This compensatory mechanism involves the

activation of Na^+ influx via NHE1 at low pH_i, which then leads to greater cellular retention of Ca^{2+} by decreasing the Na^+-driving force for Ca^{2+} extrusion via NCX. The retained Ca^{2+} is sequestered by SERCA into the SR to increase SR Ca^{2+} load and hence Ca^{2+} transient amplitude (Vaughan-Jones *et al.* 2009; Garciarena *et al.* 2013). However, many of the Ca^{2+}-handling proteins involved in the functional coupling between NHE1 and SERCA are inhibited by H^+ ions. Cytoplasmic Ca^{2+}–H^+ exchange may help to rescue this coupling in acidic microdomains by locally raising diastolic Ca^{2+} to overcome H^+ inhibition.

Slow cytoplasmic Ca^{2+} diffusivity may limit the rate of Ca^{2+} uptake into organellar stores, such as the SR and mitochondria. The additional, uphill Ca^{2+} flux evoked by an $[H^+]$ gradient could facilitate Ca^{2+} handling by improving diffusive coupling. H^+ extrusion by NHE1 will drive Ca^{2+} diffusion away from the sarcolemma, passing the SR *en route* to the more acidic myocyte core. This Ca^{2+} delivery pipeline may improve the functional coupling between Na^+-driven pH_i regulators and SR Ca^{2+} load. In another example of H^+ extrusion, the electron transport chain of the inner mitochondrial membrane acidifies the inter-membrane space, relative to the bulk cytoplasm (Xiong *et al.* 2010; Schroeder *et al.* 2013). As the outer mitochondrial membrane is freely permeable to mobile buffers, this pH gradient would be expected to facilitate Ca^{2+} delivery to mitochondria.

During periods of spatially heterogeneous blood flow, as may occur in myocardial ischaemia, impaired washout of metabolites produces gradients of CO_2 and lactic acid in the heart, particularly at so-called ischaemic border zones (Case *et al.* 1979; Cascio *et al.* 1992). Such partitioning of extracellular membrane-permeant weak acids will give rise to local intracellular acidosis, accompanied by gradients of pH_i extending out towards the normal myocardium (Spitzer *et al.* 2000; Swietach *et al.* 2005). Since HDPs can permeate gap junctional channels and because junctional channels remain open at relatively low pH_i values (Swietach *et al.* 2007*a*), cCHX is likely to be evoked among myocytes coupled over much larger spatial scales than just the single cell. Spatial Ca^{2+}–H^+ exchange is predicted to respond to pH_i non-uniformity by diverting Ca^{2+} from normal myocardium into acidic regions. Ca^{2+} delivery may be beneficial as part of a compensatory reaction, but in excess, it may contribute to the injurious and arrhythmogenic phenomenon of intracellular Ca^{2+} overload during regional ischaemia in the heart.

Cytoplasmic Ca^{2+}–H^+ exchange is not unique to the cardiac myocyte. ATP is ubiquitous in living cells and high levels of HDPs have been measured in many cells, including skeletal muscle, neurons and glia (O'Dowd *et al.* 1988, 1990). Large pH_i gradients have been demonstrated in neurons (Schwiening & Willoughby, 2002; Willoughby & Schwiening, 2002), and these may exploit cCHX for Ca^{2+} delivery and local control of signalling.

Buffer-mediated cytoplasmic Ca^{2+}–H^+ exchange adds a new paradigm to our understanding of ion transport and mechanisms of local signalling by Ca^{2+} and H^+ ions.

References

Balnave CD & Vaughan-Jones RD (2000). Effect of intracellular pH on spontaneous Ca^{2+} sparks in rat ventricular myocytes. *J Physiol* **528**, 25–37.

Baran EJ (2000). Metal complexes of carnosine. *Biochemistry (Mosc)* **65**, 789–797.

Bassani JW, Bassani RA & Bers DM (1994). Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms. *J Physiol* **476**, 279–293.

Baylor SM & Hollingworth S (1998). Model of sarcomeric Ca^{2+} movements, including ATP Ca^{2+} binding and diffusion, during activation of frog skeletal muscle. *J Gen Physiol* **112**, 297–316.

Berlin JR, Bassani JW & Bers DM (1994). Intrinsic cytosolic calcium buffering properties of single rat cardiac myocytes. *Biophys J* **67**, 1775–1787.

Berridge MJ, Lipp P & Bootman MD (2000). The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* **1**, 11–21.

Bers DM (2002). Cardiac excitation–contraction coupling. *Nature* **415**, 198–205.

Boldyrev AA (1993). Does carnosine possess direct antioxidant activity? *Int J Biochem* **25**, 1101–1107.

Boldyrev AA, Aldini G & Derave W (2013). Physiology and pathophysiology of carnosine. *Physiol Rev* **93**, 1803–1845.

Bountra C & Vaughan-Jones RD (1989). Effect of intracellular and extracellular pH on contraction in isolated, mammalian cardiac muscle. *J Physiol* **418**, 163–187.

Cannell MB & Kong CH (2012). Local control in cardiac E–C coupling. *J Mol Cell Cardiol* **52**, 298–303.

Cascio WE, Yan GX & Kleber AG (1992). Early changes in extracellular potassium in ischemic rabbit myocardium. The role of extracellular carbon dioxide accumulation and diffusion. *Circ Res* **70**, 409–422.

Case RB, Felix A & Castellana FS (1979). Rate of rise of myocardial PCO_2 during early myocardial ischemia in the dog. *Circ Res* **45**, 324–330.

Cheng H, Lederer WJ & Cannell MB (1993). Calcium sparks: elementary events underlying excitation–contraction coupling in heart muscle. *Science* **262**, 740–744.

Choi HS, Trafford AW, Orchard CH & Eisner DA (2000). The effect of acidosis on systolic Ca^{2+} and sarcoplasmic reticulum calcium content in isolated rat ventricular myocytes. *J Physiol* **529**, 661–668.

Clapham DE (2007). Calcium signaling. *Cell* **131**, 1047–1058.

Drozak J, Veiga-da-Cunha M, Vertommen D, Stroobant V & Van Schaftingen E (2010). Molecular identification of carnosine synthase as ATP-grasp domain-containing protein 1 (ATPGD1). *J Biol Chem* **285**, 9346–9356.

Garciarena CD, Youm JB, Swietach P & Vaughan-Jones RD (2013). H^+-activated Na^+ influx in the ventricular myocyte couples Ca^{2+}-signalling to intracellular pH. *J Mol Cell Cardiol* **61**, 51–59.

Hardman JG, Robison GA & Sutherland EW (1971). Cyclic nucleotides. *Annu Rev Physiol* **33**, 311–336.

House JR, Miller DJ & O'Dowd JJ (1989). Differences in the distribution of the imidazoles of rat heart between atria and ventricles. *J Physiol* **417**, 162P.

Irving M, Maylie J, Sizto NL & Chandler WK (1990). Intracellular diffusion in the presence of mobile buffers. Application to proton movement in muscle. *Biophys J* **57**, 717–721.

Junge W & McLaughlin S (1987). The role of fixed and mobile buffers in the kinetics of proton movement. *Biochim Biophys Acta* **890**, 1–5.

Kushmerick MJ (1997). Multiple equilibria of cations with metabolites in muscle bioenergetics. *Am J Physiol Cell Physiol* **272**, C1739–C1747.

Kushmerick MJ & Podolsky RJ (1969). Ionic mobility in muscle cells. *Science* **166**, 1297–1298.

Leem CH, Lagadic-Gossmann D & Vaughan-Jones RD (1999). Characterization of intracellular pH regulation in the guinea-pig ventricular myocyte. *J Physiol* **517**, 159–180.

Leem CH & Vaughan-Jones RD (1998). Out-of-equilibrium pH transients in the guinea-pig ventricular myocyte. *J Physiol* **509**, 471–485.

O'Dowd JJ, Cairns MT, Trainor M, Robins DJ & Miller DJ (1990). Analysis of carnosine, homocarnosine, and other histidyl derivatives in rat brain. *J Neurochem* **55**, 446–452.

O'Dowd JJ, Robins DJ & Miller DJ (1988). Detection, characterisation, and quantification of carnosine and other histidyl derivatives in cardiac and skeletal muscle. *Biochim Biophys Acta* **967**, 241–249.

Pavlov AR, Revina AA, Dupin AM, Boldyrev AA & Yaropolov AI (1993). The mechanism of interaction of carnosine with superoxide radicals in water solutions. *Biochim Biophys Acta* **1157**, 304–312.

Schroeder MA, Ali MA, Hulikova A, Supuran CT, Clarke K, Vaughan-Jones RD, Tyler DJ & Swietach P (2013). Extramitochondrial domain rich in carbonic anhydrase activity improves myocardial energetics. *Proc Natl Acad Sci U S A* **110**, E958–967.

Schwiening CJ & Willoughby D (2002). Depolarization-induced pH microdomains and their relationship to calcium transients in isolated snail neurones. *J Physiol* **538**, 371–382.

Spitzer KW, Ershler PR, Skolnick RL & Vaughan-Jones RD (2000). Generation of intracellular pH gradients in single cardiac myocytes with a microperfusion system. *Am J Physiol Heart Circ Physiol* **278**, H1371–H1382.

Swietach P, Camelliti P, Hulikova A, Kohl P & Vaughan-Jones RD (2010). Spatial regulation of intracellular pH in multicellular strands of neonatal rat cardiomyocytes. *Cardiovasc Res* **85**, 729–738.

Swietach P, Leem CH, Spitzer KW & Vaughan-Jones RD (2005). Experimental generation and computational modeling of intracellular pH gradients in cardiac myocytes. *Biophys J* **88**, 3018–3037.

Swietach P, Rossini A, Spitzer KW & Vaughan-Jones RD (2007*a*). H^{+} ion activation and inactivation of the ventricular gap junction: a basis for spatial regulation of intracellular pH. *Circ Res* **100**, 1045–1054.

Swietach P, Spitzer KW & Vaughan-Jones RD (2007*b*). 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* **92**, 641–653.

Swietach P & Vaughan-Jones RD (2005). Spatial regulation of intracellular pH in the ventricular myocyte. *Ann N Y Acad Sci* **1047**, 271–282.

Swietach P, Youm JB, Saegusa N, Leem CH, Spitzer KW & Vaughan-Jones RD (2013). Coupled Ca^{2+}/H^{+} transport by cytoplasmic buffers regulates local Ca^{2+} and H^{+} ion signaling. *Proc Natl Acad Sci U S A* **110**, E2064–2073.

Teufel M, Saudek V, Ledig JP, Bernhardt A, Boularand S, Carreau A, Cairns NJ, Carter C, Cowley DJ, Duverger D, Ganzhorn AJ, Guenet C, Heintzelmann B, Laucher V, Sauvage C & Smirnova T (2003). Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. *J Biol Chem* **278**, 6521–6531.

Tsai MF, Jiang D, Zhao L, Clapham D & Miller C (2014). Functional reconstitution of the mitochondrial Ca^{2+}/H^{+} antiporter Letm1. *J Gen Physiol* **143**, 67–73.

Vaughan-Jones RD, Peercy BE, Keener JP & Spitzer KW (2002). Intrinsic H^{+} ion mobility in the rabbit ventricular myocyte. *J Physiol* **541**, 139–158.

Vaughan-Jones RD, Spitzer KW & Swietach P (2009). Intracellular pH regulation in heart. *J Mol Cell Cardiol* **46**, 318–331.

Vistoli G, Carini M & Aldini G (2012). Transforming dietary peptides in promising lead compounds: the case of bioavailable carnosine analogs. *Amino Acids* **43**, 111–126.

Willoughby D & Schwiening CJ (2002). Electrically evoked dendritic pH transients in rat cerebellar Purkinje cells. *J Physiol* **544**, 487–499.

Xiong JW, Zhu L, Jiao X & Liu SS (2010). Evidence for DeltapH surface component (DeltapH(S)) of proton motive force in ATP synthesis of mitochondria. *Biochim Biophys Acta* **1800**, 213–222.

Xu L, Mann G & Meissner G (1996). Regulation of cardiac Ca^{2+} release channel (ryanodine receptor) by Ca^{2+}, H^{+}, Mg^{2+}, and adenine nucleotides under normal and simulated ischemic conditions. *Circ Res* **79**, 1100–1109.

Yamashita T, Shimada S, Guo W, Sato K, Kohmura E, Hayakawa T, Takagi T & Tohyama M (1997). Cloning and functional expression of a brain peptide/histidine transporter. *J Biol Chem* **272**, 10205–10211.

Zaniboni M, Swietach P, Rossini A, Yamamoto T, Spitzer KW & Vaughan-Jones RD (2003). Intracellular proton mobility and buffering power in cardiac ventricular myocytes from rat, rabbit, and guinea pig. *Am J Physiol Heart Circ Physiol* **285**, H1236–H1246.

Additional information

Competing interests

None declared.

Author contributions

All authors contributed to writing the paper. PS presented the work at the XXXVII Congress of the International Union of Physiological Sciences (IUPS) in Birmingham, UK.

Funding

This work was supported by the British Heart Foundation (RG/08/016 to RDVJ), Royal Society (PS) and National Institutes of Health (R37HL042873-24 to KWS)