

Pumping Ca^{2+} up H^{+} gradients:
a cytoplasmic $\text{Ca}^{2+}/\text{H}^{+}$ exchanger without a membrane

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Abstract

Cellular activity arises from the orchestrated action of proteins, many of which are modulated by small ions, such as Ca^{2+} and H^+ . Cardiac myocytes are exquisitely sensitive to these ions. By regulating the spatial and temporal distribution of intracellular $[\text{Ca}^{2+}]$ and $[\text{H}^+]$, the myocyte exercises control over its biological function. A well-established paradigm in cellular physiology is that specialised membrane-embedded channel and carrier proteins regulate ion concentration. Many carrier proteins couple the transmembrane movement of two or more types of ion, in co- or counter-transport reactions, thus linking ion concentrations among different compartments. We review evidence for a novel type of $\text{Ca}^{2+}/\text{H}^+$ coupling within cytoplasm, which produces uphill Ca^{2+} transport, energised by spatial H^+ ion gradients. This results in the cytoplasmic compartmentalization of Ca^{2+} , without the requirement for a partitioning membrane. The mechanism, demonstrated within mammalian ventricular myocytes, relies on diffusible cytoplasmic buffers, such as carnosine, homocarnosine and ATP, to which Ca^{2+} and H^+ ions bind competitively. These molecules actively recruit Ca^{2+} to acidic microdomains, in exchange for the counter movement of H^+ ions. The resulting Ca^{2+} microdomains thus have the potential to regulate function locally. Spatial cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange thus acts like a “pump” without a membrane.

The second messenger calcium is highly compartmentalized as a result of uphill transport

Calcium (Ca^{2+}) ions trigger a wide variety of intracellular signalling cascades (Clapham, 2007). In the heart, these include systems that control electrical excitation, cellular contraction, growth and development (Bers, 2002). The chemical basis for triggering is the ability of Ca^{2+} to bind to proteins and modulate their function. Unlike many other signalling agents, such as cyclic nucleotides (Hardman *et al.*, 1971), Ca^{2+} ions cannot be regulated by biosynthesis or break-down. Instead, Ca^{2+} signalling relies on the principle of compartmentalization by transport, whereby the concentration of Ca^{2+} ions is set to different levels in different regions of the cell (Berridge *et al.*, 2000). Microdomains of elevated Ca^{2+} can modulate biological function locally, and can also be the source of Ca^{2+} for release into adjacent regions, in order to produce dynamic responses (Cheng *et al.*, 1993).

The magnitude and stability of local Ca^{2+} compartmentalization is limited by the extent of dissipative back-flux of Ca^{2+} down the ensuing electrochemical gradient. This could be regulated by imposing diffusional barriers and engaging in a compensatory uphill flux of Ca^{2+} . A major barrier to the movement of highly hydrophilic Ca^{2+} ions is the lipid bilayer. Large $[\text{Ca}^{2+}]$ gradients, established and maintained by Ca^{2+} -pumping transport proteins, are observed across biological membranes such as the sarcoplasmic reticulum (SR), lysosomal and plasmalemmal membranes (Figure 1). For example, in cardiac myocytes, sarcolemmal Ca^{2+} movements on $\text{Na}^+/\text{Ca}^{2+}$ exchange (NCX) and $\text{Ca}^{2+}/\text{H}^+$ ATPase (PMCA) proteins generate a low cytoplasmic $[\text{Ca}^{2+}]$ of $\sim 10^{-7}$ M (Bassani *et al.*, 1994; Bers, 2002). In a topologically analogous manner, the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) sequesters cytoplasmic Ca^{2+} into the SR, an important Ca^{2+} store in cardiac myocytes (Bassani *et al.*, 1994; Bers, 2002).

In aqueous cytoplasm, the passive mobility of Ca^{2+} ions is reduced by volume-exclusion due to macromolecules (which increase diffusion path-length) and by binding to buffer molecules (such as troponin, calmodulin or ATP) (Kushmerick & Podolsky, 1969; Baylor & Hollingworth, 1998). Typically, only one in a hundred calcium ions is free to diffuse, and the diffusivity of the remaining calcium depends on the mobility of the buffers to which it is complexed (Berlin *et al.*, 1994; Baylor & Hollingworth, 1998). These phenomena reduce cytoplasmic Ca^{2+} diffusivity to a level that supports signalling microdomains such as calcium sparks (Cheng *et al.*, 1993), but only transiently, unless a counter-flux of Ca^{2+} ions balances diffusive dissipation. It has been commonly accepted that such an uphill counter-flux of Ca^{2+} can only occur by transport of the ion across membranes. Our recent findings challenge this notion (Swietach *et al.*, 2013). We have demonstrated that a class of small molecules dissolved in cytoplasm can mediate the exchange of Ca^{2+} and H^+ ions without the involvement of a biological membrane. As a result of this cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange, stable microdomains of Ca^{2+} can be generated that map onto spatial gradients of cytoplasmic pH. Central to this novel paradigm in ion exchange is the mechanism of cytoplasmic H^+ ion transport which we and others have studied extensively (Junge & McLaughlin, 1987; Irving *et al.*, 1990; Vaughan-Jones *et al.*, 2002; Swietach *et al.*, 2005; Swietach *et al.*, 2007b).

Diffusive exchange of protonated and unprotonated mobile buffers mediates cytoplasmic H⁺ transport

H⁺ ions, like Ca²⁺ ions, bind avidly to intracellular buffer molecules. However, there are many more cytoplasmic binding sites for H⁺ compared to Ca²⁺, such that only a negligible (~1:500,000) fraction 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), but this is not observed experimentally. 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 photolytic H⁺-uncaging) and recording the pH response in different downstream cytoplasmic regions (Vaughan-Jones *et al.*, 2002; Zaniboni *et al.*, 2003). In myocyte cytoplasm, H⁺ ions diffusion is considerably slower than in pure water, but much faster than the prediction for a compartment buffered only by immobile molecules (Figure 2Ai). These findings suggest that a sub-population of H⁺-binding buffers is mobile, i.e. of low molecular weight. Additionally, an observed increase in the apparent H⁺ diffusion coefficient as intracellular pH (pH_i) rises is consistent with an increase in the ratio of mobile-to-fixed buffering capacity (Figure 2Aii), arguing for chemically distinct populations of mobile and fixed buffers, with the former possessing a higher population-averaged pK (Swietach *et al.*, 2007b).

Carbonic (CO₂/HCO₃⁻) buffer is an important contributor to cytoplasmic H⁺ buffering (particularly at alkaline pH) and its chemical components diffuse relatively rapidly. Excluding CO₂/HCO₃⁻ 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.*, 2007b). 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 GABA) and anserine, (methylhistidine with β-alanine). These are present collectively in cardiac myoplasm at 10-20 mM levels (O'Dowd *et al.*, 1988; House *et al.*, 1989). Their small size (~240 Da), and the presence of a titratable imidazole group (pK_a close to 7), make these molecules suitable for passively transporting H⁺ ions in cytoplasm (Vaughan-Jones *et al.*, 2002). Interestingly, neonatal heart cells have lower HDP levels and display a lower cytoplasmic H⁺ diffusivity (Swietach *et al.*, 2010) in agreement with theoretical predictions (Junge & McLaughlin, 1987; Irving *et al.*, 1990). 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).

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 'carriers' 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) (Figure 2Bi). Transmembrane acetic acid entry (at the acetate-exposed end of the myocyte) and exit (from the other end of the cell) result in spatially localised H^+ -influx and H^+ -efflux, respectively, which are coupled intracellularly by longitudinal cytoplasmic H^+ diffusion. Since intracellular H^+ mobility is low, a large cytoplasmic pH gradient is formed, which provides the necessary chemical driving force to balance the transmembrane H^+ -fluxes (Figure 2Bii). In the case of buffers with rapid protonation and deprotonation kinetics, such as imidazole-derivatives, the reaction process at the H^+ ion source and sink will not normally be rate-limiting, in comparison with the rate of buffer-mediated H^+ diffusion (Swietach *et al.*, 2005). Carbonic buffer (CO_2/HCO_3^-) is unusual because of its slow spontaneous chemical conversion (time constant ~ 5 seconds) (Leem & Vaughan-Jones, 1998; Schroeder *et al.*, 2013). Carbonic anhydrase enzymes are present at only modest levels in myocyte cytoplasm, and their catalytic activity accelerates CO_2 hydration/dehydration by ~ 3 -fold (Leem & Vaughan-Jones, 1998; Schroeder *et al.*, 2013). The ability of CO_2/HCO_3^- to shuttle H^+ ions spatially can therefore be rate-limited by H^+ -releasing (CO_2 hydration) and H^+ -consuming (dehydration) chemical reactions. Although a third of total buffering capacity at equilibrium and near resting pH is accounted for by CO_2/HCO_3^- , the fraction of H^+ traffic carried by this buffer will be smaller due to its slow reaction kinetics (Swietach *et al.*, 2005).

Protonated and deprotonated forms of mobile buffer differ by one unit of charge and merely one unit of mass. The charge difference can have an effect on the electrical interactions between the mobile H^+ buffer and ions other than protons, and give rise to some surprising ionic interactions of physiological importance, as explained below.

Binding of calcium to deprotonated mobile buffers results in the spatial transport of Ca^{2+} ions up an H^+ gradient

Cytoplasmic exchange of protonated and deprotonated buffer-molecules resembles the transport cycle of membrane-embedded proteins. According to the ping-pong model of membrane transporters, translocation of an ion-bound state from one side of the membrane to the other, in exchange for an unbound state, results in net transfer of ions between adjacent aqueous compartments. If the returning unloaded state were able to bind selectively to a different type of ion, the transport cycle would result in coupled ion exchange. Many forms of active transport, such as that mediated by Na^+/H^+ or Na^+/Ca^{2+} exchange, involve such ion coupling. Following the analogy between soluble mobile buffers and membrane-embedded transporters, coupled ion exchange in cytoplasm would be possible if the deprotonated buffer-molecule were able to bind to an ion other than H^+ .

The principal cytoplasmic monovalent ions (Na^+ , K^+ , Cl^-) are only very weakly buffered, if at all, and therefore mobile H^+ buffers are not expected to carry any meaningful fraction of these ions. In contrast, divalent cations of biological importance (Ca^{2+} , Mg^{2+}) interact more strongly with organic molecules thanks

to a high polarising density. The mobile H^+ buffer, ATP, binds Mg^{2+} and Ca^{2+} ions with micromolar affinity (Kushmerick, 1997; Baylor & Hollingworth, 1998) and contributes significantly to the buffered reservoir of divalent cations. 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 μ M and 0.5-1.0 mM, respectively), Mg^{2+} -occupancy will exceed Ca^{2+} -occupancy by several orders of magnitude. Nonetheless, the total amount of Ca^{2+} carried by HDPs (1-10 μ M, over typical $[Ca^{2+}]$ range) is biologically meaningful because the total intracellular concentration of these buffers is high (10-20 mM).

On the basis of the charge difference between protonated and deprotonated forms of mobile buffer, divalent cations are expected to be chelated more stably at higher pH (Baran, 2000). In effect, H^+ and divalent cations like Ca^{2+} compete for binding to the buffer. This prediction was tested experimentally by measuring the stability of Ca^{2+} -bound buffers *in-vitro*, in response to gradual 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 (Figure 3Ai), as expected for an effective competition between Ca^{2+} and H^+ ions. A similar competition between H^+ and Mg^{2+} ions is predicted from ion binding equilibria. Thus, a fall in cytoplasmic pH is expected to decrease divalent binding to mobile buffers like carnosine and ATP, and evoke a rise in free $[Ca^{2+}]$ and $[Mg^{2+}]$ (Figure 3Aii). A *local* fall of pH in a region of cytoplasm would, additionally, drive outward diffusion of protonated buffer in exchange for inward diffusion of deprotonated buffer, and some of the latter would carry divalent cations. In this case, the dissipative flow of H^+ ions out of an acidic microdomain would drive a counter-influx of divalent cations, which would result in the formation of a local microdomain of raised $[Ca^{2+}]$ or $[Mg^{2+}]$, unless it was dissipated by a passive back-flux. An analogy can be drawn with a pump-leak steady-state across a membrane e.g. that between active membrane transport by the Na^+ pump and passive dissipation through Na^+ channels. The magnitude of divalent back-flux will depend on cytoplasmic diffusivity, which is slow in the case of Ca^{2+} because of extensive binding to buffers. Consequently, in response to an acidic microdomain, localised cytoplasmic exchange of protonated and deprotonated mobile buffers, if sustained, is predicted to produce a sustained and substantial rise of Ca^{2+} in the acidic zone (Figure 3Aiii). This prediction was tested experimentally by regional exposure of an isolated ventricular myocyte to acetate using dual microperfusion, to generate a standing intracellular pH gradient. The Ca^{2+} -sensitive fluorophore, Fluo3, reported a rise of $[Ca^{2+}]$ at the acidic end of the cell (Figure 3Bi) of ~ 40 nM Ca^{2+} initially, rising to ~ 80 nM Ca^{2+} . Importantly, the $[Ca^{2+}]$ gradient did not dissipate over time, consistent with a net uphill transport of Ca^{2+} into the acidic microdomain. It is important to note that fixed buffers alone cannot support this form of interaction, even if they exhibit competitive Ca^{2+}/H^+ binding, because adequate diffusivity is essential to complete the transport cycle.

According to the results of mathematical modelling, HDPs are the most important contributors to cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange because of their high concentration, an acid-dissociation constant that is near resting cytoplasmic pH ($\text{pK} \sim 6.8$, ensuring comparable concentrations of protonated and deprotonated forms), and adequate Ca^{2+} binding, predominantly to the deprotonated form (Swietach *et al.*, 2013). The contribution of ATP to cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange is smaller, despite a higher affinity for Ca^{2+} ($K_{\text{Ca}} 10^{-4.6}$ M), and a substantial intracellular concentration (~ 7.5 mM in cardiac myocytes). This is because its acid-dissociation constant is further away from resting pH ($\text{pK}_a 6.5$). However, ATP is a more labile chemical component of cytoplasm, highly sensitive to the balance between metabolic demand and energy supply. Thus, during periods of compromised ATP production, localised pH_i microdomains are expected to produce smaller spatial Ca^{2+} gradients (i.e. a lower $\text{Ca}^{2+}/\text{H}^+$ exchange ratio), because of the depletion of intracellular ATP. A slower spatial Ca^{2+} gradient in response to a given pH_i gradient (imposed using dual microperfusion) has been measured experimentally in ventricular myocytes, after pharmacological blockade of cellular respiration. The degree of inhibition of cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange, however, was larger than expected from the depletion of ATP_i alone (Figure 3Bii). The additional decrease in the $\text{Ca}^{2+}/\text{H}^+$ exchange ratio can be explained in terms of the excess Mg^{2+} released when ATP is hydrolysed to ADP, a nucleotide with much lower divalent cation affinity (Kushmerick, 1997). Indeed, upon acidification *in-vitro*, ADP releases much less Ca^{2+} compared to ATP (Figure 3A). The additional intracellular Mg^{2+} released from ATP during metabolic inhibition is buffered by HDPs, at the expense of reducing their Ca^{2+} -carrying capacity, hence resulting in a lower $\text{Ca}^{2+}/\text{H}^+$ exchange ratio. This effect was confirmed experimentally in metabolically normal isolated myocytes, after cytoplasmic $[\text{Mg}^{2+}]$ had been raised globally (by driving whole-cell transmembrane $\text{Na}^+/\text{Mg}^{2+}$ exchange in the outward current mode, in Na^+ -free solution). The observed end-to-end Ca^{2+} gradient in response to a longitudinal standing pH_i gradient (imposed by dual microperfusion) was now reduced (Figure 3Bii). Thus, the inhibitory effect of raising Mg^{2+}_i on cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange is comparable to the effect of competitive antagonists on membrane-bound transporter-proteins.

Intracellular Ca^{2+} gradients map stably and automatically onto pH_i non-uniformity

Compartmentalization of Ca^{2+} is the basis on which this divalent cation acts as a second messenger in key signalling cascades in cells. Our findings in ventricular myocytes demonstrate that spatial gradients of Ca^{2+} can, in principle, be formed in cytoplasm over regions of pH_i non-uniformity. Because of extensive buffering, transport of H^+ ions is dependent on the diffusive exchange of protonated and deprotonated buffer-molecules. When there is spatial non-uniformity of pH_i , greater Ca^{2+} binding to the deprotonated form results in an obligatory uphill movement of Ca^{2+} in exchange for downhill H^+ movement, energized by the cytoplasmic H^+ gradient, in a manner similar to the mechanism of secondary active transport across membranes. Thus any spatial non-uniformity of pH_i will be overlaid by a comparable spatial non-uniformity of Ca^{2+}_i . Computational modelling predicts that a 0.1 pH_i unit gradient will produce a 30 nM Ca^{2+} gradient

under resting conditions (Swietach *et al.*, 2013). Although many buffers may show a degree of competitive (or at least apparently competitive) Ca^{2+} - H^+ binding, only *mobile* buffer-molecules are able to mediate spatial cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange, because adequate buffer diffusivity is necessary to produce the necessary uphill Ca^{2+} flux. A partitioning membrane is not required for cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange because the diffusion of H^+ and Ca^{2+} ions aboard buffers that do not participate in the $\text{Ca}^{2+}/\text{H}^+$ shuttle is very slow and therefore does not short-circuit the coupled ion transport process.

The apparent stoichiometry of cytoplasmic, spatial $\text{Ca}^{2+}/\text{H}^+$ exchange evoked by an imposed H^+ gradient is $\sim 1\text{Ca}^{2+}:2\text{H}^+$ (Figure 2B vs 3B). This ratio is a complex function of the relative Ca^{2+} and H^+ affinities of the participating mobile buffers, overall H^+ and Ca^{2+} buffering capacity, and the proportion of H^+ traffic carried by divalent-binding buffers. From resting (diastolic) conditions, a 0.1 pH_i gradient can evoke a stable, 30 nM $[\text{Ca}^{2+}]_i$ gradient in the cytoplasm. Cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange can, in principle, be reversed to produce uphill H^+ transport driven by local cytoplasmic gradients of Ca^{2+} . However, the apparent $\text{H}^+/\text{Ca}^{2+}$ stoichiometry of this process is very different ie. a large Ca^{2+} gradient of several μM is required to produce a much smaller nanomolar H^+ gradient. This is because the fraction of Ca^{2+} traffic carried by H^+ -binding buffers is relatively low, and because the uphill H^+ flux needs to overcome a very high H^+ buffering capacity (Swietach *et al.*, 2013) in order to establish a pH_i gradient.

Competition between Mg^{2+} and Ca^{2+} renders cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange exquisitely sensitive to a cell's metabolic state. Indeed, local spatial zones of metabolic inhibition can also evoke $\text{Ca}^{2+}/\text{H}^+$ exchange through the diffusive exchange of ATP for ADP, and divalent-bound HDPs for protonated HDPs between regions of net ATP synthesis (uptake of H^+ and divalents) and net ATP hydrolysis (release of H^+ and divalents) (Swietach *et al.*, 2013). Local metabolic stress within a cell can therefore recruit Ca^{2+} and H^+ via the $\text{Ca}^{2+}/\text{H}^+$ exchange system.

Importance of cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange in cell signalling and function

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 binding sites within the cell. By diverting Ca^{2+} flux towards acidic regions, $\text{Ca}^{2+}/\text{H}^+$ exchange may help spatially to unify Ca^{2+} responses during periods of local pH_i non-uniformity. In ventricular myocytes, membrane acid-extrusion on NHE1 produces spatial gradients of pH_i of up to 0.1 units (subsarcolemma more alkaline than bulk cytoplasm), which can last for several tens of seconds (Swietach & Vaughan-Jones, 2005; Garciarena *et al.*, 2013). Such H^+ microdomains, by acting on cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange, will drive uphill Ca^{2+} transport towards the more acidic regions of the cell. NHE1 activity is known to increase systolic $[\text{Ca}^{2+}]_i$, which helps to support contractility during acidosis. This increase occurs via Na^+ influx on NHE, which acts on sarcolemmal NCX to enhance $[\text{Ca}^{2+}]_i$ in the vicinity of SERCA pumps, thus increasing SR Ca^{2+} loading, and subsequent SR Ca^{2+} release during an action potential (Vaughan-Jones *et al.*, 2009; Garciarena *et al.*, 2013). Many of the Ca^{2+} handling proteins involved in the functional coupling between NHE and NCX are pH_i

sensitive. Spatial $\text{Ca}^{2+}/\text{H}^+$ exchange may therefore help to rescue this coupling in acidic microdomains by locally raising diastolic Ca^{2+} to overcome inhibitory effects of reduced pH_i .

Slow cytoplasmic Ca^{2+} diffusivity may limit the ability of organellar stores, such as the SR and mitochondria, to take-up (or release) Ca^{2+} ions. 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 most 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 borderzones (Case *et al.*, 1979; Cascio *et al.*, 1992). Such partitioning of extracellular membrane-permeant weak acids will give rise local intracellular acidosis, accompanied by gradients of pH_i extending out towards the normal myocardium (Spitzer *et al.*, 2000; Swietach *et al.*, 2005). Spatial $\text{Ca}^{2+}/\text{H}^+$ exchange is predicted to respond to this pH_i non-uniformity by diverting Ca^{2+} from normal myocardium into acidic regions, as part of a compensatory reaction. Since HDPs can permeate gap junctional channels and since junctional channels remain open at relatively low pH_i values (Swietach *et al.*, 2007a), cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange is likely to be evoked among myocytes coupled over much larger spatial scales than just the single cell. The spatial exchanger may thus contribute to the injurious and arrhythmogenic phenomenon of intracellular Ca^{2+} overload during regional ischaemia in the heart.

Cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ 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; O'Dowd *et al.*, 1990). Neurons, where large pH_i gradients have been demonstrated (Schwiening & Willoughby, 2002; Willoughby & Schwiening, 2002), may exploit cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange for Ca^{2+} delivery up $[\text{H}^+]$ gradients and for local control of signalling.

Buffer-mediated cytoplasmic $\text{Ca}^{2+}/\text{H}^+$ exchange adds a new paradigm to our understanding of ion transport – in this case, without the canonical membrane – and its participation in local Ca^{2+} and H^+ ion signalling.

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Figure legends

Figure 1. *Membrane transport can compartmentalise 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). In some cases, pumping of Ca^{2+} is coupled to the movement of other ionic species (e.g. Na^+/Ca^{2+} exchange or plasmalemmal Ca^{2+}/H^+ ATPase). 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).

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. *(i)* 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 ($\sim 120 \mu m^2/s$), as reported by the pH-sensitive dye cSNARF1. *(ii)* 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. *(i)* Dual microperfusion device exposes the rat myocyte to two sharply separated microstreams of solution (black arrow indicates boundary position). Regional exposure to 80mM 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). *(ii)* 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.

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. *(i)* Stability of Ca^{2+} -complexes of carnosine (a histidyl dipeptide mobile buffer), ATP, ADP and inorganic phosphate (Pi) during acidification (photolytic H^+ uncaging), determined *in-vitro* from rise in free $[Ca^{2+}]$ in agarose-set solutions. *(ii)* Binding of Ca^{2+} and H^+ ions to carnosine and ATP shows a degree of apparent competitiveness. *(iii)* A locally imposed

[H⁺] gradient produces a spatial gradient of Ca²⁺-loaded mobile buffers. H⁺- and Ca²⁺-bound mobile buffers diffuse down their respective gradients, producing Ca²⁺/H⁺ exchange. **(B).** Rat ventricular myocyte superfused with Na⁺-free, Ca²⁺-free solutions. (i) Response of resting [Ca²⁺] (measured with Ca²⁺ dye Fluo3) during regional exposure to 80mM acetate (arrow indicates boundary between acetate-containing and acetate-free microstreams). A gradient of [Ca²⁺] maps spatially onto the imposed gradient of [H⁺]. (ii) One half of myocyte exposed to acetate. Rise of [Ca²⁺] at the proximal (acidic) end of myocyte does not dissipate towards distal end, indicating that an uphill Ca²⁺ flux is balancing the diffusive back-flux. Inset shows size of [Ca²⁺] gradient measured at 8 min of dual microperfusion under control conditions, following metabolic inhibition with rotenone (10 μM), antimycin (10 μM) and deoxyglucose (5 mM) and following a protocol that raises cytoplasmic [Mg²⁺] four-fold (superfusion with 30mM Mg²⁺-containing, Na⁺-free solution). Raised [Mg²⁺] reduces the Ca²⁺-carrying capacity of histidyl dipeptides. Metabolic inhibition depletes ATP, a key mobile buffer, and raises cytoplasmic [Mg²⁺].

Figure 4. *Cytoplasmic Ca²⁺/H⁺ exchange by the mobile buffer shuttle is a new paradigm in spatial Ca²⁺/H⁺ interactions.* Diffusive exchange of H⁺-bound mobile buffer (down a spatial [H⁺]-gradient) for Ca²⁺-bound mobile buffer, produces uphill Ca²⁺ transport that can result in the establishment of a stable spatial [Ca²⁺] 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 Ca²⁺ that can locally regulate function.

Low $[Ca^{2+}]$

High $[Ca^{2+}]$

Membrane



















