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Effects of tumour necrosis factor-α and interleukin-1β on the Ca^{2+} transient, contraction and sarcoplasmic reticulum Ca^{2+} content of ventricular myocytes

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Tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are pro-inflammatory cytokines produced during the development of an immune response. Elevated levels of these cytokines are associated with sepsis, reperfusion injury and heart failure. These experiments were carried out to assess the impact of these two cytokines on the regulation of Ca^{2+} and contraction in isolated rat ventricular myocytes. Treated cells were incubated (at 30°C) for either 1, 2 or 3 h in physiological salt solution supplemented with 0.05 ng/ml TNF-α and 2 ng/ml IL-1β and comparison made between control cells which were incubated in cytokine-free salt solution for the same durations. Cytoplasmic Ca^{2+} was measured using fura-2 or fluo-3 and cell shortening recorded optically. Cells were field stimulated at 1 Hz (30°C) and in experiments carried out on cytokine-treated cells, the superfusate included the same concentrations of cytokines used during incubation. Data are presented as mean ± SEM.

After 3 h incubation, the magnitude of the Ca^{2+} transient (control 0.24 ± 0.02, treated 0.16 ± 0.01 fluorescence ratio units) and contraction (control 3.8 ± 0.4, treated 1.9 ± 0.4 % of resting cell length) were significantly ($P<0.05$, t test, $n = 21$) decreased. There were no significant changes in the time-course of either the global Ca^{2+} transient (fura-2) or the contraction following treatment. Sarcoplasmic reticulum (SR) Ca^{2+} content, as estimated from the amplitude of Ca^{2+} transients evoked by rapid application of 20 mM caffeine, was significantly reduced (to 77 ± 7% of control, $P<0.05$, t test, $n = 21$) after 3 h of cytokine treatment, however the half-time of decay of the caffeine-evoked Ca^{2+} transients was unaffected, suggesting little effect of cytokine exposure on Ca^{2+} efflux mechanisms (e.g. Na^+-Ca^{2+} exchange).

Confocal imaging of cells (at 24°C) demonstrated the presence of Ca^{2+} sparks, the frequency of which was 0.63 ± 0.13 s^{-1} in control cells. In cytokine-treated cells, spark frequency was significantly elevated to 2.51 ± 0.17 s^{-1} ($P<0.001$, t test, $n = 19$). Cytokine-induced effects on spark frequency were abolished when cells were skinned in the continued presence of the cytokines.

These data suggest that exposure to TNF-α and IL-1β affects gating of the ryanodine receptor, enhances leak of SR Ca^{2+}, which would reduce SR Ca^{2+} content and contribute to the negative inotropic effects of TNF-α and IL-1β. Furthermore, it appears that effects on spark frequency are not mediated by the cytokines directly but by downstream mediators that are lost when cells are skinned.

Supported by The White Rose Consortium and The British Heart Foundation.

Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

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Effect of renal sympathetic nerve activity on NHE3 in the renal proximal tubule

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Na^+-H^+ exchangers (NHEs) are ubiquitous proteins with a very wide array of physiological functions. The NHE3 isoform plays a crucial role in Na^+ reabsorption, acid-base homeostasis and volume regulation and has been implicated in hypertension. Two basic regulatory mechanisms are involved in NHE3 regulation: (a) changes in turnover number and (b) changes in trafficking. The aim of this study was to evaluate whether changes in renal sympathetic nerve activity (RSNA) could modulate retraction of NHE3 from the microvillar tips to the intermicrovillar clefts and thence to endosomes. The effect of RSNA on the activity of the exchanger was also assesed.

In vivo studies were performed on chlorolose/urethane (1ml, 16.5:250mg/ml, I.P.) anaesthetised rats. Cannulae were placed in the femoral artery to measure blood pressure and in the femoral vein to infuse saline and inulin. Using flank incisions, the right ureter was cannulated; the left kidney was exposed, its ureter cannulated and it was subjected to surgical denervation. After 2 h stabilisation, two 15 min clearances were undertaken, the kidneys were excised and placed on ice. The cortices were dissected free and brush border membranes were obtained using Mg^{2+} precipitation and differential centrifugation (Weinman et al. 1987). The membranes were subjected to SDS-PAGE and, following Western blotting, NHE3 was quantified and identified. Exchange activity was assayed using the acridine orange (AO) fluorescent method in apical membrane vesicles also prepared by Mg^{2+}. Exchange activity was measured as the reappearance of AO from acid-loaded vesicles (Besse-Eschmann et al. 2004). Specific activity was expressed as the slope of the initial Na^+-dependent fluorescent increase. Data (means±S.E.M.) were subjected to Student's t test and significance was taken when $P<0.05$. Western blot analysis showed that NHE3 abundance was reduced 3-fold after denervation in relation to the innervated kidney ($n=8$). There was significantly higher ($P<0.01$) urinary Na^+ excretion in the denervated kidney (3.17±0.70mmol/min/kg) as opposed to the innervated kidney (1.16±0.24mmol/min/kg). Fractional Na^+ excretion was also significantly elevated ($P<0.01$) in the denervated kidney (0.67±0.15 vs.0.26±0.04%). GFR was similar in both the innervated and denervated kidneys at 2.99±0.24 and 3.34±0.24ml/mg/kg, respectively. There was a 17% decrease in NHE3 activity in the denervated kidney. This could possibly be explained by a RSNA-mediated retractionof exchangers to intermicrovillar cleft and endosomal regions. These data demonstrate that renal denervation decreases apical membrane NHE3 abundance and activity, independently of renal haemodynamics.

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Besse-Eschmann *et al.* (2002). *J Am Soc Nephrol* **13**, 2199.

Supported by grant PRTLI-3.

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Acidosis and cardiac excitation-contraction coupling

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When cardiac muscle is exposed to an acid solution, the strength of contraction decreases rapidly. This is due predominantly to an acidosis-induced decrease in myofilament Ca sensitivity, modulated by changes in Ca handling that also slow the decline of the Ca transient (Orchard, 1987). Despite a maintained intracellular acidosis, the decrease of contraction is followed by a slower recovery, which is accompanied by an increase in Ca transient amplitude and recovery of its time course (Orchard, 1987). The recovery of contraction appears to be due to an increase in sarcoplasmic reticulum (SR) Ca content as a result of: (i) desensitisation of the ryanodine receptor to trigger Ca. The subsequent decrease in Ca transient amplitude increases Ca influx via the Ca current, and decreases Ca efflux via Na-Ca exchange. In the absence of other changes this increases cellular, including SR, Ca content, and hence Ca release, until the amplitude of the Ca transient recovers to control levels. This is, however, achieved at a higher SR Ca content and smaller fractional release than previously (Choi et al. 2000). (ii) Activation of acid extrusion pathways, which increases intracellular [Na] and hence, via Na-Ca exchange, intracellular Ca (Bountra & Vaughan-Jones, 1989). (iii) Altered phosphorylation of the regulatory protein phospholamban (PLB), which causes PLB to unbind from the SR Ca pump, increasing its activity and SR Ca uptake (DeSantiago et al. 2004).

Despite these marked effects on excitation-contraction coupling, reducing extracellular pH from 7.4 to 6.5 has relatively modest effects on the electrical activity of atrial and ventricular myocytes isolated from rat heart when the perforated patch clamp technique is used to minimise disturbance of the intracellular milieu (Komukai et al. 2002). However ecg measurements in isolated rat heart show that acidosis markedly slows heart rate and increases the P-R interval, suggesting that the sino-atrial and atrio-ventricular nodes are particularly sensitive to pH. This has been investigated further using the optical dye RH237 to monitor electrical activity on the epicardial surface of isolated rabbit hearts. This revealed that perfusion with acidic solution (pH 6.8) caused a significant delay in the time between atrial activation and the appearance of electrical activity on the ventricular epicardial surface. The conclusion that delayed conduction at the a-v node underlies this result is supported by two further observations: (i) ventricular conduction velocity was not significantly slower; depressed ventricular conduction only became evident at pH 6.3; (ii) experiments on isolated a-v nodal preparations confirmed that the time for atrial-bundle of His (A-H) conduction is slowed by ~20% at pH 6.8. Further slowing of A-H conduction occurs at pH 6.3 to the extent that complete block is frequently observed. Thus it appears that moderate acidosis (pH 6.8) affects cardiac electrophysiological function primarily by altering the electrical activity of nodal tissue. At more acidic pH values (6.3) myocardial conduction velocity is depressed and a-v nodal conduction is dramatically prolonged.

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The authors are grateful to the British Heart Foundation for financial support.

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Molecular mechanisms of acidification-induced closure of gap junctions in the heart

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Acidification of the intracellular space leads to closure of cardiac gap junction channels. It has been suggested that this process acts as one of the substrates for the generation of malignant ventricular arrhythmias consequent to myocardial ischemia. We have studied the molecular events that lead to pH-induced closure ("pH gating") of channels formed by Connexin43 (Cx43), the most abundant cardiac gap junction protein. Our studies show that pH gating follows a particle-receptor model where the carboxyl terminal domain of Cx43, acting as a gating particle, binds to a region of the cytoplasmic loop, acting as a "receptor." Additional studies, using nuclear magnetic resonance, have allowed us to characterize the secondary structures of the fragments involved and detect the amino acids that participate in the process. With this information at hand, we have used a high-throughput assay (phage display) to identify 12-mer peptidic sequences that can interfere with this particle-receptor interaction. A screening of an estimated 2.5 billion peptides led to the identification of a group of 48 sequences that can bind to the carboxyl terminal of Cx43. Through additional screenings, we singled out a particular peptide that can interfere with the regulation of Cx43 gap junctions by pHi. Studies currently in progress aim to determine the specific mechanism of action of this peptide and its potential use in cellular models of cardiac arrhythmias.

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Proton shuttles and gap junctions: role in spatial pH regulation

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Intracellular H^+ buffers protect cells from changes in intracellular pH (pH_i) but also dramatically reduce H^+ mobility by two

orders of magnitude. Such low H^+ diffusibility may lead to the formation of pH_i microdomains. In ventricular myocytes, which exhibit strong pH_i-dependence of excitation-contraction coupling, and complex pH_i-Ca^{2+} interactions, pH_i non-uniformity will pose a threat to whole-cell homeostasis. In the myocardium, variation in capillary perfusion or metabolic rate, can produce cell-to-cell variation of pH_i and reduce the efficiency of heart function. In the pathological condition of ischaemia, sharp gradients of pH_i may exist at border zones and coincide with areas of abnormal Ca^{2+} signalling. Ventricular myocytes express a considerable concentration of cytoplasmic, low-molecular weight H^+ buffers, such as dipeptides. These compete for H^+ with the larger buffer molecules like proteins. They serve as mobile carrier molecules that facilitate H^+ movement within and between cells, producing a passive H^+ shuttle mechanism. Near resting pH_i, H^+-shuttling will keep the interior of cells well-coupled with the sarcolemma, the site of transmembrane acid/base transport. They will also dissipate local acid/base-loads released from subcellular structures such as mitochondria. In coupled cells of the intact myocardium, carrier molecules are able to facilitate H^+-flux between cells through open gap junctional channels. The ability of such molecules to spatially regulate pH_i inside the myocardium is limited by the fraction of H^+-buffering that is mobile and by the gating of gap junctions. The former rises with pH_i and is augmented by the addition of exogenous mobile buffers, such as CO_2/bicarbonate. Gap junctional gating can be controlled by a plethora of factors such as phosphorylation, Ca^{2+}-dependent proteins and pH_i. Interestingly, both acidosis and alkalosis have been shown to close gap junctions, creating a range of pH_i, defined as the permissive range, over which the junctional route for H^+-flux is large, and will exceed sarcolemmal acid/base transport if the pH_i disturbance driving junctional flux is local. These findings illustrate a novel paradigm in pH_i regulation, in which the spread of H^+, carried on shuttles, is determined by the magnitude of the acid/base disturbance. Within the permissive range, cell-to-cell pH_i variation is minimised to help coordinate pH_i-sensitive processes across the myocardium. Outside this range, gap junctions prevent the spread of the local pH_i disturbance and sarcolemmal transporters are recruited remove the excess acid or base from the pH-disturbed cell. The concepts of H^+-shuttles and their permeation across gap junctions add a new dimension to our understanding of pH_i regulation and may offer new insight into disease conditions in which pH_i changes are a characteristic feature.

This work is supported by the British Heart Foundation and the National Institutes of Health.

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Monocarboxylate (lactate) transporters in the heart – role and regulation

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Glycolysis is greatly stimulated in the ischaemic/hypoxic heart as ATP production by oxidative metabolism becomes impaired. The end product of glycolysis, lactic acid, must leave the cell if the intracellular pH is not to drop. This process is mediated by members of the monocarboxylate transporter (MCT) family - monocarboxylate/proton symporters with a broad substrate specificity that includes L-lactate, pyruvate and the ketone bodies acetate, acetoacetate and β-hydroxybutyrate. Hence MCTs may also be used to import such substrates as metabolic fuels for the heart under normoxic conditions. There are fourteen members of the MCT family, but only MCTs 1-4 have been shown to transport lactate, each with subtly different kinetics that fit the different requirements of the tissues in which they are expressed. The major MCT isoform in heart is MCT1, but some species may also express lower amounts of the higher affinity MCT2 or the lower affinity MCT4. However, the latter isoform is expressed strongly in neonatal heart cells, whose energy metabolism is more glycolytic than adult heart cells, and is up-regulated by hypoxia through a Hypoxia Inducible Factor 1α (HIF-1α) mediated increase in gene transcription. By contrast, MCT1 is up-regulated in response to increased work load by mechanisms involving both transcriptional and post-transcriptional mechanisms. MCT1 expression is confined to the plasma membrane and is not detected in Percoll-purified mitochondria that possess a separate mitochondrial pyruvate carrier.

MCTs require a single transmembrane ancillary protein, with extracellular immunological domains, for expression and function at the plasma membrane. This can be either Basigin or Embigin, the contribution of each depending on the species. Organomercurials inhibit lactate transport into cardiomyocytes by binding to a reactive thiol group on basigin, with residual inhibitor-insensitive transport reflecting the MCT1-embigin complex. Very recently, a novel class of MCT1 specific inhibitors (K_i values ~ 1nM) have been described and the effects of these on lactate transport into heart cells and on the haemodynamic behaviour of the heart under normoxic conditions and following ischaemia/reperfusion will be described.

This work was supported by the British Heart Foundation.

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pH_i-regulatory proteins as therapeutic targets in cardiac disease: focus on NHE1

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The cardiac sarcolemmal Na^+/H^+ exchanger (NHE) is a membrane glycoprotein encoded by the ubiquitously expressed NHE1/SLC9A1 gene, which is one of nine currently known members of the solute carrier 9 (SLC9) gene family. Based largely on experiments with selective pharmacological inhibitors, NHE1 activity has been identified as a causal or permissive factor in the inotropic and growth responses of myocardium to neurohormonal and mechanical stimuli, and in the development of myocardial injury during ischaemia and reperfusion and myocardial hypertrophy and remodeling during haemodynamic overload. Data from recent clinical trials with cariporide, a selective

NHE1 inhibitor, support earlier pre-clinical work and indicate an important role for NHE1 activity in myocardial injury during ischaemia and reperfusion in humans. However, serious adverse effects preclude therapeutic application of the treatment modality that was tested. Improved understanding of the molecular signaling mechanisms that regulate NHE1 activity in healthy and diseased myocardium may lead to the development of new approaches to its therapeutic manipulation, in a cardiac- and/or disease-specific manner. Recent studies in isolated myocytes indicate that extracellular signal regulated kinases 1 and 2 (ERK1/2) and their downstream effector, the 90 kDa ribosomal S6 kinase (p90RSK), play key roles in mediating increased sarcolemmal NHE activity in response to diverse stimuli, such as prolonged intracellular acidosis, oxidative stress and stimulation of Gq protein-coupled receptors. Interestingly, recent data suggest that stimulation of some Gi protein-coupled receptors (e.g. adenosine A1 receptors) inhibit the increase in sarcolemmal NHE activity that arises from stimulation of Gq protein-coupled receptors, such as α1-adrenoceptors. It appears that the dynamic regulation of NHE1 kinase and phosphatase activities and localization underlies much of the acute receptor-mediated regulation of sarcolemmal NHE activity, and investigations are ongoing to determine the molecular mechanism(s), including the critical phosphorylation sites, that are responsible. Future therapeutic approaches may target attenuation of increased myocardial NHE1 activity in response to pathological stimuli, rather than the global inhibition of ion transport by this ubiquitously expressed protein.

The author's work has been supported by the British Heart Foundation and the Medical Research Council.

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