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Symposium: Stress, disease and Ca²⁺ management: the cardiovascular challenge.

Chair: Lea M D Delbridge & David Saint

Cardiac SR Ca^{2+} release channels and adrenergic stimulation

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Adrenergic stimulation of the heart involves phosphorylation of many intracellular Ca^{2+} handling proteins including the ryanodine receptor Ca^{2+} release channels (RyRs) in the SR. It is known that RyRs can be phosphorylated at three serine residues at 2808, 2814 and 2030 (Huke & Bers, 2008) and that phosphorylation of RyRs *via* PKA causes an increase in RyR activity cardiomyocytes. However, little is known about how phosphorylation of RyRs alters their regulation by intracellular Ca^{2+} and our aim was to explore this physiologically important question.

In our experiments, RyRs were isolated from rat hearts, which had been rapidly removed, perfused with Krebs buffer in a Langendorff apparatus. One group of hearts was perfused with 1 μmol/l isoproterenol (β1- and β2-adrenergic agonist) and the other group without (control) and immediately snap frozen in liquid N_2 in order to capture their state of phosphorylation. SR vesicles containing RyRs were isolated from the heart tissues as previously described for sheep heart (Laver *et al.*, 1995). The buffers used for RyR isolation also contained 20 mmol/l NaF to prevent dephosphorylation of RyRs by endogenous phosphatases. This approach allowed the RyRs to be phosphorylated by the physiological signalling processes resulting from adrenergic stimulation of cardiomyocytes. RyRs were incorporated into artificial planar lipid bilayers and their activity was measured using single channel recording in the presence of a range of luminal and cytoplasmic [Ca^{2+}]. Western Blots were used to determine RyR phosphorylation state.

Adrenergic stimulation of rat hearts caused an increase in heart rate from 278±16 to 460±35 (n=6) which was sustained for 1 min prior to freezing. This stimulation caused an increase in phosphorylation at S2808 without any change at S2814 and S2030. The activity of RyRs from isoproterenol stimulated hearts (ISO RyRs, n=25) was 3-fold higher than control RyRs (n=24) at diastolic [Ca^{2+}] (100 nmol/l) but was not significantly different at systolic [Ca^{2+}] (>1 μmol/l). At diastolic [Ca^{2+}], addition of Protein Phosphatase1 (PP1, 5 min) reduced the activity of ISO RyRs by 98 ± 2.6% (n=4) and control RyRs by 70 ± 20% (n=4) but this treatment had no effect at systolic [Ca^{2+}]. ISO RyRs displayed a 100-fold channel-to-channel variation in activity which was larger than, and encompassed, the range of activity seen for control RyRs and PP1 treated RyRs. A subpopulation of ISO RyRs (13 of 25) were typical of control RyRs hearts and another, excited subpopulation (8 of 25), had 10-fold higher opening rates.

The effects of adrenergic stimulation on RyR2 regulation by cytoplasmic and luminal Ca^{2+} were accurately fitted by a model based on a tetrameric RyR structure with four Ca^{2+} sensing mechanisms on each subunit (Laver, 2007; Laver & Honen, 2008). Phosphorylation did not alter the ion binding affinities for these sites. Rather, it increased channel opening rate and decreased the channel closing rate associated with Ca^{2+} binding to the cytoplasmic and luminal activation sites.

The results indicate that: 1) Adrenergic stimulation causes a rapid increase in phosphorylation at S2808; 2) which increases RyR2 activity during diastole but not during systole; 3) RyRs show large channel-to-channel variations in activity most likely as a result of varying degrees of phosphorylation at S2808; and 4) adrenergic stimulation increases the proportion of phosphorylated RyRs in the SR. The increase in RyR2 activity will contribute to an increase in the frequency of the SR Ca^{2+} uptake-release cycle which in turn generates the increased heart rate seen during exercise and stress.

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Cardiac ischemic stress: Ca^{2+} and sex scenarios

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Important sex differences exist in cardiovascular heart disease, and much of this differential is cardiac specific. Pre-menopausal women are protected from ischemic heart disease compared with age-matched men, but prevalence increases steadily post-menopause. There is growing awareness of the extent to which cardiac function can be influenced by sex and sex hormones, however the fundamental mechanisms responsible for these sex differences are not well understood. Female and male cardiomyocytes exhibit markedly different calcium (Ca^{2+}) handling characteristics which reflect the influences of endogenous levels of sex steroids on myocyte Ca^{2+} transport mechanisms. Experimental studies show that, compared with males, female myocytes operate on a relatively low Ca^{2+} cycling load, with Ca^{2+} entry through L-type channels reduced and sarcoplasmic reticulum Ca^{2+} cycling downregulated. Overall, diastolic and systolic Ca^{2+} operational levels are higher in male myocytes – with endogenous estrogen and testosterone playing reciprocal regulatory roles in maintaining this difference. Ca^{2+} is a major causative factor in many of the pathologies associated with ischemia/reperfusion, including arrhythmogenesis, contractile dysfunction and multiple forms of cardiomyocyte death. Ca^{2+} overload triggers hypercontracture and activates calpain, leading to sarcolemmal rupture and a loss of cell integrity. It also promotes mitochondrial Ca^{2+} loading, causing the mitochondrial permeability transition pore to open. Subsequent mitochondrial swelling leads to cytochrome c release and caspase-mediated apoptosis. With more severe ischemic insults, an uncoupling of the mitochondria depletes ATP levels and necrotic injury occurs. Evidence suggests Ca^{2+} also triggers autophagy, though whether this is responsible for ischemia-induced autophagy is yet to be resolved. Limiting Ca^{2+} loading in ischemia/reperfusion substantially improves post-ischemic outcomes. The extent of Ca^{2+} overload is partly mediated by the actions of Ca^{2+}/calmodulin-dependent protein kinase (CaMKII). Responsive to fluctuations in Ca^{2+}, CaMKII functionally modulates many ion channels and transporters within the cardiomyocyte. Hence, an initial rise in Ca^{2+} levels during ischemia activates CaMKII, augmenting Ca^{2+} entry and increasing intracellular Ca^{2+}. Male only studies have shown that inhibiting CaMKII during ischemia/reperfusion reduces Ca^{2+} overload and attenuates apoptotic and necrotic cardiomyocyte death. We hypothesized that the lower operational levels of Ca^{2+} in female cardiomyocytes may limit the influence of CaMKII in ischemia/reperfusion injury and mediate the cardioprotection afforded to female hearts. We have recently shown CaMKII-mediated injury in simulated ischemia/reperfusion is attenuated in female myocytes. CaMKII inhibition (KN93) markedly enhanced male myocyte survival after a simulated ischemic event, but had only marginal effects on the more resilient female myocytes. Further studies will discern the fundamental mechanisms of this sex differential and how it may be modulated in complex disease settings (cardiac hypertrophy, diabetes).

Cardiomyopathies: When is Ca^{2+} the culprit?

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The coordinated contraction and relaxation of the heart that allows it to function as a pump arises from transient changes in the concentration of Ca^{2+} within the myocytes. Influx of Ca^{2+} during the cardiac action potential triggers release from the intracellular Ca^{2+} store (the sarcoplasmic reticulum, or SR), rapidly increasing the cytosolic [Ca^{2+}] ~10-fold. Ca^{2+} then activates cross-bridge cycling, and force production, by binding to the regulatory sites on troponin C. Relaxation takes place when the [Ca^{2+}] returns again to resting levels as it is removed from the cytosol by two principle transport mechanisms: re-uptake into the SR by the Ca^{2+}-ATPase (SERCA2a); and transport across the sarcolemma (SL) by the Na^{+}/Ca^{2+} exchanger (NCX). Given the key role of Ca^{2+} in the mechanical activity of the heart, it is not surprising that Ca^{2+} mis-handling is often implicated in cardiomyopathies where force production is compromised. However, many other changes also occur in hearts *en route* to failure, such as extracellular matrix remodelling and increased ß-adrenergic stimulation. The impact of these changes on a beat-to-beat basis remains unclear, particularly since most studies only examine force and Ca^{2+} during steady-state, or single beat responses. In this study, we utilised an animal model of hypertensive failure to gain insights into Ca^{2+} homeostasis in the recovery from non-steady-state interventions in isolated left ventricular preparations.

Measurements of isometric force and $[Ca^{2+}]_i$ were made at 37°C in left ventricular trabeculae from failing spontaneously hypertensive rat (SHR) hearts, and their normotensive Wistar-Kyoto (WKY) controls. At 1Hz, peak stress was reduced in SHR (14.5 ± 2.4 mN mm^{-2} *versus* 22.5 ± 6.7 mN mm^{-2} for WKY), although the Ca^{2+} transients were bigger (peak $[Ca^{2+}]_i$ 0.60 ± 0.08 μM *versus* 0.38 ± 0.03 μM for WKY) with a slower decay of fluorescence (time constant 0.105 ± 0.005 s *versus* 0.093 ± 0.002 s for WKY). Two experimental protocols were used to potentiate force as a probe of dynamic Ca^{2+} cycling: (i) an interval of 30s rest, and (ii) a 30s train of paired-pulses, and the recirculation fraction (RF) calculated for recovery to steady-state. No difference was found between rat strains for RF calculated from either peak force or Ca^{2+}, although the RF was dependent on potentiation protocol. Since SR uptake is slower in SHR, the lack of change in RF must be due to a parallel decrease in trans-sarcolemmal Ca^{2+} extrusion. This view was supported by a slower decay of caffeine-induced Ca^{2+} transients in SHR trabeculae. Confocal analysis of LV free wall showed t-tubules were distorted in SHR myocytes, with reduced intensity of SERCA2a and NCX labelling in comparison to WKY.

Defining the roles of Ca^{2+} entry in endothelin-1 and thromboxane A_2 receptor mediated vascular contractile responses

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Introduction: Ca^{2+} is an important mediator of vascular contractility, which can enter the cytosol through voltage-gated L- and T-channels and intracellular SR Ca^{2+} release. Upon depletion of the SR Ca^{2+} store, Ca^{2+}-release activated Ca^{2+} (CRAC) channels, which are composed of plasma membrane bound Orai1 and SR-bound STIM1, form functional channels, allowing store refilling. Although endothelin-1 (ET-1) and thromboxane A_2 are both potent vasoconstrictors implicated in various vascular disease states, they mediate Ca^{2+} entry and vasoconstriction through uniquely different mechanisms.

Aim and Method: To identify the role of IP3 receptors, CRAC-, L- and T-channels in ET-1 and thromboxane A_2-mediated vasoconstriction. Using an *in vitro* rat artery model, functional vascular myography coupled with patch clamp analysis were used to identify the activation and inhibition of Ca^{2+} entry pathways mediated by agonists and pharmacological inhibitors of ion channels, respectively.

Results: Brief sequestration of extracellular Ca^{2+} using EGTA (5mM) revealed that approximately 20% ET-1-mediated vasoconstriction involved IP3-mediated SR Ca^{2+} ($p<0.05$; n=4). Following SR Ca^{2+} depletion using cyclopiazonic acid (10mM) (a SERCA pump inhibitor) and 2-aminoethyl diphenyl borate (100mM) (which is known to block IP3 receptors, CRAC channels and potentially non-selective cation channels), vascular contractility was abrogated ($P<0.05$; n=4), indicating a role for both IP3 receptors and CRAC channels. Blocking extracellular Ca^{2+} entry using combined L-/T-channel blockers, mibefradil (1mM) ($p<0.05$; n=7) and efonidipine (0.021mM) ($p<0.05$; n=13) attenuated approximately 65% ET-1-mediated vasoconstriction in the microvasculature (Ball *et al*,, 2009). Patch clamp analysis of I_{CRAC} has revealed that in addition to blocking L- and T-channels, both mibefradil and efonidipine also inhibited CRAC channels. In contrast, thromboxane A_2-mediated vasoconstriction only involved Ca^{2+} entry through L-channels and RhoA-Rho kinase Ca^{2+}-independent sensitization and does not involve IP3 receptors, T or CRAC channels.

Conclusion: In the microvasculature, ET-1 mediates Ca^{2+} entry *via* L, T, IP3 receptors and CRAC channels. In contrast to the traditional L-type Ca^{2+} channel blockers, the more recently developed combined L-/T-channel blockers may provide additional benefit through blockade of CRAC channels, which may effectively enable clinical modulation of SR Ca^{2+} release.

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