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578-Pos Board B457
Voltage-Dependent Modulation of Cardiac Ryanodine Receptors (RyR2) by Protamine
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Early studies showed that protamine (>20μg/ml) inhibits skeletal RyR1 regardless of the cytosolic Ca^{2+} levels (Koulen et al., Mol. Biol. Cell. 11:2213–9, 2000). We extended these studies to cardiac RyR2 reconstituted into planar lipid bilayers. We found that protamine (0.2μg/ml) added to the cytosolic surface of RyR2 decreased channel activity. This effect did not depend on cytosolic Ca^{2+} levels or on the presence/absence of agonists. The action of protamine was complex and involved transition to several substates as well as full block of the channel. Substates did not seem to represent protamine binding to a ryanoid site as they were observed in ryanodine-modified channels. At Vm > 0mV (SR lumen - cytosol) block events were rare and only the substate with the highest level of conductance had high probability. As expected, increasing Vm from 0 to +80mV, decreased the apparent on-rate of protamine-induced substate and increased its off-rate. Unexpectedly, the I-V relationships for the full openings and for the protamine-induced substate were parallel (i.e., the current amplitude drop induced by protamine had the same magnitude at 0 and +80mV). This is not the case with most known conductance modifiers, which induce a drop in the current amplitude that changes proportionally with the amplitude of the full opening. In contrast, the modifications in the RyR2 conduction pathway induced by protamine (conformational changes or partial occlusion of the pore) heavily depended on the electric field. Analogous electrostatic interactions between neighboring RyRs and/or with associated proteins may play a role in the heterogeneity of RyRs current amplitudes frequently observed in multichannel recordings (Supported by NIH R01 GM078665 to JAC).

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SAM Regulation of RyR2
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RyR2 channel activity is subject to allosteric and posttranslational regulation. S-adenosyl-L-methionine (SAM), the primary methyl group donor for enzyme-mediated methylation of proteins and other biological targets, activates RyR2 via an unknown mechanism. To determine if activation of RyR2 by SAM requires methyltransferase activity, cardiac sarcoplasmic reticulum (CSR) vesicle [^{3}H]ryanodine binding was performed in media containing 150 mM KCl, 20 mM PIPES pH 7.0, 3 μM Ca^{2+}, 1 mM SAM, and 0–1 mM sinefungin, a competitive methyltransferase inhibitor. Sinefungin did not alter CSR vesicle [^{3}H]ryanodine binding, and the SAM-induced increase in [^{3}H]ryanodine binding was not altered by sinefungin. To investigate further whether activation of RyR2 by SAM involves RyR2 methylation, RyR2 was immunoprecipitated from CSR vesicles pretreated for 30 min at 37°C with 3 μCi; 285 nM [^{3}H]SAM plus or minus 500 fold excess cold SAM or 1 mM SAH, an inhibitor of methyltransferase activity, followed by centrifugation through sucrose. Radioactivity incorporated into pretreated CSR and immunoprecipitated RyR2 was determined by liquid scintillation counting. Although the amount of radioactivity incorporated into [^{3}H]SAM pretreated CSR vesicles was reduced from 6.54 to 1.86 and 1.69 pmol [^{3}H]SAM/mg protein in the presence of SAH and excess cold SAM respectively, the amount of radioactivity recovered by immunoprecipitation with anti-RyR was not increased over control (immunoprecipitation without RyR specific antibody). Because SAM contains an adenosine moiety and may activate RyR2 via interaction with the channel's adenine nucleotide binding site(s), the affects of SAM and ATP on RyR2 activity were compared. The SAM and ATP concentration dependence of CSR vesicle [^{3}H]ryanodine binding virtually overlapped with no differences at any concentration tested. This work suggests SAM does not methylate RyR2 and the similarities between ATP- and SAM-induced RyR2 activation support allosteric regulation of RyR2 by SAM.

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Targeted Stabilisation Of The RyR2 I-Domain Restores Ca^{2+} Handling And Intercellular Synchrony In Ouabain-disrupted Cardiac Cell Monolayers
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Stabilisation of cardiac ryanodine receptors (RyR2) has emerged as an important approach for normalising Ca^{2+} cycling dysfunction in cardiac disease. Here, we targeted the I-domain of human RyR2, a region critically involved in RyR2 intramolecular rearrangement following channel activation. The I-domain is a hot-spot for arrhythmia-linked mutations that destabilise the activated channel. Putative functional motifs within the I-domain were mapped using a bioinformatics approach and the synthesised using an in vitro expression system. The efficacy of affinity-purified fragments in normalising Ca^{2+} handling and intercellular coupling was screened using a cardiac-cell model of ouabain-induced dysynchrony. Under defined experimental conditions, ouabain perturbs normal intracellular Ca^{2+} cycling and ablates the synchronous intercellular coupling in super-confluent HL-1 monolayers. A 146 amino acid fragment, termed ID^B, normalised ouabain-induced Ca^{2+} dysfunction and resulted in the re-synchronisation of Ca^{2+} transients across the monolayer. In the same ouabain-disrupted model , bacterially-synthesised ID^B increased the extent of intercellular synchrony to levels greater than those measured in naïve (non-ouabain treated) spontaneously contractile HL-1 cells. The fortuitous presence of overlapping, truncated recombinant fragments that spanned the entire ID^B sequence and co-purified with intact ID^B from bacterial culture may have contributed to these effects. Importantly, ID^B-mediated normalisation of Ca^{2+} handling and intercellular synchronisation within the ouabain-treated monolayer extended to distant cell populations that had not been transduced with the recombinant protein. In experiments using cyan-yellow bio-engineered RyR2 (CYBER) probes that report intra-RyR2 conformational rearrangement, we showed that the ID^B-mediated stabilisation of the RyR2 channel directly correlated with the normalisation of ouabain-induced Ca^{2+} dysfunction. Our data provides evidence that a specific epitope-targeting strategy can stabilise RyR2 and that this approach may have remarkable therapeutic utility in normalising channel abnormalities associated with acquired and genetic cardiac disease.

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The Effect of Volatile Anaesthetics on the Cardiac Ryanodine Receptor
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Although volatile anaesthetics serve a crucial role in preventing pain they continue to have side effects such as their ability to excite Ca^{2+} release from the sarcoplasmic reticulum (SR) via the ryanodine receptor calcium release channels (RyR). Here we report the first detailed investigation on the effects of volatile anaesthetics on the function of cardiac RyRs.
RyRs were isolated from sheep hearts and incorporated into artificial lipid bilayers and subjected to single channel recording. Clinical doses of halothane and isoflurane increased RyR activation by luminal and cytoplasmic Ca^{2+} by increasing channel open time and opening frequency. The K_a's for halothane and isoflurane were 1 mM and 3 mM, respectively. However, the maximal effect of halothane (5-fold increase in $placeP_o$) was ~3-fold larger than that for isoflurane. These agents activated RyRs by interacting with cytoplasmic domains distinct from the ATP activating sites.
The effects of halothane on RyR regulation by luminal and cytoplasmic Ca^{2+} and Mg^{2+} were accurately fitted by a luminal-triggered calcium feedthrough model involving four Ca^{2+} sensing mechanisms on each RyR subunit; two activation sites (luminal *L*-site, 40 μM affinity; cytoplasmic *A*-site, 1 μM affinity) and two cytoplasmic inactivation sites (I_1-site, 10 mM affinity; I_2-site, 1 μM affinity). Halothane did not appear to 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 two activation sites.
The potentiating effect of halothane on luminal Ca^{2+} activation of cardiac RyRs was due to 1) an increase on opening frequency because of synergistic actions of the luminal and cytoplasmic (L and A) sites and 2) an increase in open time because of Ca^{2+} feedthrough to the A-site.

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Iron (II) Modulation of the Cardiac Ryanodine Receptor (RyR2)
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Cardiomyopathies and arrhythmias are major causes of mortality in chronic iron overload. There is evidence that iron overload impairs cardiomyocytes Ca^{2+} homeostasis (Baptista-Hon *et al*, 2005). However its molecular substrates remain unknown. Cardiac ryanodine receptors (RyR2) dysfunction is implicated in several diseases where Ca^{2+} homeostasis is lost. We therefore wanted to investigate RyR2 role as a potential target for iron-induced cardiomyopathies.
We isolated heavy sarcoplasmic reticulum (HSR) vesicles containing RyR2 from sheep hearts. RyR2 were reconstituted into L-α-phosphatidylethanolamine (PE) bilayers. Unitary currents were measured under voltage-clamp with 250mM Cs^+ as the charge carrier and 10μM activating Ca^{2+}. High affinity [^{3}H]ryanodine binding of the native vesicles was detected by liquid scintillation counting. Non-specific binding determined by incubations with 100x cold ryanodine.
Fe^{2+} reduced RyR2 open probability and conductance in a dose dependent manner. Lifetime analysis revealed 5 shut times components and 3 open times components in control. Fe^{2+} caused an extra-shut component. Furthermore, there

was a dose dependent shift in the open time constants towards the faster components. The binding assays revealed a [Fe^{2+}] dependent, co-operative reduction in [^{3}H]ryanodine binding to HSR vesicles. Preliminary data of [^{3}H]ryanodine binding in increasing [Ca^{2+}] showed a rightward shift in the presence of Fe^{2+}.
The results presented here show for the first time that Fe^{2+} is a potent inhibitor of RyR2. The mechanism of this inhibition may be due to competition with Ca^{2+} for RyR2 activation sites. Suppression of RyR2 activity by Fe^{2+} may therefore be one of the mechanisms involved in iron-induced cardiomyopathies.
References
Baptista-Hon, D, Díaz, M. E., and Elliot, A. C. Acute exposure to iron (II) alters calcium handling in isolated rat ventricular myocytes. Journal of Molecular and Cellular Cardiology 39, 179. 2005.

583-Pos Board B462
Increased Expression of Ryanodine Receptors and the Iron Transporter DMT1 in Hippocampal Neurons by Brain Derived Neurotrophic Factor (BDNF), NMDA or Spatial Memory Training
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Ryanodine receptors (RyR) mediate skeletal and cardiac muscle contraction and amplify via CICR postsynaptic calcium signals generated by activity-dependent calcium influx through NMDA receptors (NMDAR) in hippocampal glutamatergic synapses. We have recently shown that reactive oxygen species (ROS) and iron, which as shown here promotes ROS generation in neurons, stimulate RyR-mediated calcium release when added to primary hippocampal neurons. Here, we report that 5 min incubation of hippocampal cells in primary culture with NMDA (50 μM), induced RyR-mediated calcium signals that were inhibited by pre-incubation with the iron chelator desferroxamine. Incubation with NMDA also enhanced >2-fold the expression (measured 24 h later) of the iron transporter DMT1 (IRE form), while incubation with BDNF (50 ng/ml) increased >5-fold RyR expression. Additionally, we investigated if spatial memory training of male rats in a Morris water maze affected RyR and DMT1 expression. The hippocampus was dissected 6 h after the last behavioral task (5d, 2d rest, 1d platform free) and samples from tissue were prepared for Western blot and RT-PCR experiments. We found that spatial memory training increased the mRNA and protein expression of DMT1, RyR2 and RyR3. Our results confirm enhanced RyR2 expression following spatial memory training and correlate for the first time enhanced in vivo expression of the iron transporter DMT1 and RyR3 with spatial memory acquisition/consolidation. We propose that iron-induced ROS production stimulates the emergence of RyR-mediated intracellular calcium signals that promote RyR and DMT1 expression during the spatial memory task.
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Increased Levels Of Type 2 Ryanodine Receptor (RyR2) In Rat Heart Mitochondria During Diabetes
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Diabetes reduces myocardial contractility. Studies attribute this defect in part to a dysfunction of cardiac mitochondria. However, molecular mechanisms responsible for mitochondrial dysfunction during diabetes remain incompletely defined. The present study was designed to determine whether diabetes alter expression and activity of cardiac mitochondrial ryanodine receptor (mRyR). Type 1 diabetes was induced in male Sprague-Dawley rats using streptozotocin (STZ). Two and eight weeks after STZ injection, diabetic rats were sacrificed, hearts harvested, and cardiac mitochondria were purified using differential and Percoll gradient centrifugations. In Western blots, bottom and middle Percoll fractions from control hearts immuno-reacted with VDAC and COX IV, but not with SERCA2 antibodies. These fractions also contained a protein of $M_w \approx 500$kDa that immuno-reacted with RyR2 but not with RyR1 antibodies. Trypsin digestion followed by mass spectroscopic analysis revealed this high molecular weight protein to be RyR2 (mRyR2). After two weeks of diabetes, mRyR2 protein level in bottom fraction increased 1.8 fold, as was total [^{3}H]ryanodine bound (11.9 ± 4.8 fmol [^{3}H]ryanodine bound/mg protein for control *vs* 17.5 ± 2.0 fmol/mg protein for diabetic at 900μM Ca^{2+}). After 8 weeks of diabetes, mRyR2 protein level remained elevated. Interestingly, the activity of mRyR2 as assessed from [^{3}H]ryanodine bound increased 5-fold (from 46.9 ± 8.1 fmol/mg protein in control to 262.7 ± 40.1 fmol/mg protein at 200μM Ca^{2+}). Two weeks of insulin-treatment initiated after 6 weeks of diabetes, normalized expression and activity of mRyR2 to near control values. These data are the first to show mRyR2 expression increases in heart during diabetes. This increase in expression of mRyR2 during diabetes could perturb mitochondrial Ca^{2+} homeostasis resulting in disrupting of ATP production and a reduction in myocyte function. (Supported in part by NIH grants to S-SS and KRB)

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Intracellular Calcium Release Channels Mediate Their Own Countercurrent: The Ryanodine Receptor Case Study
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The ryanodine receptor (RyR) and inositol trisphosphate receptor (IP3R) calcium release channels mediate large calcium release events lasting >5 ms from intracellular calcium storage organelles. For these channels to mediate such a long-lasting calcium efflux, a countercurrent of other ions is necessary to prevent the membrane potential from rapidly (<1 ms) reaching the calcium Nernst potential. A recent model of ion permeation through a single, open RyR channel is used here to show that the vast majority of this countercurrent is likely conducted by the release channel itself. Consequently, changes in membrane potential are minimized locally and instantly, assuring maintenance of a calcium driving force. This auto-countercurrent is possible because of the poor calcium selectivity and high conductance for both monovalent and divalent cations of the calcium release channels. For example, the RyR model suggests that in normal cellular ionic conditions this auto-countercurrent clamps the membrane potential near 0 mV within ~150 micros. Consistent with experiment, this model demonstrates how RyR calcium current is defined by luminal calcium concentration, surrounding permeable ion composition, pore selectivity and conductance. Since the RyR and IP3R channels have homologous pores and permeation characteristics, we predict this will also be true for IP3R-mediated calcium release as well. If so, then auto-countercurrent may be essential to nearly any RyR or IP3R mediated calcium release event observed in cells.

Calcium Signaling Proteins

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Essential Roles for Coiled-coil Domains in STIM1 Oligomerization and CRAC Channel Activation
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The calcium release-activated calcium (CRAC) channel is activated by depletion of Ca2+ from the ER. Store depletion causes the ER Ca2+ sensor, STIM1, to translocate to sites of close ER-plasma membrane apposition, where it interacts with Orai1, the pore-forming subunit of the CRAC channel and activates Ca2+ entry. STIM1 self-associates in resting cells (Baba et al., PNAS 103:16704, 2006) and further oligomerizes after store depletion (Liou et al., PNAS 104:9301, 2007), an event that triggers the self-assembly and activation of STIM1-Orai1 clusters at ER-PM junctions (Luik et al., Nature 454:538, 2008). STIM1 has several protein interaction domains, including a lumenal sterile alpha motif (SAM) and two putative cytosolic coiled-coil regions. The isolated lumenal EF hand - SAM region is known to oligomerize upon Ca2+ removal in vitro (Stathopulos et al., JBC 281:35855, 2006), but the roles of the coiled-coil domains in the functions of STIM1 in situ are not as well understood. Using fluorescence recovery after photobleaching, co-immunoprecipitation, and blue native PAGE analysis on truncated mutants of STIM1 we show that the two coiled-coil domains of STIM1 affect STIM1 oligomerization in different ways. The ER-proximal coiled-coil is sufficient for the self-association of STIM1 in resting cells but does not by itself support oligomerization in response to store depletion. The distal coiled-coil is required for depletion-induced oligomerization. Mutation of specific residues within the predicted hydrophobic interface of the distal coiled-coil prevents the formation of STIM1 puncta and the activation of CRAC channels. These results reveal an essential role for the distal coiled-coil of STIM1 in the oligomerization step that controls store-operated Ca2+ entry.

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Atomic Force Microscopy of Copine I and Annexin A1 on Supported Phospholipid Bilayers: Structure and Synergism
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