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
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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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The time- and calcium-dependent association of recombinant human copine I or annexin A1 with supported lipid bilayers composed of 25% brain PS and 75% DOPC was monitored by atomic force microscopy. Neither protein bound to featureless areas of the bilayer but both rapidly bound to small domains that appeared to be 0.5 to 0.8 nm lower than the rest of the bilayer. These domains may be enriched in PS and/or have a more disordered lipid structure. Copine I assembled into a reticular pattern made of 40nm linear elements that appeared to be one or two molecules high. In vivo such copine arrays might form a scaffold for the assembly of signalling proteins bound by copine I. Annexin A1 did not form ordered structures but appeared to promote the growth of the domains of lowered height to which it was bound. These enlarged domains created by annexin A1 provided binding sites for copine I when it was added subsequently. Therefore, in vivo, annexin A1 might recruit C2 domain-containing proteins like copine to membranes by modulating membrane structure.

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Depletion Of Intracellular Cholesterol Disrupts Carbachol But Not PTH-mediated Ca^{2+} Signals In HEK293 Cells

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In HEK cells stably expressing receptors for type 1 parathyroid hormone (PTH), PTH increases the sensitivity of IP_3 receptors (IP_3R) to IP_3 via a cAMP-dependent mechanism, thereby potentiating Ca^{2+} signals evoked by muscarinic M_3 receptors that stimulate IP_3 formation. The effect of PTH results from cAMP binding directly to a low-affinity site on either the IP_3R itself or a protein tightly associated with it. cAMP appears to pass directly from AC to IP_3R via an association we have termed an AC-IP_3R junction, formed selectively by AC6 and IP_3R2. Here we show disruption of cholesterol-rich lipid microdomains differentially disrupts M_3R signaling in HEK cells.

In the absence of extracellular Ca^{2+}, stimulation of HEK cells with a maximal concentration of carbachol (CCh, 1mM) caused an increase in $[Ca^{2+}]_i$ of 249 ± 33nM which returned to basal within 60–70s. Subsequent addition of PTH, in the continued presence of CCh, evoked further concentration-dependent (EC_{50} = 59 ± 15nM) increases in $[Ca^{2+}]_i$. Treatment with the cholesterol-depleting agent MβCD (2h; 22°C) caused an 86 ± 3% decrease in the response to CCh whilst having no significant effect on the response to PTH. Single-cell imaging revealed that treatment with MβCD caused a 30% decrease in the number of cells responding to CCh and a 45% decrease in amplitude of the Ca^{2+} signal in cells that did respond. Filipin staining of free cholesterol confirmed that MβCD caused depletion of cellular cholesterol.

Depletion of intracellular cholesterol with MβCD disrupts CCh but not PTH signalling in HEK293 cells. We hypothesise this may be due to the existence of either two different M_3R populations in the PM or differential distributions of IP_3R isoforms in the ER.

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Increased Store-Operated Ca^{2+} Entry in Skeletal Muscle with Knockdown of Calsequestrin

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Malignant hyperthermia (MH) is a life-threatening syndrome triggered by volatile anesthetics, in which uncontrolled elevation of myoplasmic Ca^{2+} leads to hypercontracture of skeletal muscle and elevation of body temperature. Our recent study showed that azumolene, an analog of dantrolene used to treat MH, inhibits a component of store-operated Ca^{2+} entry (SOCE) coupled to activation of the ryanodine receptor in skeletal muscle (*JBC* **281**: 33477, 2006). Given our previous observation that overexpression of calsequestrin-1 (CSQ1) suppressed SOCE in skeletal muscle (*JBC* **278**: 3286, 2003), here we tested the hypothesis that reduced CSQ1 expression would enhance an azumolene-sensitive SOCE in this tissue. A shRNA probe specific for CSQ1 (*JBC* **281**: 15772, 2006) was introduced into flexor digitorum brevis (FDB) muscles of living mice using electroporation. Individual transfected FDB muscle fibers labeled with a fluorescent marker were isolated for SOCE measurements using Mn-quenching of Fura-2 fluorescence. At room temperature (20–22°C), SOCE induced by caffeine/ryanodine was significantly enhanced in CSQ1-knockdown muscle fibers (in 10^{-4} ΔF_{360}/s, 9.36 ± 1.31) compared to those transfected with control (4.71 ± 1.29, $p<0.05$). Pre-incubation with azumolene (20 μM) completely inhibited the elevated SOCE detected in CSQ1-knockdown fibers (1.26 ± 0.38, $p<0.01$). To prevent muscle contraction, we used *N*-benzyl-*p*-toluene sulfonamide (BTS, 40 μM), a specific myosin II inhibitor. When temperature of the bathing solution was increased to 40°C, muscle fibers with knockdown of CSQ1 displayed a significant elevation in cytosolic Ca^{2+} over that seen in control fibers. Thus reduced CSQ1 expression is likely coupled to elevation of cytosolic Ca^{2+} due to increased SOCE function at higher temperatures. These results suggest that elevated SOCE activity in skeletal muscle may be linked to the pathophysiology of MH and the heat-sensitivity of MH-susceptible animals.

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Role of the Ryanodine Receptor/Calcium Release Channel in Beta-adrenergic Receptor Blocker Treatment of Heart Failure

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To explore the role of protein kinase A (PKA) phosphorylation of the cardiac ryanodine receptor (RyR2)/calcium release channel in the treatment of heart failure (HF) using beta-adrenergic receptor blockers (beta-blockers) we generated a knock-in mouse with aspartic acid replacing serine at residue 2808 in RyR2 (RyR2-S2808D). This mutation mimics constitutive PKA hyperphosphorylation of RyR2, a condition that occurs during HF. RyR2-S2808D+/+ mice developed an age-dependent cardiomyopathy characterized by moderate cardiac dysfunction and mild left ventricular dilatation indicating that PKA hyperphosphorylation of RyR2 alone can cause cardiac dysfunction. Following myocardial infarction (MI), RyR2-S2808D+/+ mice exhibited increased mortality compared to WT littermates. Treatment with the rycal S107, a 1,4-benzothiazepine derivative that inhibits PKA hyperphosphorylation-induced depletion of calstabin2 from the RyR2 complex, for 4 weeks significantly reduced HF progression in WT and RyR2-S2808D+/+ mice, confirming the important role of calstabin2 binding to RyR2 in preventing HF progression. In contrast, following MI, treatment with the beta-adrenergic receptor blocker (beta-blocker) metoprolol improved cardiac function in WT but not in RyR2-S2808D+/+ mice, indicating the important role of inhibition of PKA hyperphosphorylation of RyR2 as a key mechanism underlying the beneficial effects of beta-blockers in HF. Taken together, these data show that chronic RyR2 PKA hyperphosphorylation alone can cause a cardiomyopathy, preventing calstabin2 depletion from the RyR2 macromolecular complex can inhibit HF progression, and PKA phosphorylation of RyR2 is an important determinant of the therpaeutic efficacy of beta-blocker therapy of HF.

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Structural Basis for Calcium Sensing by GCaMP2

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Genetically encoded Ca2+ indicators are important tools that enable the measurement of Ca2+ dynamics in a physiologically relevant context. GCaMP2, one of the most robust indicators, is a circularly permutated EGFP (cpEGFP)/M13/Calmodulin (CaM) fusion protein, that has been successfully used for studying Ca2+ fluxes in vivo in the heart and vasculature of transgenic mice. Here we describe crystal structures of bright and dim states of GCaMP2 that reveal a sophisticated molecular mechanism for Ca2+ sensing. In the bright state, CaM stabilizes the fluorophore in an ionized state similar to that observed in EGFP. Mutational analysis confirmed critical interactions between the fluorophore and elements of the fused peptides. Solution scattering studies indicate that the Ca2+-free form of GCaMP2 is a compact, pre-docked state, suggesting a molecular basis for the relatively rapid signaling kinetics reported for this indicator. These studies provide a structural basis for the rational design of improved Ca2+-sensitive probes.

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Interference In Coiled-coil Mediated Coupling Between Stim1 And Orai Channels

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STIM1 and ORAI1, the two limiting components in the CRAC signalling cascade, have been reported to couple tightly upon store-depletion culminating in CRAC current activation. Based on the homology within the ORAI protein family, an analogous scenario might be assumed for ORAI2 as well as ORAI3 channels as both are activated in a similar store- and STIM1-dependent manner. A combined approach of electrophysiology and confocal Förster Resonance Energy Transfer (FRET) microscopy revealed a general mechanism in the communication of STIM1 with ORAI proteins that involved the predicted second coiled-coil motif in STIM1 C-terminus and the conserved putative coiled-coil domain in the respective ORAI C-terminus. Of the latter, a much higher coiled-coil probability is predicted for ORAI2 as well as ORAI3 than for ORAI1, compatible with our observation that a single point coiled-coil