$[Ca^{2+}]_r$ of triadin-null cells to levels similar to Wt cells, suggesting either an additional Ca^{2+} entry pathway, insensitive Cd^{2+} and La^{3+}, or an enhanced Ca^{2+} release. Inhibition of Ca^{2+} release by ryanodine resulted in a significant reduction of $[Ca^{2+}]_r$ in triadin-null cells but not in Wt, indicating that triadin-null cell also have increased baseline RyR1 channel activity. Western blot analysis and lipid bilayer studies revealed that calcium channels from triadin-null cells have reduced FKBP-12 binding and increased subconductance states, respectively. Accordingly, over expression of FKBP-12.6 caused a significant reduction in $[Ca^{2+}]_r$ in triadin-nulls but did not affect Wt cells. Overall these data support the idea that elevated resting free calcium levels observed in triadin-null myotubes are the combined effect of at least two things: (i) an increase in calcium entry mediated by Cd^{2+} and La^{3+} sensitive channels, but insensitive to nifedipine, and (ii) an augmented basal SR calcium release as the result of enhanced RyR1 channel activity induced by a deficiency in RyR1/FKBP-12 binding.

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2641-Pos

The Leak State of the RyR1 is Regulated by RyR1/DHPR Interaction, Controlling the Cytosolic Free-Ca^{2+} Concentration and the SR Ca^{2+} Content at Rest

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The sarcolemmal L-type Ca^{2+} channel (DHPR), is known to both positively and negatively regulate RyR1 in skeletal muscle. The mechanism for the negative control is unknown. To assess this we measured resting intracellular Ca^{2+} concentration ($[Ca^{2+}]_{rest}$) using Ca^{2+}-selective microelectrodes in dysgenic (MDG) and Wt myotubes. The $[Ca^{2+}]_{rest}$ in MDG and Wt myotubes were 158 ± 2.5 nM (mean ± SE, n = 9) and 117 ± 2.1 nM (n = 10), respectively ($p<0.001$). Bastadin 5 (B5), which suppresses the leak state of RyR1, decreased $[Ca^{2+}]_{rest}$ to 99 ± 0.7 nM (n=16) and 103 ± 1.3 nM (n=16) in Wt and MDG myotubes, respectively. After FK506 treatment, $[Ca^{2+}]_{rest}$ increased to 139 ± 2.4 nM (n=10), 217 ± 3.0 nM (n=10) in Wt and MDG myotubes, respectively and the addition of B5 in FK506 treated cells had negligible effect: 129 ± 1.8 nM (n=10) and 202 ± 3.5 nM (n=10) respectively. These experiments clearly show that B5 treatment equalizes the $[Ca^{2+}]_{rest}$ in all the three cell types tested while FK506 abolishes its action. To estimate the SR Ca^{2+} content, we measured Ca^{2+} release elicited by 20 mM caffeine, using Fluo5N. The area under the curve of the Fluo5N signal was ~4 times smaller in MDG (25 ± 2.3 arbitrary units (a.u.), n=69) compared to Wt (107 ± 8.5 a.u, n=66) and B5 increased the Ca^{2+} release only in MDG cells: 76 ± 16 a.u. (n=21) MDG vs 101 ± 12 a.u. (n=60) Wt. These data demonstrate that the negative control exerted by the DHPR is at least in part due to the ability of the DHPR to control the percent of RyR1s in the leak state. Supported by NIH/NIAMS R01AR43140 (to PDA and INP)

2642-Pos

Increased Sensitivity of RyR2 to Activation by Ca^{2+} and cADP-Ribose during Diabetes

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In prior studies others and we found an increase in spontaneous Ca^{2+} release in ventricular myocytes isolated from streptozotocin-induced diabetic rat hearts. To date, molecular mechanisms underlying this phenomenon remains poorly defined. This study tests the hypothesis that the sensitivity of RyR2 to be activated by intrinsic modulators is altered during diabetes. Diabetes was induced in male Sprague-Dawley rats using streptozotocin. Ten weeks after injection, diabetic rats were divided into two groups: one group was treated with insulin for two weeks while the other group received no treatment. At the end of this time, cardiac and myocyte functions were assessed using echocardiography and high-speed video edge detection. Evoked Ca^{2+} transients were also assessed using confocal microscopy. Expression of RyR2 was assessed using RT-PCR and Western blots, while RyR2 activity and responsiveness to Ca^{2+}, cADP-ribose and AMP-PCP were assessed using [^{3}H]ryanodine binding and single channel analyses. After 12 weeks of diabetes, ejection fraction and fraction shortening were reduced by >25%. Rate of evoked Ca^{2+} release was slowed as was the time to peak myocyte contraction. Expression of RyR2 and the total amount of [^{3}H]ryanodine bound at 200μM Ca^{2+} were reduced by ≈ 40%. However, the K_d for ryanodine remained essentially unchanged (5.2nM). Interestingly, in [^{3}H]ryanodine binding and single channel assays, diabetic RyR2 was activated to a greater extentby low Ca^{2+} (0.53 μM and 1 μM), AMP-PCP (1mM) and cADP-ribose (1 μM). Two-weeks of insulin, initiated after 10 weeks of diabetes, treatment blunted these changes. These new data indicate that the increase in spontaneous Ca^{2+} release seen in diabetic myocytes stems in part from alterations in the responsiveness of RyR2 to activation by intrinsic ligands. Funded in part by NIH HL085061 and the Nebraska Redox Biology Center.

2643-Pos

Effects of Divalent Current Carriers on Voltage-Dependence of RyR2 Channels

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Voltage-dependent modulation of cardiac ryanodine receptors (RyR2) was studied in planar lipid bilayers. Different earth alkaline cations (M^{2+}: Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}) were utilized as current carriers. When added to the cytosolic surface of the channels, Ca^{2+} (EC_{50} ~2μM) or Sr^{2+} (EC_{50} ~20μM) interacted with cytosolic high affinity (HA) sites and activated the channels. Neither Mg^{2+} nor Ba^{2+} activated RyR2 but only Mg^{2+} interfered with Ca^{2+}/Sr^{2+} action. Fully activated RyR2 (100-200μM cytosolic Ca^{2+}) were inhibited with low affinity (LA) by all M^{2+} (similar IC_{50}, ranging 3.6-5.7mM) suggesting that LA inhibitory sites do not distinguish M^{2+} identity. We found that RyR2 were much more active with lumenal Ca^{2+}/Sr^{2+} versus lumenal Ba^{2+}/Mg^{2+}. However increasing lumenal→cytosolic M^{2+} flux by increasing lumenal holding voltage (Vm) never increased open probability (Po). In contrast, increasing Vm decreased Po in half of the RyR2 exposed to lumenal Ca^{2+}/Sr^{2+} and in all RyR2 exposed to lumenal Ba^{2+}/Mg^2. This suggests that lumenal flux does not reach HA but LA sites. An effect of Vm is evident in all channels displaying modal gating (low and high Po mode) but not in RyR2 where high Po dominates. Indeed, high Po mode is much more abundant with lumenal Ca^{2+}/Sr^{2+}. Moreover, increasing cytosolic Ca^{2+} activated all channels and removed the effect of Vm. In summary, modulation of RyR2 gating by M^{2+} flux is complex and seems to reflect lumenal M^{2+}-dependent stabilization of high Po and low Po mode (the latter being voltage-dependent). Supported by NIH R01 GM078665 to JAC.

2644-Pos

Molecular Cloning and Expression of the Ryanodine Receptor Type 2 (RyR2) from Rat Cerebral Artery Smooth Muscle

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Ryanodine receptors (RyRs) are a family of Ca^{2+} release channels found in intracellular Ca^{2+} storage/release organelles and participate in a variety of important Ca^{2+} signaling phenomena. Here, we set to clone and sequence full-length cDNA encoding the type 2 RyR (RyR2) from smooth muscle cells of *Rattus norvegicus* cerebral arteries. Middle cerebral and basilar arteries were isolated and de-endothelized, and total RNA was purified for RT-PCR. RyR2 cDNA was divided in two parts of similar size; 5' segment: 1-7486 bp, and 3' segment: 7487-14862 bp. Specific primers were designed to obtain both 5' and 3'-terminals. Following insertion of both terminals in the mammalian expression vector pCI-neo, we characterized several clones by restriction analysis, and confirmed the full-length cDNA sequence by automated sequencing. The rat cerebral artery smooth muscle RyR2 cDNA contains 14862 bp and encodes a deduced protein of 4953 amino acids with a M_r of 562451.3 Da. Nucleotide blast analysis indicates that the cerebral artery smooth muscle RyR2 shows 100% identity with recombinant RyR2 cloned from rat cardiac muscle (Accession NM_032078). RyR2 cDNA expression was determined after transfection of HEK293 cells with the insert into the pCMV6-AN-tagGFP vector. Membrane insertion of cerebral artery myocyte RyR2 was determined by immunolabeling with polyclonal antibodies. To our knowledge, this is the first time that a RyR is cloned from rat cerebral artery smooth muscle, its functional characteristics being currently studied. *Supported by Grant AA11560 (AMD).*

2645-Pos

Bacterial Expression of the Ryanodine Receptor Pore Forming Region and a Potassium Channel Chimera

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Several lines of evidence exist to support the proposal that RyR2 contains a pore composed of structural elements analogous to the pore forming regions (PFR) of K+ channels. Our analogy model, constructed using the bacterial

potassium channel KcsA as the template, encompasses transmembrane helices 8-10 and provides an excellent description for the pore domain of RyR2 with the overall structure and arrangement of key structural elements of the model closely resembling those of KcsA (Biophysical Journal 2004, 87, 2335-2351). Although good progress has been made in understanding ion handling capabilities in RyR2 the exact mechanisms remain elusive and controversial. To test and define the analogy model we have constructed and expressed both the PFR of RyR2 alone and a KcsA_RyR2_PFR chimera whereby the 22 residue N-terminal helix and 41 residue C-terminal domain of KcsA have been added by primer extension to the RyR2 PFR region and cloned into a modified pET expression vector containing an N-terminal hexa-His tag. Just as voltage sensor modules are transferable among potassium channels, our chimera will give us information on the functionality and transferability of the RyR2 PFR. Preliminary results indicate that both the chimera and PFR constructs express in large amounts in rosetta bacterial cells and are targeted to the membrane with no detectable protein in the soluble fraction. Detergent trials have identified LDAO as the best candidate for solubilisation from the membrane although other commonly used detergents are also capable of solubilisation, albeit to a lesser degree. Both constructs were purified as tetramers following nickel-affinity and gel filtration chromatography. This suggests strongly that both the RyR2 PFR and chimera are functional proteins capable of tetramerisation.
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2646-Pos
Enhanced RyR1 Channel Activity by the Knock-In Mouse that Expresses Human Malignant Hyperthermia Mutation T4826I
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Malignant hyperthermia (MH) is a life-threatening pharmacogenetic syndrome of skeletal muscle associated with mutations in the Ca^{2+} release channel - ryanodine receptor type 1 (RyR1). A genetically engineered knock-in mouse that expresses the human C-terminal MH mutation T4826I RyR1 was created. Both RyR1-T4826I/WT (HET) and RyR1-T4826I/T4826I (HOM) mice survive and thrive if unchallenged with triggering agents. Sarcoplasmic reticulum membranes from HET, HOM, and wild type (WT) mice were prepared to study the biochemical and biophysical properties of RyR1complexes using [^{3}H]ryanodine ([^{3}H]Ry) binding analysis, western blotting, and single channels incorporated in bilayer lipid membranes. The results from this study reveal: **(1)** Significantly elevated [^{3}H]Ry binding in the preparations with rank order HOM >>HET>WT), **(2)** significant diminution in the inhibitory potency of Ca^{2+} and Mg^{2+} for both HOM and HET, **(3)** initial rates of [^{3}H]Ry binding with rank order HOM>>HET>WT when measured at either 25 °C or 37°C, **(4)** Significantly greater open probability, longer mean open dwelling time and shorter mean closed times with HOM and HET channels. These data indicate that the T4826I RyR1 mutation confers significantly destabilizes the closed channel conformation of RyR1, and the altered channel properties may be mostly responsible for the abnormal intracellular Ca^{2+} homeostasis and the MH susceptibility. (Words: 202).

2647-Pos
AICAR Prevents Heat-Induced Death in Mice with Malignant Hyperthermia
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The pharmacogenetic disorder malignant hyperthermia (MH) is serious and potentially life-threatening illness caused by mutations in the skeletal muscle Ca^{2+}-release channel (ryanodine receptor, RyR1) located in the membrane of the sarcoplasmic reticulum. An MH episode is triggered by general anesthetics, heat and exercise in warm conditions. Signs of an MH crisis are: elevated core temperature, arrhythmias, hyper-metabolism and rhabdomyolysis. The MH mice created in our laboratory with a $Y524S^{+/-}$ mutation in RyR1 ($RyR1^{Y524S+/-}$) die upon exposure to elevated environmental temperatures (37°C) (Chelu et al 2006). The muscle relaxant dantrolene is the only known treatment for an MH crisis, but its adverse side-effects preclude prophylactic usage. We find that the treatment of the $RyR1^{Y524S+/-}$ mice with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an activator of AMP-activated protein kinase (AMPK), prevents heat-induced sudden death. Less than 5% of untreated $RyR1^{Y524S+/-}$ mice survive a heat-challenge, but treatment with AICAR leads to 100% survival. Muscle AMPK phosphorylation ($AMPK\alpha^{Thr172}$) levels are not significantly altered after 10 min subcutaneous injection with AICAR compared to saline. This suggests that the rescue may not be due solely to AMPK activation, but may also involve off-target effect(s) of AICAR. Adenine nucleotides are known channel agonists and AICAR is a precursor of the AMP analog, ZMP. We tested the effects of AICAR on ^{3}H -ryanodine binding in the presence of non-hydrolysable ATP (AMP-PCP). We find that AICAR is a partial agonist of RyR1 and prevents full activation of RyR1 in the presence of cellular concentrations of ATP in both $RyR1^{Y524S+/-}$ and wild-type mice. AICAR prevents heat-induced death in mice with MH and, since it is thought to have few side-effects, it is a potential prophylactic treatment for heat induced death associated with some MH mutations.

2648-Pos
Effects of a Y522S-RyR1 Mutation on Cerebellar Purkinje Cell Function
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The devastating consequences of calcium dysregulation are exemplified by the skeletal muscle diseases malignant hyperthermia (MH) and central core disease (CCD). These diseases result from mutations in the 'skeletal muscle-isoform' of the ryanodine receptor calcium release channel (RyR1). To investigate the etiology of MH and CCD, mouse models have recently been generated (Chelu et al. 2005). Skeletal muscle harboring the Y522S-RyR1 knock-in mutation exhibit Ca^{2+} leak from internal stores, basal cellular stress, and ultimately progressive mitochondrial and cellular damage (Durham et al. 2008). Although the effects of this MH-causing mutation have been characterized in skeletal muscle, the effects in the nervous system have not been documented. RyR1 is expressed highly in cerebellar Purkinje neurons. Y522S-RyR1 mice do not exhibit gross neurological defects suggesting that the defects may be subtle or that neuronal function is spared due to compensatory changes.
We have initiated studies investigating 3 main aspects of Y522S-RyR1 mouse Purkinje cells: intracellular calcium release, cellular organization/morphology, and cellular stress. In preliminary studies using imaging techniques with the calcium indicator dye Fura2-AM, RyR-mediated calcium release in Y522S-RyR1 Purkinje neurons exhibited a negative shift in the apparent EC_{50} for the agonist caffeine. In addition, the sensitivity of RyR to other activators (temperature, voltage, ryanodine) and intracellular calcium store content were investigated. Since calcium signaling is important during neuronal development and maturation, the morphology of Y522S-RyR1 Purkinje cells was examined using immunohistochemistry and confocal microscopy. Moreover, we have initiated studies to determine if altered RyR1 function results in basal cellular stress using Western blot and immunohistochemical analyses. Y522S-RyR1 mutant mice provide an excellent tool to address calcium dysregulation, its pathological consequences, and potential approaches for compensation of altered calcium signaling in the central nervous system.

2649-Pos
Distinct Properties of CPVT Mutations Located in the Central Domain of Human RyR2
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The cardiac ryanodine receptor (RyR2) Ca^{2+} release channel plays a central role in the rapid calcium release from the sarcoplasmic reticulum that is essential for muscle excitation-contraction coupling. Discrete mutations that have been discovered within the RyR2 associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) are found to cluster in distinct regions of RyR2. These regions may represent important regulatory domains of the molecule that either directly or indirectly can affect channel gating. In response to a physiological trigger, RyR2 mutations are believed to cause diastolic Ca^{2+} leak which results in arrhythmia, making them an important potential therapeutic target.
We have expressed the RyR2 central domain CPVT-associated region and examined parameters which contribute to the structural and functional stability. A wild-type construct was compared with three constructs each containing a different CPVT mutation (P2328S, N2368I or A2387P). Circular dichroism spectroscopy revealed that none of the mutations significantly altered the percentage ellipticity of the protein, indicating that the overall conformation of the polypeptide backbone is only slightly affected by the mutations. Chemical denaturation using guanidine hydrochloride and monitoring tryptophan fluorescence suggested that the P2328S mutation was less stable than the wild-type or the other two mutations, indicated by a lower free energy change upon unfolding. A predicted ATP binding motif has previously been proposed in this domain and using fluorescence spectroscopy for the wild-type construct, ATP binding was observed with a K_d ~0.03mM.