extent with 1.0 μM Ca^{2+} than control RyR2 (P_o was 5X greater in diabetic RyR2). Two weeks of insulin treatment blunted the enhanced Ca^{2+} responsiveness. When added to the *cis* chamber the potent reactive carbonyl species (RCS), 80 μM methylglyoxal increased the open probability (P_o) of RyR2 3-fold (0.05 to 0.16) within 10 min and this increase was independent of holding potential. Increasing [MGO] further to 160 μM, reduced the conductance of RyR2 by 25% without changing P_o. Incubating RyR2 with MGO (5-500 μM with 200 μM free Ca^{2+} in buffer) dose-dependently reduced its ability to binding [3H]ryanodine. Singly mutating R1611, R2190 and K2888 to W or Y, to mimic adducts previously found on them during diabetes, resulted in gain-of-function of RyR2 (P_o increased >2-fold at 3.3 μM Ca^{2+}). Mutating c-terminal R4462, and R4683 to W or Y resulted in loss-of function of RyR2. We conclude that modification of RyR2 by RCS during diabetes is responsible in part for its dysregulation. (This work was funded by NIH and Nebraska Redox Biology Center)

1573-Pos

Gating of the Purified Human Cardiac Ryanodine Receptor (hRyR2) in the Absence of Regulatory Accessory Proteins

Saptarshi Mukherjee, Nia Lowri Thomas, Laila Guzadhur, Chloe E. Maxwell, Alan J. Williams.
Cardiff University, Cardiff, United Kingdom.

The cardiac ryanodine receptor (RyR2) mediates Ca^{2+} efflux from intracellular stores to effect myocyte contraction during excitation-contraction coupling. Mutations in this channel perturb Ca^{2+} release function, leading to triggered arrhythmias that may cause sudden cardiac death (SCD). The exact molecular mechanisms by which SCD-linked RyR2 dysfunction occurs constitutes a burgeoning area of cardiac research. Most studies so far have concentrated on the secondary effects of mutation on channel function by virtue of affecting channel modification by phosphorylation and accessory protein binding, with no great emphasis on elucidating the gating mechanisms of the channel itself. Our aim is to elucidate the mechanistic basis of wild-type (WT) hRyR2 activation by its primary activating ligand, Ca^{2+}, under precisely controlled conditions in the absence of any accessory proteins with a view to determining the effect of mutation on hRyR2 gating in the same way. hRyR2 channels, recombinantly expressed in HEK293 cells, were purified and studied at the single channel level in symmetrical 210mM KCl under reducing conditions. *Trans* (luminal) Ca^{2+} was buffered at 50nM using EGTA, while *cis* (cytosolic) Ca^{2+} buffering was stringently controlled using EGTA, HEDTA and NTA to achieve free Ca^{2+} concentrations in the range of 0-500 μM. Preliminary data obtained from sigmoidal dose-response curves of P_o vs pCa for 10 WT hRyR2 channels yields an EC_{50} of 3.25 ± 1.04 μM, resulting in a maximum P_o greater than 0.8 (in 5 out of 10 channels). This increase in P_o resulted from an increase in the frequency of channel openings, until P_o of 0.8 - above which any increases in P_o resulted from an increase in open times. Likely gating mechanisms will be discussed with a view to mutant channel analysis.
Supported by the British Heart Foundation

1574-Pos

S-Adenosyl-L-Methionine Activation of Cardiac Ryanodinr Receptors is Associated with an Increased Frequency of Subconductance Sates

Angela J. Kampfer, Edward M. Balog.
Georgia Institute of Technology, Atlanta, GA, USA.

The biological methyl group donor, S-adenosyl-L-methionine (SAM) activates the cardiac ryanodine receptor (RyR2). Previously we investigated the mechanism underlying SAM regulation of RyR2 with [^{3}H]ryanodine binding to cardiac SR vesicles. SAM enhances Ca^{2+} -activation of RyR2 and increases the apparent affinity of ryanodine for the channel. Notably, methyltransferase inhibitors have no effect on SAM-activation, and SAM-mediated methylation of RyR2 is not detected. Furthermore, the concentration dependence for SAM and ATP-induced increase in [^{3}H]ryanodine binding overlap. Presently, we investigated the affect of SAM on native RyR2 channels incorporated into planar lipid bilayers. Channel were grouped according to initial P_o values under control conditions (10 μM cytosolic Ca^{2+}), those with $P_o < 0.2$ (n=7), and $P_o > 0.2$ (n=5). For channels with an initial $P_o < 0.2$, SAM caused a rapid (within seconds) increase in P_o ($p < 0.05$). The SAM-induced increase in P_o was due primarily to an increase in mean open time ($p<0.05$; n=3). Interestingly, SAM activation was associated with an increased frequency of subconducatance states. In contrast, the increase in channel P_o caused by 2mM ATP was not associated with the appearance of subconductance states. Thus, the effect on subconductance states appears specific to SAM. This work highlights the complexity underlying SAM regulation of RyR2. The data suggest ligand binding is among the multiple mechanisms responsible for SAM-activation of RyR2.

1575-Pos

Molecular Interplay between the Heart Lim Protein (HLP) and RyR2 in Murine Heart

Dong Woo Song, So Yeon Park, Do Han Kim.
Gwangju Institute Science and Technology, Gwangju, Korea, Republic of.

HLP is a heart-specific LIM-only protein having two LIM domains each consisting of two zinc fingers. Through the bacterial 2 hybrid screening and a following LC-MS/MS study, we have found that HLP interacts with the cytosolic divergent region of mouse heart RyR2. The direct interaction between RyR2 and HLP was confirmed by GST pull-down and co-immunoprecipitation assays. HLP was partially co-localized with RyR2 in HL-1 cells and rat adult cardiomyocytes. siRNA or Adenovirus-mediated knock-down of HLP in HL-1 cells and neonatal cardiomyocytes led to more than 70% decrease in the expression of HLP, without a concomitant change of other Ca^{2+} handling proteins (e.g. SERCA, RyR2, calsequestrin and DHPR). Ca^{2+} transient measurement of fura2-loaded cardiomyocytes by 1Hz field stimulation demonstrated that silencing of HLP decreased the peak amplitude of Ca^{2+} transient (~ 15%) in HL-1 cells and in neonatal cardiomyocytes. Currently, various deletion-mutants of LIM protein are being used to characterize the RyR2 binding sites in HLP. (This work was supported by the Korean Ministry of Science and Technology grant, Systems Biology Research Grant, M1050301001-6N0301-0110, and the 2009 GIST Systems Biology Infrastructure Establishment Grant).

1576-Pos

Mitsugumin-29 Regulates RyR1 Activity In Mouse Skeletal Myotubes

Ji-Hye Hwang, Jin Seok Woo, Eun Hui Lee.
The Catholic Univ. of Korea, Seoul, Republic of Korea.

Canonical-type transient receptor potential cation channel type 3 (TRPC3) in plasma membrane allows the entry of Ca^{2+} ions into various cells. In skeletal myotubes, functional interaction between TRPC3 and RyR1 (ryanodine receptor1, a Ca^{2+} channel in sarcoplasmic reticulum (SR) membrane) regulates the gain of skeletal excitation-contraction coupling (*J. Biol. Chem*., 2006). Mitsugumin-29 (MG29) is a four membrane-spanning protein and is found in both plasma and SR membrane. MG29 has been known as a TRPC3-interacting protein in skeletal myotubes (*Biochem. J.*, 2008).
To identify critical region(s) of MG29 that participate in binding to TRPC3 or the role of MG29 in skeletal muscle, N-terminus, three intervenient loops among four transmembrane regions, and C-terminus of MG29 were expressed in E. coil as N-terminal GST-fused forms, and subjected to co-immunoprecipitation assay with intact TRPC3 from rabbit skeletal muscle. Cytoplasmic N-terminus and a loop between first and second transmembrane domains of MG29 effectively bound to TRPC3. Two deletion mutants of MG29 (missing the TRPC3-binding sites: deleting the N-terminus only or longer N-terminus covering the loop region) was expressed in mouse skeletal myotubes, and the myotubes was subjected to the measurement of Ca^{2+} transients with Fura-2 or Fluo-4. The later mutant showed significantly decreased responsiveness of RyR1 to caffeine, suggesting that MG29 may be a mediator between the functional interaction between TRPC3 and RyR1.

1577-Pos

Phosphorylation of Excitation-Contraction Coupling Components in a Guinea-Pig Model of Heart Failure

Mark L. Bannister[1], Mark Scoote[2], Martyn P. Kingsbury[1], Mark A. Turner[1], Jolanda van der Velden[3], Desmond J. Sheridan[1], Alan J. Williams[4], Sian E. Harding[1].
[1]Imperial College, London, United Kingdom, [2]Colchester Hospital University, Colchester, United Kingdom, [3]VU University Medical Centre, Amsterdam, Netherlands, [4]Wales Heart Research Institute, Cardiff, United Kingdom.

Phosphorylation status appears to be a key determinant of excitation-contraction coupling ion channel and pump function. Dysfunction of the ryanodine receptor (RyR) secondary to catecholaminergic drive and phosphorylation has been proposed as a factor in contractile dysfunction and arrhythmia patho-physiology in the failing heart. The phosphorylation states of RyR, along with those of phospholamban and troponin I have been investigated by immunoblotting, and quantitated by comparing levels in failing hearts with basal levels, minimum levels after beta-blocker treatment and maximal levels achieved by ex vivo treatment with isoprenaline. We found that RyR residue Ser2809 was phosphorylated to 124 ± 11 % ($n = 5$, $P > 0.05$) of control (sham-operated, basal) in heart failure under basal conditions and 143 ± 12 % ($n = 6$, $P < 0.05$) with isoprenaline treatment, and residue Ser2030 was 94 ± 10 % ($n = 8$, $P > 0.05$) for heart failure and 199 ± 9 % (n=6, $P < 0.05$) for isoprenaline treatment. Phosphorylation levels at Ser16 of phospholamban were higher: 159 ± 17 % (heart failure, $n = 7$, $P < 0.05$) and 366 ± 95 % (isoprenaline treatment, $n = 5$, $P < 0.05$). At Ser23/24 of troponin I there is no significant

change in heart failure ($n = 5$, $P < 0.05$) but a 230 ± 92 % increase with isoprenaline treatment ($n = 6$, $P < 0.05$). Basal levels of phosphorylation are thus relatively low at RyR Ser2030, phospholamban Ser16, and troponin I Ser23/24, and are not significantly increased in heart failure, but are substantially increased by isoprenaline treatment. In contrast, the phosphorylation level at Ser 2809 is already high and can be increased only moderately by isoprenaline.

1578-Pos

Inhibition of RyR2-S2814 Phosphorylation Prevents Heart Failure by Reducing SR Ca Leak

Ralph J. van Oort, Na Li, Jonathan L. Respress, Angela DeAlmeida, **Xander H. Wehrens**.
Baylor College of Medicine, Houston, TX, USA.

Abnormal regulation of RyR2 by Ca2+/calmodulin-dependent protein kinase 2 (CaMKII) has been suggested as a cause of sarcoplasmic reticulum (SR) Ca2+ leakage and contractile dysfunction in heart failure. We hypothesized that CaMKII phosphorylation of RyR2 is crucial for heart failure development. We generated RyR2 knockin mice in which CaMKII phosphorylation site S2814 was mutated to alanine (S2814A) to prevent phosphorylation. Cardiac function and dimensions monitored by echocardiography were similar in S2814A and WT mice up to 12 months of age. WT (n=13) and S2814A mice (n=9) were subjected to transverse aortic constriction (TAC) to induce pressure overload. At 8 weeks after TAC, S2814A mice displayed a similar hypertrophic response and decrease in ejection fraction (EF) as WT mice. At 16 weeks after TAC, however, EF was significantly lower in WT (32.5 +/- 3.4%) compared to S2814A mice (43.0 +/- 2.9%) suggesting inhibition of heart failure development in the latter. This rescue effect was further verified by a lower lung-weight-to-tibia-length ratio and a decrease in expression levels of the cardiac stress genes ANF and BNP in S2814A mice compared to WT mice at 16 weeks after TAC. Ca2+ imaging in cardiomyocytes, isolated from S2814A and WT mice at 16 weeks after sham or TAC surgery, demonstrated an decreased incidence of spontaneous SR Ca2+ release (SCR) events in S2814A (29.7% of myocytes) compared to WT (61.3% of myocytes; $P<0.01$). Whereas CaMKII inhibitor KN93 reduced the incidence of SCR events in WT to 36.1% ($P<0.01$ vs. WT TAC), KN93 did not have an effect of SCR in S2814A myocytes (30.0%; P=NS vs. S2814A TAC). Together, our results demonstrate that blocking CaMKII phosphorylation of RyR2 prevents SR Ca2+ leak, and inhibits or delays the progression to congestive heart failure in S2814A mice.

1579-Pos

Luminal Regulation of Single RyR2 Channels by Cardiac Calsequestrin

Marcia Cortes-Gutierrez[1], Heather R. Orrell[1], Simon Williams[2], Patricio Velez[1], **Ariel L. Escobar[1]**.
[1]UC Merced, Merced, CA, USA, [2]TTUHSC, Lubbock, TX, USA.

We examined the hypothesis that calsequestrin (CSQ2) can regulate the RyR2 activity using a combination of single channel electrophysiology and lanthanide resonance energy transfer (LRET). Under steady-state, open probability (Po) of RyR2 of cardiac SR fractions from dog is modulated by luminal [Ca2+]. Po increased when luminal [Ca2+] was increased from 6 μM to 2 mM at a fixed cytosolic [Ca2+] of 2 μM. This effect on RyR2 appears to be mediated by luminal sites. Interestingly, RyR2 Po also increases when 2 mM Mg2+ was added to the luminal side. To gain mechanistic insights on the Casq2-mediated luminal regulation, we used the binding of Casq2 to Tb+3 as a functional assay. Luminescence produced by LRET between the tryptophan's of purified dog Casq2 and the lanthanide increased as function of the [Tb+3]. This fluorescence was reduced as [Ca2+] increased suggesting that Ca2+ binds to purified Casq2 by displacing tightly bound Tb+3 from a common binding site. To further explore the specificity of this regulation, we expressed recombinant dog Casq2 in E coli. The specificity of this interaction was assessed by LRET lifetime measurements. Interestingly, we found that the displacement of Tb3+ by Ca2+ was not significantly different than the displacement of Tb3+ by Mg2+ (~ 2.5 mM) suggesting that Ca2+ and Mg2+ share a common binding site. Finally, we explore the hypothesis that Tb3+ bound to Casq2 was able to modulate the RR2 activity. At fixed cytosolic [Ca2+] of 2 μM, Po of single RyR2 increased as a function of luminal [Tb+3 (KD ~ 500 nM, Hill coeff. ~4). These results are consistent with the idea that a multimeric form of Casq2 acts as luminal divalent cation sensor and translates it into changes in RyR2 gating. Supported by NIH R01-HL-084487 to AE.

1580-Pos

Ca SR Leak is Modulated by CaMKII Phosphorylation on RyR2-S2814

Yi Yang[1], **Laetitia Pereira[1]**, Ralph J. van Oort[2], Xander H.T. Wehrens[2], Donald M. Bers[1].
[1]UC Davis, Davis, CA, USA, [2]Baylor College of Medicine, Houston, TX, USA.

CaMKII has been shown to increase cardiac SR Ca leak through RyRs. RyR2-S2814 has been suggested as the phosphorylation site responsible for SR Ca leak triggering cardiac arrhythmias in heart failure. Here we test the requirement of S2814 for these effects, in knock-in mice expressing only RyR2-S2814A or -S2814D. Ca spark frequency (CaSpF) and SR load were studied in intact and permeabilized cardiomyocytes using confocal microscopy. At baseline CaSpF is higher in S2814D vs. WT (8.7 ± 0.4 (n=9) vs 6.44 ± 0.3, n=8, $p<0.01$) without altered SR Ca load. Activation of endogenous CaMKII (1.2 μM Ca-calmodulin) in WT increases CaSpF as described by Guo et al (Circ. Res, 2006), but only to the level seen in S2814D at baseline. Moreover, CaMKII did not further increase CaSpF in S2814D myocytes. In RyR2-S2814A CaMKII activation produces a very small CaSpF increase vs WT (19 vs 60%), and that response in S2814A is secondary to increased SR Ca load (by 15%, n=10, $p<0.01$). In intact myocytes (as above), basal CaSpF was highest in S2814D vs. WT and S2814A (which were similar). Baseline Ca transients were not different among the groups. The cAMP activated GTP exchange factor Epac may activate CaMKII to induce SR Ca leak (Pereira et al, J Physiol, 2007). In WT mice the Epac activator 8-CPT enhanced CaSpF and decreased both SR Ca load and Ca transient amplitude (consistent with a primary effect on SR Ca leak). However, 8-CPT had no effect on any of these parameters in either S2814A or S2814D myocytes. These data indicate that RyR2-S2814 is the critical RyR2 site responsible for CaMKII-dependent enhancement of SR Ca leak and potential arrhythmogenesis in heart failure, and confirm that Epac signaling can work through this same pathway.

1581-Pos

RyR2 NH2-terminal Mutations Associated with Cardiomyopathies Reduce the Threshold for Ca2+ Release Termination

Yijun Tang.
Department of Physiology and Pharmacology, University of Calgary, CALGARY, AB, Canada.

Naturally occurring mutations in the cardiac Ca2+ release channel/ryanodine receptor (RyR2) have been linked not only to cardiac arrhythmias and sudden death, but also to cardiomyopathies. The causal mechanisms underlying RyR2-associated cardiomyopathies are unknown. We have previously shown that RyR2 mutations linked to catecholaminergic polymorphic ventricular tachycardia (CPVT) reduce the threshold for store overload induced Ca2+ release (SOICR), also known as spontaneous Ca2+ release during Ca2+ overload. To determine the impact of RyR2 mutations associated with cardiomyopathies, we generated stable, inducible HEK293 cells expressing the RyR2 wt and the exon-3 deletion, R420W, and L433P mutants, and monitored the luminal Ca2+ dynamics in these wt and mutant cells using the fluorescence resonance energy transfer (FRET)-based luminal Ca2+ sensing protein, D1ER. Consistent with other CPVT mutations, the exon-3 deletion, R420W, and L433P mutations reduce the SOICR threshold. Interestingly, we found that these mutations also lower the critical luminal Ca2+ level at which Ca2+ release is terminated (the termination threshold). To further assess the role of the NH2-terminal region of RyR2 in Ca2+ release termination, we deleted the first NH2-terminal 305 amino acid residues and found that this NH2-terminal deletion also lowers the termination threshold for Ca2+ release. Our data demonstrate that the NH2-terminal region of RyR2 plays an important role in Ca2+ release termination, and suggest that alterations in the termination of Ca2+ release via RyR2 may cause cardiomyopathies (Supported by NIH and CIHR).

1582-Pos

Modulation of Neuroscretory Granule Mobilization and Neuropeptide Release by Ryanodine Receptors in Neurohypophysial Terminals

Jose R. Lemos, Edward Custer, James McNally, **Sonia Ortiz-Miranda**.
Univ. of Massachusetts Medical School, Worcester, MA, USA.

The neuropeptides oxytocin (OT) and vasopressin (AVP) are contained in large dense core vesicles (LDCV) and are released at the neurohypophysis (NH). The mobilization and translocation of vesicles to exocytotic release sites is modulated by cytosolic Ca^{2+}. Intracellular structures and organelles able to store and release Ca^{2+} are significant contributors of cytosolic Ca^{2+}. The presence of ryanodine receptors (RyR) in LDCV of NH terminals, coupled with the demonstration that pharmacological activation of these receptors can induce spontaneous focal Ca^{2+} transients, make them ideal modulators of cytosolic levels of Ca^{2+}, and therefore, vesicle mobilization and subsequent neuropeptide (NP) release.

To test this hypothesis, the association of LDCV in an area within 0.45 μm of the plasma membrane was assessed using immunolabeling of Neurophysin I (OT) and II (AVP) along with high stringency deconvolution techniques in isolated NH terminals. We found that the total amount of membrane associated NP-immunoreactivity varies significantly between terminal type; significantly higher in OT than in AVP terminals. This membrane associated distribution