provide the relationship between the distribution of amplitudes in focus and in xyt images (xyt-to-xyzt correction), thus unifying the three possible modes of spark sampling. The relative merits and shortcomings of the three modes will be discussed from this unified viewpoint.
Funded by NIAMS, NHLBI and NCRR (NIH).

3035-Pos Board B140
Isoproterenol Widens the Source of Release Flux Underlying Ca Sparks
Demetrio J. Santiago, Eduardo Rios, Thomas R. Shannon.
Our previous work [Biophys. J. 98(10):2111-20; Biophys. J. 98(3):102a] suggested that the diastolic ryanodine receptor (RyR) mediated leak (Jleak) from the sarcoplasmic reticulum (SR) of intact ventricular myocytes occurs in spark and non-spark forms. We further showed that the fraction of spark-mediated Jleak increases upon isoproterenol treatment in intact rabbit ventricular myocytes, suggesting that the effective sensitivity to cytosolic Ca is increased by RyR phosphorylation [Biophys. J. 98(3):102a]. We now present an extension of this work, focused on aspects of individual sparks taken from cells at matched [Ca] and SR load. Events were wider in isoproterenol (8.5% greater FWHM of the F/F0 profile) but had similar amplitudes than control. A backward reconstruction of the release flux density, when applied to average sparks assumed to be spherically symmetric, rendered a source that was wider for the isoproterenol event, indicating the recruitment of peripheral RyRs. A forward release flux reconstruction which recapitulates the steps of spark formation could not simultaneously fit the amplitudes and sizes of any of the two average sparks when using realistic radii for the junctional SR. This result may be interpreted as implying the existence of RyRs, peripheral to and perhaps outside the couplon. Compounded with the increased CICR sensitivity upon isoproterenol treatment (see above), the greater spark width of isoproterenol events may increase the probability of Ca wave generation.

3036-Pos Board B141
Activation of Calcium Sparks in Resting Cardiomyocytes by β-Adrenergic Stimulation May Involve CaMKII and nNOS
Daniel Gutierrez, Jakob Ogrodnik, Ernst Niggli.
It has been reported that during β-adrenergic stimulation of cardiac myocytes, phosphorylation of Ca^{2+} release channels (ryanodine receptors, RyRs) by PKA and/or CaMKII may result in arrhythmogenic diastolic Ca^{2+} leak (as elementary Ca^{2+} release events, Ca^{2+} sparks) from intracellular Ca^{2+} stores (the sarcoplasmic reticulum, SR). Using confocal Ca^{2+} imaging, we have recently shown that β-adrenergic stimulation by 1 μM isoproterenol (ISO) increases the Ca^{2+} spark frequency several-fold in quiescent, whole-cell voltage-clamped guinea-pig myocytes, without altering SR Ca^{2+} content. As this occurs without variations of the diastolic intracellular Ca^{2+} concentration, this observation suggests a sensitization of the RyRs. Experiments with protein kinase inhibitors (KN-93 and H89) indicated an involvement of CaMKII in the change of spark frequency. Surprisingly, but in line with the kinase inhibitor experiments, increasing cAMP production and PKA activity by direct stimulation of adenylate cyclase with forskolin (1 μM) did not significantly elevate Ca^{2+} spark frequencies under the same experimental conditions. Further experiments revealed that the change in sensitivity of the RyRs upon β-adrenergic stimulation may be linked to nitric oxide (NO), as pre-incubation of the cells with the NOS inhibitor L-NAME (500 μM) prevented the increase of the Ca^{2+} spark frequency without dramatic changes of SR Ca^{2+} content. Using the nNOS specific inhibitor AAAN (100 μM) resulted in analogous observations, suggesting that the nNOS isoform, located in close proximity of the RyRs, may be involved in this signaling pathway. Taken together, the results suggest the presence of a non-classical pathway linking β-adrenergic stimulation of cardiac myocytes to enhanced activity of the RyRs. Preliminary pharmacological evidence indicates that the pathway includes both, CaMKII and nNOS as important components. Supported by SNF.

3037-Pos Board B142
B-Adrenergic Stimulation Accelerates Local Recovery of Cardiac Ca^{2+} Release
Ona Liu, Hena Ramay, Eric A. Sobie.
In cardiac myocytes, Ca^{2+} sparks terminate reliably and exhibit time-dependent refractoriness after termination. Compelling evidence suggests that dynamic local changes in SR [Ca^{2+}] play an important role in the control of these processes. We examined Ca^{2+} spark refractoriness by exposing fluo-3 loaded quiescent rat ventricular myocytes to 50 nM ryanodine, recording Ca^{2+} sparks with a confocal microscope, and analyzing the repeated sparks that were produced at a limited number of ryanodine receptor (RyR) clusters. Previous experiments showed that altering RyR sensitivity (caffeine or tetracaine) influenced the time between consecutive sparks but did not affect the recovery of spark amplitude (time constant = ~100 ms in all cases). Here we examined repeated Ca^{2+} sparks after application of 100 nM isoproterenol to determine how β-adrenergic stimulation influences spark restitution. Isoproterenol dramatically decreased the median interval between consecutive sparks (192 ms vs. 280 ms in control) and led to faster recovery of Ca^{2+} spark amplitude (time constant = 58 ms). Mechanisms underlying these results were explored through simulations with an established mathematical model of the Ca^{2+} spark. Simulations showed that faster SR refilling led to earlier triggering of Ca^{2+} sparks due to the greater flux of Ca^{2+} through each open RyR, but this effect was insufficient to explain the experimental data. The results could be reproduced if we assumed that isoproterenol both increased the rate of local SR refilling and increased RyR sensitivity. Together, our results indicate that β-adrenergic stimulation influences both: 1) Ca^{2+} spark amplitude recovery, through changes in the time course of local SR refilling; and 2) Ca^{2+} spark triggering, through changes in both refilling and RyR sensitivity.

3038-Pos Board B143
Beta-Adrenergic Stimulation Increases the Intra-Sarcoplasmic Reticulum Ca Threshold for Spontaneous Ca Waves
Timothy L. Domeier, Joshua T. Maxwell, Lothar A. Blatter.
Beta-adrenergic signaling induces positive inotropic effects on the heart that frequently associate with spontaneous arrhythmogenic Ca release events including Ca waves. It remains unclear if the greater incidence of Ca waves is due to increased sarcoplasmic reticulum (SR) Ca content ([Ca]SR) or a change in the function of ryanodine receptors. To address this controversy we utilized dynamic [Ca]SR measurements (fluo-5N) to test if beta-adrenergic stimulation alters the [Ca]SR level where Ca waves initiate (wave threshold) during rest after action potential stimulation. Under control conditions [Ca]SR was progressively increased to the wave threshold via incremental increases in pacing frequency in a high extracellular Ca (7 mM) environment. In the presence of the beta-adrenergic agonist isoproterenol (ISO, 1 microM) [Ca]SR increased and Ca waves were observed. When [Ca]SR was subsequently lowered using low extracellular Ca (1 mM) and SERCA inhibition (3 microM cyclopiazonic acid), Ca waves were no longer observed, even at [Ca]SR levels above the control wave threshold. In parallel experiments we found that resting cytosolic [Ca] (indo-1) was similar between the respective experimental conditions. Indirect assessment of [Ca]SR using the amplitude of the cytosolic Ca transient induced by 10 mM caffeine confirmed our observation that in the presence of ISO Ca waves only occur when [Ca]SR is above the control wave threshold. Furthermore, spontaneous Ca spark measurements (fluo-4) showed a tendency towards spark inhibition in the presence of ISO at experimentally matched [Ca] SR. Together, these data show that acute beta-adrenergic stimulation increases the [Ca]SR threshold for Ca waves, and therefore the primary cause of Ca waves is the robust increase in [Ca]SR above this higher threshold level. Elevation of the [Ca]SR wave threshold may be interpreted as a protective mechanism against pro-arrhythmogenic Ca release during beta-adrenergic stimulation.

3039-Pos Board B144
β-Adrenergic Receptor Stimulation of ROS Production Generates Spontaneous Ca^{2+} Waves in Rabbit Ventricular Myocytes
Elisa Bovo, Stefan R. Mazurek, Stephen L. Lipsius, **Aleksey V. Zima**.
Stimulation of β-adrenergic receptors (β-AR) leads to positive inotropic effects, but also can generate pro-arrhythmogenic spontaneous Ca^{2+} waves. We investigated the role of reactive oxygen species (ROS) production in the generation of Ca^{2+} waves during β-AR stimulation in rabbit ventricular myocytes. In electrically stimulated myocytes, isoproterenol (ISO; 0.1 μM) increased Ca^{2+} transient amplitude during systole, sarcoplasmic reticulum (SR) Ca^{2+} load and the occurrence of spontaneous Ca^{2+} waves during diastole. These effects, however, developed at different time points during ISO application. While SR Ca^{2+} release and load reached maximum after 3 min, Ca^{2+} waves did not appear until 6-12 min after ISO application. Measurements of intra-SR free Ca^{2+} ($[Ca^{2+}]_{SR}$) with Fluo-5N showed an initial increase of SR Ca^{2+} load from 0.9 to 2.1 mM followed by a gradual decline to 1.4 mM after 12 min of ISO application. This decline of $[Ca^{2+}]_{SR}$ was not due to decreased SERCA activity, but instead was the result of increased SR Ca^{2+} leak in the form of Ca^{2+} waves. SR Ca^{2+} leak, measured as a decline of $[Ca^{2+}]_{SR}$ after SERCA inhibition, was increased by 30% after 6-12 min of ISO application. Moreover, ISO significantly increased ROS production. ROS scavenger Tiron and superoxide dismutase mimetic MnTBPA abolished the ISO-mediated ROS production. Tiron (10 mM) or MnTBPA (20 μM) significantly decreased the occurrence of Ca^{2+} waves during ISO application and partially prevented ISO-mediated SR Ca^{2+} leak, but did not affect ISO-mediated increase in SR Ca^{2+} load or Ca^{2+} transient amplitude. ROS donor t-butyl peroxide (100 μM) elicited Ca^{2+} waves that were dependent on elevated SR Ca^{2+} load. These results demonstrate that β-AR-mediated ROS production acts in

conjunction with elevated SR Ca^{2+} load to generate spontaneous Ca^{2+} waves in rabbit cardiomyocytes.

3040-Pos Board B145

Regulation of Sarcoplasmic Reticulum [Ca^{2+}] during Rest in Rabbit Ventricular Myocytes

Elisa Bovo, Aleksey V. Zima.

During diastole, ryanodine receptor Ca^{2+} release channels are not completely quiescent, thus providing a pathway for significant sarcoplasmic reticulum (SR) Ca^{2+} leak. Cytosolic Ca^{2+} can be pumped back into the SR by the SR Ca^{2+}-ATPase (SERCA) or extruded by Na^{+}-Ca^{2+} exchanger (NCX). Therefore, the activity of Ca^{2+} transport systems during diastole plays a critical role in setting SR Ca^{2+} load under normal conditions and in disease states. Using confocal microscopy, we studied mechanisms that control intra-SR free Ca^{2+} ($[Ca^{2+}]_{SR}$) at rest in rabbit ventricular myocytes. We compared the rate of $[Ca^{2+}]_{SR}$ decline (with Fluo-5N) after rest from electrical pacing in control conditions and after SERCA inhibition with thapsigargin (TG; 10 μM). We found that the rate of $[Ca^{2+}]_{SR}$ decline increased only ~30% after SERCA blockade compared to control conditions (from 10.9 in control to 14.1 μM/s in the presence of TG). Similar results were obtained by measuring the rate of decline of total SR Ca^{2+} content, estimated from caffeine-induced Ca^{2+} transient amplitude (with Fluo-4). Inhibition of NCX by Ni^{2+} (5 mM) or by 0 $[Na^{+}]$/0$[Ca^{2+}]$ solution significantly slowed $[Ca^{2+}]_{SR}$ decline during rest (by 3.4 times), but did not prevent it. Simultaneous inhibition of NCX with 0 $[Na^{+}]$/0$[Ca^{2+}]$ solution and plasmalemmal Ca^{2+} ATPase with La^{3+} (1 mM) completely prevented $[Ca^{2+}]_{SR}$ decline during rest. These results indicate that in rabbit ventricular myocytes the predominant mechanism for cytosolic Ca^{2+} removal during rest is NCX but not SERCA-mediated Ca^{2+} uptake. These data are compatible with a model in which the majority of SR Ca^{2+} leak occurs through clusters of ryanodine receptors in the junctional SR that closely oppose NCX in the dyadic cleft.

3041-Pos Board B146

Increased Myofilament Ca^{2+} Sensitivity Decreases Sarcomere Length and Increases Spark-Spark Interactions

Ye Chen-Izu, Tamas Banyasz, Shaden Khabbaz, Stephanie Edelmann, Charles Payne, Jil C. Tardiff, **Leighton T. Izu**.

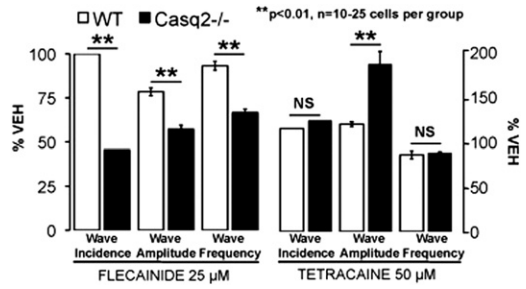
People with familial hypertrophic cardiomyopathy (FHC) harboring mutations of cardiac troponin T (cTnT) are often at a high risk of sudden cardiac death. Transgenic mice harboring some of these cTnT mutations show increased myofilament sensitivity to Ca^{2+} and also shortened diastolic sarcomere length (SL). Our computational studies predicted that decreasing the distances between Ca^{2+} release units (CRUs) of the sarcoplasmic reticulum (SR) by decreasing SL can destabilize the Ca^{2+} control system and increase the probability of spontaneous Ca^{2+} waves. Destabilization results from enhanced crosstalk between neighboring CRUs. In this study we mimic the greater myofilament Ca^{2+} sensitivity conferred by cTnT mutations using the myofilament Ca^{2+} sensitizer EMD 57033 (EMD). At concentrations up to 3 μM, EMD had no effect on either the peak Ca^{2+} transient or the diastolic Ca^{2+} levels and did not alter the SR Ca^{2+} load. To test the prediction that SL shortening increases the coupling between CRUs, we loaded myocytes with Di8-ANEPPS and Fluo-4 and simultaneously measured SL and Ca^{2+} sparks in 2 spatial dimensions using the Zeiss 5 Live high-speed 2-D scanning confocal microscope. EMD (1.5 μM) decreased SL significantly compared to the control cells in normal Tyrode (1.58 μm vs.1.69 μm, $p<0.05$). The spark coupling strength measures the influence of one CRU on another and is derived from an analysis of the spatiotemporal distribution of Ca^{2+} sparks. EMD treatment significantly increased the spark coupling strength 2.5 fold. The enhanced spark-spark coupling as diastolic SL decreases may contribute to increased frequency of spontaneous Ca^{2+} waves during diastole that can lead to triggered arrhythmias and sudden cardiac death in FHC.

3042-Pos Board B147

RyR2 Channel Activity Determines the Potency of State-Dependent RyR2 Blockers for Suppressing Arrhythmogenic Calcium Waves

Eleonora Savio Galimberti, Bjorn C. Knollmann.

Mutations in ryanodine receptors (RyR2) or calsequestrin (casq2) cause catecholaminergic- polymorphic ventricular tachycardia (CPVT). We previously reported that the RyR2 open-channel blocker flecainide (FLEC) suppresses Ca2+ waves and prevents CPVT in mice and humans. Here we test the hypothesis that the open-state block by FLEC significantly contributes to FLEC efficacy in CPVT. We reasoned that FLEC would preferentially affect myocytes lacking casq2 (casq2-/-), which have higher rates of spontaneous RyR2 channel openings compared to WT channels. To test this hypothesis, we compared FLEC with tetracaine, a RyR2 channel blocker that has no state dependence and binds equally well to closed RyR2 channels. We found that FLEC reduced the incidence, amplitude and frequency of Ca2+ waves with significantly higher potency in casq2-/- myocytes compared to WT myocytes (Figure). In contrast, tetracaine did not suppress Ca2+ waves and had equal potency in WT and casq2-/- myocytes (Figure). Conclusion: RyR2 channel activity likely determines the potency of open-state RyR2 blockers such as FLEC for suppressing arrhythmogenic Ca2+ waves, a mechanism likely relevant to FLEC antiarrhythmic efficacy in CPVT. NIH HL88635 & HL71670.



3043-Pos Board B148

FKBP12.6 'Stabilises' Cardiac SR Ca^{2+}-Release by Antagonising High-Affinity Reversible Activation of RyR2 by FKBP12

Elena Galfrè, Mano Sitsapesan, Samantha J. Pitt, Elisa Venturi, Stephen O'Neill, Rebecca Sitsapesan.

FKBP12.6 is thought to play an important cardioprotective role, however, the underlying mechanism is not understood. Since FKBP12 is structurally similar to FKBP12.6 but is found at much higher levels (1-3 μM), we investigated the effects of both FKBP12 and FKBP12.6 on RyR2 single-channel function and on SR Ca^{2+}-release in rat isolated permeabilised cardiomyocytes. FKBP12 increased RyR2 open probability (Po) in a concentration-dependent, reversible manner (EC_{50} 51 nM). Physiological levels of FKBP12 (3 μM) increased Po from 0.187 ± 0.051 to 0.657 ± 0.111 (SEM; n=14; $P<0.001$). FKBP12.6 (200 nM), itself, did not significantly alter RyR2 Po, but was a very effective antagonist of FKBP12, shifting the FKBP12 EC_{50} to 4 μM. In permeabilised myocytes perfused with Fluo-5F, spontaneous waves of Ca^{2+}-induced Ca^{2+}-release were induced by 234 nM Ca^{2+} in the mock cytosolic solution. Perfusion with FKBP12 (3 μM) increased wave frequency from 0.34 ± 0.04 Hz to 0.52 ± 0.07 Hz (SEM; n=14; $p<0.03$). 10 mM caffeine produced a larger Ca^{2+}-transient in control (2.21 ± 0.11; F/Fo) than in FKBP12 (1.47 ± 0.16; n=6; $p<0.003$) indicating lower SR Ca^{2+}-content. Perfusion with FKBP12.6 (200 nM) alone, had no significant effect yet it reduced the ability of FKBP12 to increase wave frequency (49.9 ± 5.8% increase over control in the absence of FKBP12.6 vs. 16.2 ± 2.1% in the presence). Our single-channel experiments demonstrate that FKBP12 is a high affinity, potent activator of RyR2. FKBP12.6 acts as an antagonist of FKBP12 at RyR2 but itself possesses minimal efficacy. Our cellular experiments suggest that this is the underlying mechanism by which FKBP12.6 acts to 'stabilise' or reduce SR Ca^{2+}-release in cardiac cells. Thus, the balance between the opposing actions of FKBP12 and FKBP12.6 on RyR2 gating may be crucial for normal EC-coupling in cardiac cells.

British Heart Foundation Funding

3044-Pos Board B149

Increased Levels of Micrornas miR-1 and miR-133 in Failing Heart Underlie Dissociation of Phosphatase Activity from RyR2 Complex Resulting in Enhanced RyR2 CaMKII-Dependent Phosphorylation and Cardiac Arrhythmias

Andriy E. Belevych, Sarah E. Sansom, Radmila Terentyeva, Mickey M. Martin, Cynthia A. Carnes, Terry S. Elton, Sandor Gyorke, **Dmitry Terentyev**.

Increased propensity of ventricular myocytes to arrhythmogenic spontaneous SR Ca release and afterdepolarizations in heart failure (HF) has been linked to abnormally high activity of RyR2. Growing evidence supports hyperphosphorylation of RyR2 at the CaMKII site S-2814 as a potential mechanism for altered RyR2 function. However, the specific molecular mechanisms underlying RyR2 hyperphosphorylation remain poorly understood.

MicroRNAs are small noncoding RNAs that regulate protein expression by interfering with mRNAs of target genes. We recently reported that 2-fold overexpression of microRNA miR-1 enhances CaMKII-dependent RyR2 phosphorylation by disrupting protein phosphatase 2A scaffolding to the RyR2, resulting in increased activity of the channel and Ca-dependent afterdepolarizations in myocytes. In the present study, we used a canine model of nonischemic HF to test the hypothesis that the HF-related alterations in RyR2 phosphorylation levels are caused by a decrease in phosphatase activity localized to RyR2 due to enhanced expression of two most abundant muscle-specific microRNAs miR-1 and miR-133. qRT-PCR studies revealed that the