TRPM2 is activated by irradiation (IR) and $Ca^{2+}$ entry via this channel contributes to irreversible loss of salivary gland function (Liu et al. 2013, Nat. Commun., http://dx.doi.org/10.1038/ncomms2526). Here we have examined the possible mechanism(s) involved in long-term disruption of salivary gland cells. IR of HSG cells caused an increase in mitochondrial reactive oxygen species (mtROS) which remained elevated up to 24 hours post-IR. The elevation in mtROS was attenuated by removing extracellular $Ca^{2+}$ during IR or by blocking the mitochondrial permeability transition pore (MPTP) with cyclosporin A (CsA) or suppression of mitochondrial $Ca^{2+}$ uniporter (MCU) with siMCU. Consistent with this, the mitochondrial $[Ca^{2+}]$ was significantly increased by IR and this increase was attenuated in cells treated with siMCU or by removing external $Ca^{2+}$ during IR. Additionally, mitochondrial membrane potential ($\Psi m$) was depolarized in irradiated HSG cells during first 2h and gradually returned to normal. IR-induced decrease in $\Psi m$ was blocked by treating cells with CsA, siMCU, or Gd3+. Thus, IR-induced increase in plasma membrane $Ca^{2+}$ permeability, primarily via TRPM2, leads to elevation in $mtCa^{2+}$ and mtROS as well as a decrease in $\Psi m$. Furthermore, IR also induced a time-dependent activation of Caspase 3 and a decrease in cell viability. Treatment of cells with siMCU or Caspase 3 inhibitor, zVAD, conferred significant protection of cell viability up to 96 hours post-IR. Together, our data indicate that mitochondria are likely a major target for IR with mitochondrial disruption and caspase activation leading to irreversible changes in cell function.

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**Diversity of Mitochondrial Ca (2+) Signaling: Evidence from Genetically Encoded Probes**

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It remains somewhat controversial the extent to which mitochondrial $Ca^{2+}$ signaling contributes to cardiac excitation-contraction coupling (ECC). Existing evidence suggests that mitochondria modify ECC only slightly by buffering or storing cytosolic $Ca^{2+}$. However, little direct evidence shows mitochondrial release of $Ca^{2+}$. To examine the mitochondrial $Ca^{2+}$ signaling in cardiac myocytes, we developed and/or used 4 different genetically engineered mitochondrial $Ca^{2+}$ probes based on inverse periCaM or circularly permuted GCamp. All probes carried mitochondrial pre-sequence (MPS) and were virally infected into cultured rat neonatal cardiomyocytes, producing confocal images of fluorescence in characteristic mitochondrial patterns. The ability to detect mitochondrial $Ca^{2+}$ release appeared to depend on Kd of the probe. GCamp6-based probe, Kd of 240 nM, mostly produced strong $Ca^{2+}$ uptake signals but also detected $Ca^{2+}$ release signals in small fraction of mitochondria. The GCamp3-based probe, Kd $\cong$ 1μM, produced mitochondrial $Ca^{2+}$ uptake signals that were transient in response to cytosolic rises of $Ca^{2+}$, suggesting both uptake and release of $Ca^{2+}$. The high-affinity mitycam probe (Kd $\cong$ 200 nM) produced small and very slow fluorescence signals. The mutated mitycam probe (E31Q), Kd $\cong$ 2μM, produced concurrent $Ca^{2+}$ release and uptake signals (albeit with different kinetics) in different mitochondrial populations in response to rise cytosolic $Ca^{2+}$. We conclude that mitochondria not only buffer cytosolic $Ca^{2+}$, but also release $Ca^{2+}$ during caffeine-induced and spontaneously occurring cytosolic $Ca^{2+}$ transients. The ability to detect these releases is compromised unless the Kd of the probe is $\geq$ 1μM, suggesting that $Ca^{2+}$ concentration in mitochondria must reach such levels before rapid release can be initiated.

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**Analysis of ATP Production Efficiency of Beat-To-Beat Calcium Fluctuations in Cardiac Mitochondria**

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Pressure-volume work of the heart increases with increases in rate, stroke volume and afterload. The cardiac $[Ca^{2+}]_i$ transient is a key cellular signal that activates contraction and links electrical activity to the mechanical response of the heart. It also influences the mitochondrial calcium ($[Ca^{2+}]_m$) that is thought to be a major regulator of mitochondrial ATP production by stimulating the three calcium-dependent dehydrogenases of tricarboxylic acid cycle and the $F_1F_0$ ATPase. Here we examine this $Ca^{2+}$ hypothesis of mitochondrial ATP production using a mathematical model of mitochondrial function. However, the magnitude of $[Ca^{2+}]_m$fluctuations resulting from cytosolic $Ca^{2+}$variations is actively investigated by many groups and remains controversial. Our starting position was ourearlier model that examined mitochondrial energy production and ionic homeostasis(Nguyen et al, 2007).We reduced the assumed $Ca^{2+}$ fluxes into and out of the mitochondria so that they were consistent with recent publications from our group (Boyman et al 2014). This revised model thus produced smaller variations of $[Ca^{2+}]_m$ during a beat and was thus consistent with our recent analysis of calcium uniporter fluxes from diverse investigations (Williams et al 2013). Our simulations showed that although the new formulation results in a smaller fractional rise in ATP production during pacing, it showed a 10-fold higher efficiency of ATP production (when normalized tosubstrate entry). The model suggests that this improved efficiency is due to a reduction in mitochondrial membrane potential depolarization due to the $Ca^{2+}$ influx and a reduction in the dissipation of the mitochondrial proton gradient across the inner membrane. This analysis suggests that there is profound energy benefit to the myocardium when mitochondrial ATP production is regulated by small calcium transients in the mitochondria ($[Ca^{2+}]_m$ ) with each heartbeat.

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**In vivo Temperature Sensitivity of the Calcium Affinity of fluo-5F and mag-fluo4**

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The role of calcium ion ($Ca^{2+}$) in diverse cellular signaling pathways has been identified and investigated principally by using fluorescent chelators as indicators. Using the biophysical characteristics of a fluorescent $Ca^{2+}$ indicator allows investigators to convert measured fluorescence intensity into values of free $Ca^{2+}$ concentration ($[Ca^{2+}]$). Troublingly, the characteristics of $Ca^{2+}$-indicators are dependent on numerous environmental factors. Determining the behavior of each indicator *in vivo* under comparable experimental conditions is necessary for quantitative measurements. Temperature is one environmental factor that strongly affects $Ca^{2+}$ indicator behavior. A change in temperature can change the intrinsic physical characteristics of the indicator (e.g., $Ca^{2+}$ affinity, quantum efficiency, and fluorescence lifetime), as well as change the cellular processes that affect indicator performance (e.g., extrusion by transporters, cellular pH). Therefore, experiments were designed to estimate the disassociation constants ($K_D$) of the $Ca^{2+}$ indicators fluo-5F and mag-fluo4 (loaded into the cytosol and sarcoplasmic reticulum, respectively) at room and physiological temperatures in murine ventricular myocytes. $Ca^{2+}$ access to cytosolic compartment was established using a $Ca^{2+}$ ionophore, while sarcoplasmic reticulum (SR) access was established by sarcolemma permeabilization with saponin in the presence of caffeine. Once access was established, buffers with known $[Ca^{2+}]$ were rapidly applied using a micro-perfusion system with temperature feedback control. We observed that, increasing from room temperature to 36°C caused a modest decrease in the $K_D$ of both fluo-5F and mag-fluo4. Both indicators also displayed a decrease in dynamic range when heated. Additionally, temperature-dependent extrusion was observed for indicators loaded into the cytosol, but not those loaded into the SR. These findings are important in enabling quantitative interpretation of measurements in living cells under physiological conditions.

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**A Novel Red Fluorescence Calcium Indicator for Functional Analysis of GPCRs and Calcium Channel Targets**

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Calcium flux assay is widely used for monitoring GPCRs and calcium channels. However, most common calcium indicators (such as Fluo-3 and Fluo-4) have green fluorescence, making them impossible to be used with GFP cells or multiplexed with another green fluorescent dye. Although the rhodamine-based calcium indicators (such as Rhod-2 AM) could help alleviate this limitation, the undesired mitochondrial localization makes it much less sensitive. Cal-590 AM, a new long wavelength calcium indicator, has been developed for monitoring calcium with red fluorescence (Ex/Em= 570/590 nm). It has a significantly improved signal to background (S/B) ratio and longer intracellular retention than Rhod-2 AM. Cal-590 AM is non-fluorescent, and once enter the cells, the lipophilic AM blocking groups are cleaved by intracellular esterase, resulting in a negatively charged fluorescent dye that is well retained in cells. When cells are stimulated with a bioactive compound, the receptor signals release intracellular calcium. As Cal-590 binds calcium inside cells, its fluorescence is greatly enhanced with no overlap with GFP or fluorescein-labeled targets. In this study, the signal intensity and S/B ratio of Cal-590 AM was evaluated with different receptor signaling pathways using HEK, CHO-K1 and GFP cell lines. Unlike Rhod-2 that is easily pumped out by organic-anion transporters, Cal-590 AM has much better cell retention ability

in addition to its significantly higher S/B ratio. It requires minimal amount of organic-anion transporter inhibitor (such as probenecid) in its assay system. In conclusion, Cal-590 AM is a greatly improved red fluorescent indicator for measuring intracellular calcium change. The red-shifted wavelength makes Cal-590 AM a robust tool for evaluating GPCR and calcium channel targets, multiplexing analysis of cellular functions with a green fluorescent dye as well as screening agonists and antagonists with GFP cells.

**550-Pos Board B330**

**Coupling Interactions of the Dimeric SOAR Unit of STIM1 with Orai1 Channels**

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The STIM-Orai activating region (SOAR) of STIM1 is the minimal sequence necessary for Orai1 channel activation. Our previous work revealed that replacement of Phe-394 in SOAR with the dimensionally similar but polar histidine head group prevents Orai1 binding and gating. In the current study, we constructed YFP-SOAR-SOAR concatemers with a 21 amino acid linker between the SOAR units which allowed folding into functional dimers. We introduced the F394H mutation (FH) into either the first (N-terminal) or second (C-terminal) SOAR unit within the dimeric concatemer constructs. We found that each of these single-mutated concatemers was able to both bind to Orai1 and gate the Orai1 channel normally, the same as the wild-type concatemer. In contrast, a concatemer in which both SOAR units contained the F394H mutation (YFP-SOAR$^{FH}$-SOAR$^{FH}$), was devoid of Orai1 binding and gating activity. To ensure that the single mutated concatemers were not forming inter-concatemer dimers, we equally co-expressed the CFP-SOAR$^{WT}$-SOAR$^{WT}$ and YFP-SOAR$^{FH}$-SOAR$^{FH}$ concatemeric constructs in the same cell. While the CFP-SOAR$^{WT}$-SOAR$^{WT}$ bound to and activated Orai1, it did not pull the YFP-SOAR$^{FH}$-SOAR$^{FH}$ with it. Thus, the latter remained in the cytosol in these cells. This result is in contrast to co-expression of the monomeric CFP-SOAR$^{WT}$ with YFP-SOAR$^{FH}$ constructs. In this case, while the YFP-SOAR$^{FH}$ expressed alone does not bind to or activate Orai1, the CFP-SOAR$^{WT}$ clearly dimerizes with YFP-SOAR$^{FH}$mutant and pulls it to the PM. This also proves that he F394H mutation does not prevent SOAR-SOAR dimer formation. Overall, the results reveal a surprising new facet of the STIM1-Orai1 coupling interaction. Thus, although the SOAR dimer is likely the functional Orai1-actiavting unit, only one of the SOAR units within the SOAR dimer needs to be able bind to and activate the Orai1 channel.

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**Translocation between PI(4,5)$P_2$-Poor and PI(4,5)$P_2$-Rich Microdomains During Store Depletion Determines STIM1 Conformation and Gating of Orai1**

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Shmuel Muallem[1], Jozsef Maléth[1,2,4], Seok Choi[1,3,4], and Malini Ahuja[1]
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Receptor-stimulated $Ca^{2+}$ influx, a critical component of the $Ca^{2+}$ signal, is mediated in part by Orai1, which is activated by STIM1 in response to $Ca^{2+}$ release from the ER. Orai1 then undergoes slow $Ca^{2+}$-dependent inactivation (SCDI) that is mediated by binding of SARAF to STIM1. We use SCDI by SARAF as reporter of the conformation and microdomain localization of the Orai1-STIM1 complex. Interaction of STIM1 with the C terminus of Orai1 and the STIM1 K-domain are required for interaction of SARAF with STIM1 and Orai1 SCDI. Interaction of SARAF with STIM1 required the presence of STIM1-Orai1 in a PM/ER microdomain that is tethered by E-Syt1, stabilized by Septin4 and enriched in PI(4,5)$P_2$. Notably, selective targeting of STIM1 to PI(4,5)$P_2$-rich or to PI(4,5)$P_2$-poor microdomains revealed that SCDI by SARAF is observed only when the STIM1-Orai1 complex is within the PI(4,5)$P_2$-rich microdomain. Most notably, measuring the dynamics of STIM1-Orai1 complex localization in live cells using PI(4,5)$P_2$-rich or PI(4,5)$P_2$-poor microdomain probes revealed that store depletion is followed by transient STIM1-Orai1 complex formation in the PI(4,5)$P_2$-poor microdomain where the channel is fully active, which then translocates to the PI(4,5)$P_2$-rich domain to recruit SARAF and initiates SCDI. These findings reveal the role of the PM/ER tethers in the regulation of Orai1 function and $Ca^{2+}$ influx and describe a new mode of regulation by PI(4,5)$P_2$ involving translocation between PI(4,5)$P_2$ microdomains, rather than by PI(4,5)$P_2$ synthesis and breakdown.

# Cardiac Smooth and Skeletal Muscle Electrophysiology I

**552-Pos Board B332**

**Differential Effects of Antiarrhythmic Drugs Vernakalant and Flecainide on Human Two-Pore-Domain $K^+$ Channels**

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Atrial fibrillation (AF) contributes significantly to cardiovascular morbidity and mortality. The growing epidemic is associated with cardiac repolarization abnormalities and requires the development of more effective antiarrhythmic strategies. Cardiac two-pore-domain $K^+$ channels repolarize action potentials and represent potential targets for AF therapy. However, electropharmacology of K2P channels remains to be investigated in detail. This study was designed to elucidate human K2P channel regulation by antiarrhythmic drugs vernakalant and flecainde.

Two-electrode voltage clamp and whole-cell patch clamp electrophysiology was used to record K2P currents from Xenopus oocytes and Chinese hamster ovary (CHO) cells. The class III antiarrhythmic compound vernakalant activated K2P17.1 currents in oocytes and in mammalian cells (EC50,CHO = 40 μM) in frequency-dependent fashion. K2P17.1 open rectification characteristics and current-voltage relationships were not affected by vernakalant. In contrast to K2P17.1, vernakalant reduced K2P4.1 and K2P10.1 currents, in line with K2P2.1 blockade reported earlier. The class I drug flecainide did not significantly modulate K2P currents.

In conclusion, vernakalant activates K2P17.1 background potassium channels. Pharmacologic K2P channel activation may be employed for personalized rhythm control in patients with AF-associated reduction of $K^+$ channel function.

**553-Pos Board B333**

**Classifying the Electrophysiological Effects of Chronotropic Drugs on Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes using Voltage Sensitive Dyes and Supervised Machine Learning**

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The emergence of human induced pluripotent stem (hiPS) cell technology has expanded the possibilities for sourcing human cardiomyocytes (hiPS-CMs). Novel microscopy and analysis methods serve to accelerate development and validation of *in vitro* hiPS-CM models for drug screening. Voltage sensitive dyes (VSD) allow non-invasive, non-destructive, and longitudinal assessment of hiPS-CM electrophysiology at the sub-cellular membrane scale. In this study, we successfully use 2-photon microscopy to capture VSD signal at the cellular membrane scale generated from actively beating hiPS-CMs exposed to the chronotropic drugs, propranolol ($10^{-5}$ M) and isoproterenol ($10^{-7}$ M). We use SimFCS software, developed at the Laboratory for Fluorescence Dynamics at the University of California, Irvine, to remove motion artifact and assess the resultant signal over time. We are able to generate a waveform of VSD fluorescence that is representative of the changing membrane potential (i.e. the depolarization of an action potential). A number of characteristics of these waveforms are defined (upslope, maximum height, plateau height, downslope, peak width, and beat rate), compared across treatments, and shown to be significantly different between treatments. A supervised machine learning algorithm is then trained, validated, and the algorithm accuracy quantified using these data along with their known drug treatments. The algorithm that results can be used to predict which drugs hiPS-CMs have been exposed to given only their respective VSD waveforms. This study tests the hypothesis that VSDs may be used in conjunction with supervised learning to train an algorithm that is capable of automatically and accurately assessing, classifying, and predicting the membrane depolarization effects of chronotropic drugs.

**554-Pos Board B334**

**Sparfloxacin, a Fluoroquinolone Antibiotic, Slows Inactivation of L-Type $Ca^{2+}$ Current in Neonatal Rat Ventricular Myocytes**

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**Introduction**: The proarrhythmic effects of quinolone antibiotics used clinically have been assessed by measuring the $I_{Kr}$ antagonist potency. However, the gaps between clinically reported proarrhythmic effects and $I_{Kr}$ antagonist