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 (Ψm) was depolarized in irradiated HSG cells during first 2h and gradually returned to normal. IR-induced decrease in Ψm 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 Ψm. 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