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ES-derived cardiomyocytes to identify specific perturbations in the Ca^{2+} signal 'fingerprint' evoked by a panel of CV-active drugs (1nM to 100μM). We calculated four indices that describe the spatial and temporal organisation of drug-evoked Ca^{2+} cycling: 1) inter-transient noise (ITN), the amplitude variability of Ca^{2+} signals occurring between Ca^{2+} transients; 2) peak regularity index (PRI), a statistical assessment of Ca^{2+} transient peak-to-peak variability; 3) height regularity index (HRI), the amplitude heterogeneity between successive Ca^{2+} transients and 4) synchrony- the extent of synchronization of Ca^{2+} transients across the syncytia. These four parameters were integrated, together with an assessment of cellular contractility, into a single non-biased safety score. In operator-blinded analyses, the rank order of drug hazard was confirmed as astemizole > flecainide > cisapride = sotalol > thioridazine > valdecoxib >> nadolol = control. This assessment is remarkably similar to the established hazard profile of these drugs. Our data support the utility of this mechanistically agnostic framework for the rapid and discriminatory evaluation of CV drug toxicity.

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A Novel Fluorescent Epac-Agonist Reveals Epac1 and Epac2 Subcellular Localization in Cardiomyocytes

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Epac (Exchange proteins directly activated by cAMP) exist in two isoforms, Epac1 and Epac2. In cardiomyocytes, evidence is building that Epac2 mediates CaMKII and ryanodine receptor related arrhythmia whereas Epac1 overexpression leads to cardiac hypertrophy. This suggests potential subcellular localization differences for Epac1 and 2, but localization information in myocytes has been limited by antibody specificity. Here, we used a novel fluorescent cAMP-derivate Epac agonist 8-[Pharos-575]-2'-O-Me-cAMP (Φ-O-Me-cAMP) to identify Epac1 and Epac2 cardiac localization. Φ-O-Me-cAMP exhibits peak excitation at 543 nm and emission at 610 nm and increases several-fold when bound to Epac. Its Epac specificity was verified in double Epac1/Epac2 knockout mouse myocytes (DKO), and by competition with the non-fluorescent Epac agonist, 8-CPT (Fluorescence= 1,695 ± 162 for Φ-O-Me-cAMP (n=13) vs. 394 ± 41 with 100 μM 8-CPT (n=20) and 439 ± 16 in DKO (n=52)). The apparent affinity of Φ-O-Me-cAMP (K_d) for Epac was 9.6 ± 0.7 μM (n=14). Φ-O-Me-cAMP binding to PKA (a known cAMP target) is excluded because of the DKO results above and its lack of effect on PKA activity (assessed by a PKA-FRET-reporter) and a lack of effect of 100 μM Rp-cAMP (a PKA specific activator) on Φ-O-Me-cAMP signals in cells. Binding of 10 μM Φ-O-Me-cAMP in intact cells rose to a maximum with τ=30 min. Localization of Φ-O-Me-cAMP binding in Epac1-KO mice (i.e. Epac2 location) was prominently T-tubular (tracking Di-8-ANEPPS), whereas Epac1 distribution (in Epac2-KO cells) was non-striated and concentrated around the nucleus. In conclusion, Φ-O-Me-cAMP is a valuable fluorescent Epac-specific agonist. We conclude that Epac2 is T-tubular whereas Epac1 is more nuclear, which parallels their effects on Ca sparks and nuclear signaling, respectively.

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Functionally Isolated Sarcoplasmic Reticulum Model: Intrinsic Regulation of SR Ca^{2+} Release and Tetracaine Effect

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The fraction of the sarcoplasmic reticulum (SR) Ca^{2+} content released at a twitch (fractional release, FR) in cardiomyocytes is regulated by the L-type Ca^{2+} current (ICa,L) and the SR Ca^{2+} content ([Ca^{2+}]SR). In the experimental model of the functionally isolated SR (FISRM), cardiomyocytes are perfused with Na^+, Ca^{2+}-free solution, which makes the cells unexcitable and thermodynamically inhibits the sarcolemmal Na^+/Ca^{2+} exchanger. Intracellular free Ca^{2+} concentration ([Ca^{2+}]i) was measured with fluo-3 and Ca^{2+} transients due to SR release were evoked by 100 ms-long caffeine pulses. FISRM was used to study the intrinsic relationship between FR and [Ca^{2+}]SR in the absence of ICa,L, the physiological trigger for SR Ca^{2+} release. After SR depletion by prolonged caffeine application, 1-20 electrical stimuli were applied to attain different [Ca^{2+}]SR (from 55.3 ± 10.0 to 123.0 ± 10.3 μM). The variation in total [Ca^{2+}] (Δ[Ca^{2+}]T) during transients was estimated from [Ca^{2+}]i and Ca^{2+} buffering parameters. FR (Δ[Ca^{2+}]T/[Ca^{2+}]SR) showed a non-linear relationship with [Ca^{2+}]SR (from 5.4 ± 5.4 to 82.8 ± 7.2%, $p < 0.05$), as previously observed when SR Ca^{2+} release was triggered by ICa,L. This behavior was reproduced with a mathematical model of FISRM, after inclusion of intra-SR and cytosolic Ca^{2+} buffers, which resulted in more realistic simulations. In addition, FISRM was used to study the inhibitory effect of tetracaine on FR. The tetracaine concentration that caused 50% inhibition of FR (IC50) was 58.9 ± 5.7 μM. The FISRM may be a useful experimental model to investigate in intact cells the selective effects on SR Ca^{2+} release of drugs that also affect sarcolemmal currents, such as tetracaine.
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Fluorescence Signal Kinetics of a New Ca^{2+} Probebound to Cardiac Ryanodine Receptors Point to Micro-Domain Detection of Ca^{2+}

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To probe the micro-domains of Ca^{2+}-signaling, we constructed a probe, calstabi-cam, targeted to cardiac ryanodine receptors (RyR2) by FKBP12.6 (calstabin 2) incorporating calmodulin and EYFP as Ca^{2+}-sensor. 48-72h after infection of cultured rat neonatal cardiomyocytes (rNCM), global measurements of calstabi-cam fluorescence were accompanied by simultaneous recordings of Ca^{2+} channel (I_{Ca}) and Na^+/Ca^{2+}-exchanger (I_{NCX}) and compared to measurements of fluo-4 Ca-transients. rNCMs continued spontaneous beating after dialysis with Cs^+-based internal solutions (0.1-0.2mM EGTA at −50mV) as they generated calstabi-cam fluorescence signals that coincided with I_{NCX} oscillations, like those measured by fluo-4 that were blocked by 10μM ryanodine. Activation of I_{Ca} (~5pA/pF) triggered slowly relaxing Ca-transients of calstabi-cam fluorescence ($\Delta F/F_0 = -0.11$; n=14). Similarly, short caffeine pulses also activated long lasting calstabi-cam signals ($-\Delta F/F_0$=0.15 ± 0.012; n=28) accompanied by I_{NCX} (1.57 ± 0.13pA/pF; n=28). Calstabi-cam signals activated by rapid Na^+ withdrawal was significantly delayed (~230ms) compared to those triggered by I_{Ca} (~13ms) or caffeine (~35ms). Similar results were found in feline adult cardiac myocytes where 1μM Isoproterenol increased I_{Ca} significantly without enhancing the associated calstabi-cam transients. These results suggest that the new probe responds more effectively to Ca^{2+} that is released into dyadic clefts by RyR2 than by sarcolemmal Ca^{2+} influx via Na/Ca exchanger. The extremely slow decay kinetics of Calstabi-cam signal is consistent with the notion that [Ca^{2+}] in the dyadic cleft may rise to much higher values than those of cytosol.

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Calcium-Induced Redox Microdimains at the ER-Mitochondrial Interface

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Diverse interactions between Ca^{2+} and reactive oxygen species (ROS) signalling are thought to exist and be of both physiological and pathophysiological relevance. We hypothesized that ROS and Ca^{2+} interact locally at the ER-mitochondrial interface to induce mitochondrial Ca^{2+}-overload and subsequent permeability transition. To test the relationship between ROS and Ca^{2+}, we used genetically-encoded tools to selectively generate and measure ROS at confined locations within HepG2 and MEF cells. Using Killer Red (KR) targeted to the ER-mitochondrial interface or nucleus, we selectively controled the oxidation of these compartments with illumination. When KR was targetted to the ER-Mitochondrial interface, illumination caused a >3-fold increase in cytoplasmic Ca^{2+} response to a low-dose IP3-linked agonist as measured with fura 2, whereas illumination of the KR targeted to the nucleus was ineffective. These data demonstrate that ROS generated at the ER-mitochondrial interface are capable of sensitizing Ca^{2+} release, whereas more distant ROS was not. To test if (patho)physiological conditioins could oxidise the ER-mitochondrial interface in the same way, we utilized the genetically-encoded ratiometric sensor HyPer and the redox de-sensitized derivative SypHer to control for non-specific changes. These probes, targeted to the ER-mitochondrial interface or nucleus, are positioned to assess redox changes at a local level. When permeabilized MEF cells were subject to mitochondrial Ca^{2+} overload, a cyclosporine A-sensitive decrese in mitochondrial membrane potential, consistent with permeability transition, was observed. This was accompanied by a pronounced burst of H2O2 at the ER-mitochondrial interface but not the nucleus. These data demonstrate that the ER-mitocondrial interface may host a local redox microdomain and that ROS generated at locations immediately apposed to Ca^{2+}-transport proteins, such as the IP3 receptor, sensitize Ca^{2+} release whereas distant ROS did not. These observations underline the critical role of spatial organization in ROS-Ca^{2+} crosstalk in mitochondrial signalling.