the SR and connecting compartments. Fluo-5N accumulates in transverse structures that align with Z-discs, consistent with the location of a major compartment of the SR in adult skeletal muscle. That these structures are primarily SR in nature is indicated by the fact that fluo-5N fluorescence decreases when fibers are exposed to caffeine. FRAP experiments demonstrated similar recovery constants for SR-trapped fluo-5N to those we have previously recorded in rat cardiac myocytes. We will be using fluo-5N to study the organization of the SR, the changes in Ca2+ dynamics in the SR during EC coupling, and the role of different proteins of the SR in regulating SR [Ca2+]i.

2779-Pos Board B749

Indo-1 Hybrid Biosensors For Calcium Monitoring In Cellular Organelles

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The central role of calcium in signal transduction depends on the precise spatial and temporal control of its concentration. The existing possibilities to detect fluctuations in Ca2+ concentration with adequate temporal and spatial resolution and in specific cellular organelles, are limited. We have developed a method to measure Ca2+ concentrations in defined subcellular locations that uses derivatives of the dye Indo-1 covalently bound to fusions of “SNAP-tag” (a multiply mutated version of human alkylguanyl DNA alkyltransferase) expressed inside cells. SNAP-Indo1 conjugates retained the Ca2+- and Indo-1-labile properties of Indo-1 in vitro. One of the derivatives of Indo-1 displayed a four-fold higher fluorescence after coupling to SNAP-tag, which improves specificity of Ca2+ sensing in living cells. In a proof-of-principle experiment, local Ca2+ sensing was demonstrated in muscle cells of mice expressing a SNAP fusion localized to nuclei. [Ca2+]i inside nuclei ([Ca2+]i)n was measured by SEER (shifted excitation and emission ratioing) of confocal microscopic images of fluorescence of the sensor. After permeabilizing the plasma membrane, changes to bathing solutions containing different [Ca2+-i] induced corresponding changes in [Ca2+]i in that were readily detected and used for a preliminary calibration of the technique. Similar hybrid sensors using Indo-1 but targeted to the mitochondrial matrix and the SR were also constructed. In principle, these hybrid sensors should combine the spatial specificity of biosensors with the superior kinetics and dynamic range of small synthetic fluorescent reporters.

2780-Pos Board B750

Voltage-Dependent Ca2+ Channels Are Clustered But Not Constitutively Active In Smooth Muscle

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The organization and distribution of Ca2+ signals derived from depolarization-evoked Ca2+ entry has been studied in voltage-clamped single vascular and gastrointestinal smooth muscle cells using widefield epi-fluorescence with near simultaneous (2 ms) total internal reflection fluorescence microscopy. Depolarization activated a voltage-dependent Ca2+ current (ICa) and evoked a rise in [Ca2+]i in the subplasma membrane space and bulk cytoplasm. The rise which occurred in various regions of the bulk cytoplasm ([Ca2+]b) was approximately uniform; that of the subplasma membrane space ([Ca2+]psm) had a wide range of amplitudes and times courses. The [Ca2+]psm variations presumably reflected an uneven distribution of active Ca2+ channels (clusters) across the sarcolemma. Constitutive activity in clusters of voltage-dependent Ca2+ channels has been proposed to determine bulk average [Ca2+]i. In the present study, channels are not constitutively active. The repetitive localised [Ca2+]psm rises (‘Ca2+ sparklets’) which characterize constitutively-active channels were observed rarely (<1 in 50 cells). Nor did constitutively-active voltage-dependent Ca2+ channels regulate the bulk average [Ca2+]i. A dihydropyridine blocker of voltage-dependent Ca2+ channels, nifedipine, which blocked ICa and accompanying [Ca2+]i rise, reduced neither the resting bulk average [Ca2+]i, (at ~70 mV) or the rise in [Ca2+]i, which accompanied an increased electrochemical driving force on the ion by hyperpolarization (~130 mV). Activation of protein kinase C with indolactam-V did not induce constitutive channel activity. Thus while voltage-dependent Ca2+ channels appear clustered on the plasma membrane, constitutive activity in the channel is unlikely to play a major role in regulation [Ca2+] i. The voltage-dependent activity of the clustered channels may enable selective activation of various cellular processes by generating a localised rises in subplasma membrane [Ca2+]i. Supported by the Wellcome Trust and British Heart Foundation.

2781-Pos Board B751

Mitochondria Act Within InsP,R Clusters To Maintain Ca2+ Release In Smooth Muscle

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Many smooth muscle activities including contraction, transepitelial growth and apoptosis are regulated by transient inositol 1,4,5-trisphosphate (InsP3)-mediated increases in cytosolic Ca2+ concentration ([Ca2+]c). InsP3, binds to receptors (InsP3R) present on the sarcoplasmic reticulum to evoke Ca2+ release. InsP3R exist in clusters and Ca2+ released from one receptor may activate neighboring receptors within this cluster in a CICR-like process to evoke a ‘puff’. Ca2+ released may also diffuse to adjacent clusters to trigger further Ca2+ release and generate a Ca2+ rise throughout the cell. Mitochondrial Ca2+ uptake limits a negative feedback process operative on InsP3R to maintain Ca2+ release. Inhibition of mitochondrial Ca2+ uptake decreases InsP3-mediated Ca2+ waves by ~50%. We addressed whether mitochondria act to maintain release by operating within or between InsP3R clusters. Ca2+ puffs were evoked by localized photolysis of InsP3 in single voltage-clamped colonic smooth muscle cells in which [Ca2+]i and [Ca2+]psm were measured simultaneously. EGTA, a slow Ca2+ buffer, was used to functionally uncouple puff sites to prevent the formation of Ca2+ waves. EGTA was used at a concentration ([300 μM]) which does not affect the magnitude or kinetics of Ca2+ puffs. InsP3-evoked Ca2+ puffs had amplitudes of 0.5-5.0 F/F and durations of ~200 ms at half-maximum amplitude. Puffs were abolished by the InsP3R inhibitor 2-APB. The protonophore CCCP and the mitochondrial inhibitor rotenone, each used with oligomycin, depolarized the mitochondrial membrane potential (ΔΨm) and prevented mitochondrial Ca2+ uptake. Depolarizing ΔΨm with CCCP attenuated Ca2+ puffs by ~65% while rotenone inhibited them by ~60%. These results indicate mitochondrial Ca2+ uptake occurs quickly enough to influence InsP3R communication at the intra-cluster level. Supported by the Wellcome Trust and British Heart Foundation.

2782-Pos Board B752

Imaging The Individual and Concerted Activity Of IP3R Ca2+ Release Channels In Intact Mammalian Cells

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Cellular signaling mediated by the inositol trisphosphate (IP3) messenger pathway involves hierarchical Ca2+ liberation from the endoplasmic reticulum (ER), whereby local ‘elementary’ Ca2+ transients (Ca2+ puffs) serve autonomous signaling functions and as well as constituting the building blocks from which global cellular Ca2+ waves are constructed. These channels are inaccessible to single-channel study by patch-clamp in intact cells, and excised organelle and bilayer reconstitution systems disrupt the Ca2+-induced Ca2+ release (CICR) process that mediates channel-channel coordination. We report here the use of total internal reflection fluorescence (TIRF) microscopy to image single-channel Ca2+ flux through individual and clustered IP3R’s in intact mammalian cells. This enables a quantal dissection of calcium puffs involving stochastic recruitment of an average of 6 active IP3R clusters within <400 nm. IP3R gating kinetics during puffs indicate rapid (~10 ms) recruitment by Ca2+-induced Ca2+ release (CICR), followed by a similarly rapid inhibition process that is crucial for terminating puffs by suppressing re-openings of channels in the face of continued high local [Ca2+]c. Single-channel imaging methodology thus provides nanoscale information of the architecture and dynamic interactions between Ca2+ release channels in the cellular environment; information previously inaccessible by electrrophysiological patch-clamp techniques.

Supported by grant NIH GM 40871

2783-Pos Board B753

Analysis of Localized Calcium Alteration During Neural Cell Death

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Fluctuations in intracellular calcium ion ([Ca2+]i) levels are believed to participate in a myriad of physiological and pathological intracellular events. In an attempt to investigate localized alterations in Ca2+ dynamics in a cell-based neurodegeneration model, we used Fura-2/AM dye to monitor Ca2+ ion levels in the human SH-SY5Y neuroblastoma cells induced to undergo apoptosis with 500 mM staurosporine (STS) over a 24 h period. Using rapid illumination frequency at 5 Hz per 340/380 nm excitation wavelength pair, streaming image acquisition and analysis of 12 very small regions of interest (ROI) of ~86.5 μm in either peri-nuclear (PN) or distal (DIST) cytoplasmic locations, we captured
micro-regional signals ("Ca\(^{2+}\) ripples") at selected eight time points that were analyzed for dynamic changes in peak Ca\(^{2+}\)_i, amplitudes and frequencies across both STS exposure times and CCh treatment with standard methods consisting of nonlinear curve-fitting and fast Fourier transform, respectively, and with a novel method of recurrence quantification analysis. Their results, although at times mixed or conflicting, showed that there may indeed be alterations in local Ca\(^{2+}\)_i signaling/dynamic which are biologically valid and sometimes differ from the global Ca\(^{2+}\)_i signaling/dynamic of whole cytoplasms.

I propose, then, that, at least for intrinsic apoptosis, an early activation of caspases leads to cleavages of increasingly more IP3 receptors and sarco-/endoplasmic reticulum Ca\(^{2+}\) ATPase of ER which may enhance Ca\(^{2+}\)_i release by the former and inhibit uptake by the latter. Ultimately, this results in the accumulation of the ion that starts locally and heterogeneously until spreading by waves and becoming more apparent at the whole cytoplasmic scale.

2784-Pos  Board B754
External Sodium Affects SOCE Activation In Mouse FDB Muscle Fibers
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We report experiments carried out to study the possible contribution of the Na\(^{+}/Ca^{2+}\) exchanger activity to [Ca\(^{2+}\)]\(_{\text{myo}}\) levels during SOCE activation in mouse muscle fibers. Enzymatically dissociated FDB fibers, loaded with FURA-2 AM, and treated with 5 \(\mu\)M CPA, were submitted to a SR Ca\(^{2+}\) store depletion procedure, in the absence of external Ca\(^{2+}\). Exposure to external Ca\(^{2+}\) (2nM) activated SOCE under two different modalities. In 9 fibers, [Ca\(^{2+}\)]\(_{\text{myo}}\) increased at a rate of 0.9 \(\pm\) 0.1 nM/s showing saturation. In 7 other fibers the rate of [Ca\(^{2+}\)]\(_{\text{myo}}\) increase was 1.5 \(\pm\) 0.3 (\(p<0.05\)), with no sign of saturation at 115 \(\pm\) 11 s after SOCE activation. Exposure to a 0Na\(^{+}\) solution caused a fast, partial reversal of the SOCE dependent [Ca\(^{2+}\)]\(_{\text{myo}}\) increase. Activation of SOCE was much depressed in the absence of external Na\(^{+}\); subsequent re-exposure to Na\(^{+}\) greatly increased [Ca\(^{2+}\)]\(_{\text{myo}}\) and its rate of rise. TRIS was about twice more effective than Li\(^{+}\) as Na\(^{+}\) substitute. Thus, [Ca\(^{2+}\)]\(_{\text{myo}}\) decreased by 76.6 \(\pm\) 6.4 or 33.8 \(\pm\) 21.4 nM, (\(p<0.05\)) in the presence of TRIS or Li\(^{+}\) respectively. In both cases however, the effect occurred with similar time constants: 32.3 \(\pm\) 2.2 vs 29.9 \(\pm\) 3.6 s\(^{-1}\). When TRIS was used, Na\(^{+}\) dependent SOCE activation showed a Kd of about 25 mM. Preliminary experiments with the Na\(^{+}\) sensitive compound benzofuran isophtalate (SBFI) indicate that simultaneously with SOCE activation there is an increase in the myoplasmic Na\(^{+}\) concentration. If so, the possibility arise that high myoplasmic Na\(^{+}\) could activate the sarcolemmal Na\(^{+}/Ca^{2+}\) exchanger in its reverse mode, or even the mitochondrial Na\(^{+}/Ca^{2+}\) exchanger. An alternate possibility could be a direct modulatory effect of Na\(^{+}\) on SOCE activation machinery (FONACIT G-2001000637).