the SR and connecting compartments. Fluo-5N accumulates in transverse structures that align with Z-disks, 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+].

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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 alkyl transferase) expressed inside cells. SNAP-Indo-1 conjugates retained the Ca2+−sensing ability 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+] inside nuclei ([Ca2+]n) was evaluated 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+] induced corresponding changes in [Ca2+]n 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 monitors. Factors that tended to limit their performance in initial experiments include targeting specificity of SNAP fusions and unspecific staining by the Indo-1 not reacted with SNAP-tag. Overall, the hybrid biosensor approach is a promising tool for organellar Ca2+ imaging. Support: NIAMS/NIH grants to E.R., MDA to J.Z. and a Marie-Curie Fellowship (EC) to M.B.

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Voltage-Dependent Ca^{2+} Channels Are Clustered But Not Constitutively-Active In Smooth Muscle

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The organization and distribution of Ca^{2+} signals derived from depolarization-evoked Ca^{2+} 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 Ca^{2+} current (I_{Ca}) and evoked a rise in $[Ca^{2+}]$ in the subplasma membrane space and bulk cytoplasm. The rise which occurred in various regions of the bulk cytoplasm ($[Ca^{2+}]_c$) was approximately uniform; that of the subplasma membrane space ($[Ca^{2+}]_{PM}$) had a wide range of amplitudes and time courses. The $[Ca^{2+}]_{PM}$ variations presumably reflected an uneven distribution of active Ca^{2+} channels (clusters) across the sarcolemma. Constitutive activity in clusters of voltage-dependent Ca^{2+} channels has been proposed to determine bulk average $[Ca^{2+}]_c$. In the present study, channels are not constitutively active. The repetitive localized $[Ca^{2+}]_{PM}$ rises ('Ca^{2+} sparklets') which characterize constitutively-active channels were observed rarely (<1 in 50 cells). Nor did constitutively-active voltage-dependent Ca^{2+} channels regulate the bulk average $[Ca^{2+}]_c$. A dihydropyridine blocker of voltage-dependent Ca^{2+} channels, nimodipine, which blocked I_{Ca} and accompanying $[Ca^{2+}]_c$ rise, reduced neither the resting bulk average $[Ca^{2+}]_c$ (at -70 mV) or the rise in $[Ca^{2+}]_c$ 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 Ca^{2+} channels appear clustered on the plasma membrane, constitutive activity in the channel is unlikely to play a major role in regulation $[Ca^{2+}]_c$. The voltage-dependent activity of the clustered channels may enable selective activation of various cellular processes by generating a localized rises in subplasma membrane $[Ca^{2+}]$.
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Mitochondria Act Within $InsP_3R$ Clusters To Maintain Ca^{2+} Release In Smooth Muscle

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Many smooth muscle activities including contraction, transcription, growth and apoptosis are regulated by transient inositol 1,4,5-trisphosphate ($InsP_3$)-mediated increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). $InsP_3$ binds to receptors ($InsP_3R$) present on the sarcoplasmic reticulum to evoke Ca^{2+} release. $InsP_3R$ exist in clusters and Ca^{2+} released from one receptor may activate nearby $InsP_3R$ within this cluster in a CICR-like process to evoke a 'puff'. Ca^{2+} released may also diffuse to adjacent clusters to trigger further Ca^{2+} release and generate a Ca^{2+} rise throughout the cell. Mitochondrial Ca^{2+} uptake limits a negative feedback process operative on $InsP_3R$ to maintain Ca^{2+} release. Inhibition of mitochondrial Ca^{2+} uptake decreases $InsP_3$-mediated Ca^{2+} waves by $\geq$50%. We addressed whether mitochondria act to maintain release by operating within or between $InsP_3R$ clusters. Ca^{2+} puffs were evoked by localized photolysis of $InsP_3$ in single voltage-clamped colonic smooth muscle cells in which $[Ca^{2+}]_c$ and $\Delta\Psi_M$ were measured simultaneously. EGTA, a slow Ca^{2+} buffer, was used to functionally uncouple puff sites to prevent the formation of Ca^{2+} waves. EGTA was used at a concentration ([300 μM]) which does not affect the magnitude or kinetics of Ca^{2+} puffs. $InsP_3$-evoked Ca^{2+} puffs had amplitudes of 0.5-5.0 F/F_0 and durations of ~200 ms at half-maximum amplitude. Puffs were abolished by the $InsP_3R$ inhibitor 2-APB. The protonophore CCCP and the mitochondrial inhibitor rotenone, each used with oligomycin, depolarized the mitochondrial membrane potential ($\Delta\Psi_M$) and prevented mitochondrial Ca^{2+} uptake. Depolarizing $\Delta\Psi_M$ with CCCP attenuated Ca^{2+} puffs by ~65% while rotenone inhibited them by ~60%. These results indicate mitochondrial Ca^{2+} uptake occurs quickly enough to influence $InsP_3R$ communication at the intra-cluster level. Supported by the Wellcome Trust and British Heart Foundation.

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Imaging The Individual And Concerted Activity Of IP_3R Ca^{2+} Release Channels In Intact Mammalian Cells

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Cellular signaling mediated by the inositol trisphosphate (IP_3) messenger pathway involves hierarchical Ca^{2+} liberation from the endoplasmic reticulum (ER), whereby local 'elementary' Ca^{2+} transients (Ca^{2+} puffs) serve autonomous signaling functions and as well as constituting the building blocks from which global cellular Ca^{2+} 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 Ca^{2+} induced Ca^{2+} release (CICR) process that mediates channel-channel coordination. We report here the use of total internal reflection fluorescence (TIRF) microscopy to image single-channel Ca^{2+} flux through individual and clustered IP_3R's in intact mammalian cells. This enables a quantal dissection of calcium puffs involving stochastic recruitment of an average of 6 active IP_3Rs clustered within <400 nm. IP_3R gating kinetics during puffs indicate rapid (~10 ms) recruitment by Ca^{2+}-induced Ca^{2+} 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 $[Ca^{2+}]$. Single-channel imaging methodology thus provides nano-scale information of the architecture and dynamic interactions between Ca^{2+} release channels in the native cellular environment; information previously inaccessible by electrophysiological patch-clamp techniques.
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Analysis of Localized Calcium Alteration During Neural Cell Death

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Fluctuations in intracellular calcium ion ($Ca^{2+}{}_i$) levels are believed to participate in a myriad of physiological and pathological intracellular events. In an attempt to investigate localized alterations in $Ca^{2+}{}_i$ dynamics in a cell-based neurodegeneration model, we used Fura-2/AM dye to monitor $Ca^{2+}{}_i$ ion levels in the human SH-SY5Y neuroblastoma cells induced to undergo apoptosis with 500 nM 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^2 in either peri-nuclear (PN) or distal (DST) cytoplasmic locations, we captured