confocal microscope. In situ calibration determined the half signal of fluo-5N and rhod-5N to be 335 and 872 μM, respectively. Rhod-5N was selected for ongoing experiments. Chronic depletion of $[Ca^{2+}]$SR with caffeine reduced $[Ca^{2+}]$t-sys to 0.1 mM via chronic activation of store-operated Ca^{2+} entry (Launikonis et al 2003, PNAS). We then exposed Ca^{2+}-depleted preparations to 0-800 nM $[Ca^{2+}]$cyto in 50 mM EGTA. At $[Ca^{2+}]$cyto $>$ 100 nM the $[Ca^{2+}]$t-sys reached a plateau at 1.8-1.9 mM after 3-5 s. At $[Ca^{2+}]$cyto $<$ 100 nM the $[Ca^{2+}]$t-sys did not always reach this plateau and showed a biphasic uptake of Ca^{2+}. At the plateau $[Ca^{2+}]$t-sys lowering $[Ca^{2+}]$cyto to $<$ 1 nM did not cause a significant loss of $[Ca^{2+}]$t-sys. There was an apparent absence of effect of removing $[Na^{+}]$cyto on these results. Mathematical modeling of these results suggests that the plasma membrane CaATPase (PMCA) with its low Km for Ca^{2+} is the major protein responsible for t-system Ca^{2+} uptake in the resting muscle, despite the higher transport capacity of the Na-Ca exchanger.

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Possible Role for the Phoshorylated Tail in Retaining CSQ2 to Specific Sites Within the Secretory Pathway

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Each of the two calsequestrin (CSQ) genes expresses muscle-type specificity; CSQ1 is present only in skeletal muscle, while CSQ2 is found in cardiac tissue and slow-type skeletal muscle. The two forms of the protein share about 60% identity and have similar 3D structures. Yet, differences in distribution of negative surface charges lead to differences in intracellular polymerization and localization. A second difference in structure is the CSQ2-specific C-terminal extension, a tail that contains a cluster of protein kinase CK2-sensitive serines. In this study, we examined the subcellular localization of CSQ1 and CSQ2 following their acute overexpression in adult rat cardiomyocytes, using isoform specific antibodies. The native rat CSQ2 showed the expected junctional SR distribution, co-staining with ryanodine receptor. Canine and human CSQ2 localized within 48 h to the same puncta as the native protein, with subtle differences in distribution. In contrast to all CSQ2 proteins, CSQ1 was not observed in jSR, rather accumulating in punctae near the cell surface. To test whether the C-terminal 23-mer extension on CSQ2 (cT) underlied this difference we constructed a chimeric cDNA encoding CSQ1-cT. This protein accumulated in yet a third pattern, distinct from both the transverse jSR localization (CSQ2 compartment) and the subsarcolemmal accumulation (CSQ1 compartment). CSQ1-cT appeared to assemble in an array of punctae aligned longitudinally across the myoplasm. We conclude that CSQ2, following its perinuclear biosynthesis, encounters junctional SR sites where it can accumulate by polymerization. In contrast, CSQ1 proceeds to cortical sites along microtubules to concentrate near the cell surface. Thus, the cardiac-specific cT may interact with an upstream sorting protein, suggesting a possible mechanism of CSQ retention beyond its polymerization. Studies in cultured non-muscle cells support the hypothesis for cT-dependent CSQ retention.

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Microtubule Integrity is Essential to Junctional SR Protein Delivery

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Cardiac muscle contraction has special reliance upon ER subcompartments to regulate Ca^{2+} cycling and homeostasis. A unique ER subcompartment, the junctional sarcoplasmic reticulum (jSR) plays a critical role in this process, through the concentration of a unique group of resident proteins that regulate Ca^{2+} release. Impaired function of jSR has been shown to lead to cardiac pathologies; however, cellular mechanisms and pathways via which proteins traffic to jSR remain undiscovered. To investigate their delivery mechanisms, canine forms of junctin (JCT) or triadin (TRD) were overexpressed in adult rat cardiomyocytes, and their accumulation was visualized by confocal fluorescence microscopy using species-specific antibodies. Both JCT and TRD exhibited patterns of juxtanuclear buildup after 24h. By 48h, higher-intensity punctae were detected across the width of the cell. With microtubule depolymerization, anterograde movement ceased, and newly synthesized proteins did not migrate away from the nucleus. CSQ2-DsRed forms a polymeric complex that localizes early in biosynthesis to juxtanuclear cisternae. To determine whether all jSR proteins follow a common biosynthetic and transport route to jSR, TRD or JCT were co-overexpressed with CSQ2-DsRed. Both TRD and JCT transport from juxtanuclear sites was prohibited. In contrast, triadin in which CSQ2 binding sites were deleted proceeded to the cell periphery. These data suggest that TRD and JCT are biosynthesized at juxtanuclear sites and can interact in situ with CSQ2 at early stages in biosynthesis, and are delivered to jSR sites along a microtubule dependent pathway that lies close to Z-lines, with little or no accumulation at other sarcomeric locations. We conclude that TRD and JCT share the same direct microtubule dependent route, not via free SR compartments.

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Proton Fluxes Across the Tubular (T-) System Membrane of Rat Fast-Twitch Fibres

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Cytoplasmic pH has major effects on most cellular processes in skeletal muscle, including its ability to develop force. Protons are continuously extruded from the cytoplasm against their electrochemical gradient as shown by the considerably more alkaline pH in the resting muscle than the predicted pH value if protons were distributed passively. We aimed to determine the contribution of the t-system proton extrusion mechanisms to this gradient and the diffusive proton flux of the t-system. To do this we trapped 10 mM of the pH-sensitive dye HPTS inside the tubular (t-) system of mechanically skinned fibres from the rat extensor digitorum longus muscles and continuously imaged dye fluorescence during changes in internal solution pH, $[Na^{+}]$, $[K^{+}]$ and [ATP] by confocal microscopy. Calibrations using monensin showed that in normally polarized fibres with 36 mM $[Na^{+}]$cyto that pHt-sys was 7.50 ± 0.12 (n=3), 7.91 ± 0.20 (n=3) and 8.31 ± 0.29 (n=4) at pH-cyto of 6.8, 7.2 and 7.5, respectively. In the presence of 162 mM $[Na^{+}]$cyto, with or without amiloride, the pHt-sys and $[Na^{+}]$t-sys were similar to cytoplasmic values. The addition of 50 μM amiloride to normally polarized fibres with 36 mM $[Na^{+}]$cyto increased pHt-sys further indicating that the Na^{+}-H^{+} exchanger (NHE) was the major protein responsible for extruding protons from the cytoplasm. The pH difference across the t-system membrane at rest is reduced by NHE activity, which moves protons against the inward diffusive proton flux of the resting muscle fibre. We calculated the diffusive proton flux across the t-system to be 2.7 +/- 0.9 e-4 m/s (mean +/- SEM, n=3).

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Multiple Regions of Junctin and Triadin Interact with Calsequestrin 1 and 2 in Skeletal Muscle Cells

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The release of Ca^{2+} in skeletal muscle fibers is regulated by several proteins, which are organized in a multi-molecular complex localized at the junctional sarcoplasmic reticulum (jSR). This complex includes the intracellular Ca^{2+} channel ryanodine receptor (RyRs) and additional proteins, including triadin, junctin and calsequestrin, which have been shown to form a quaternary complex. In FRAP experiments, we found that, in differentiated myotubes, these proteins display specific dynamic properties suggesting the existence of distinctive protein-protein interactions among j-SR proteins. To better define the molecular bases of protein association in the multi-molecular complex assembled around the RyRs, we performed experiments aimed to identify the specific sequences that mediate the interactions among jSR proteins in the lumen of the SR. We expressed GST fusion proteins covering distinct intraluminal domains of triadin and junctin. The purified GST-fusion proteins were incubated with detergent-solubilized SR vesicle from mouse skeletal muscle and probed for their interaction with distinct SR proteins. Experiments were also performed with recombinant proteins expressed in HEK293 cells. We found that different regions in the intraluminal domain of junctin and triadin can bind calsequestrin-1 and/or calsequestrin-2 and that both proteins appear to bind preferentially calsequestrin-1 rather than calsequestrin-2. In addition, calsequestrin-1 and calsequestrin-2 appeared to display distinct binding affinities for junctin and triadin. The correlation between the in vitro protein-protein interactions and the dynamic properties of jSR proteins will be verified.

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Temperature Effects on the Myoplasmic Free Ca^{2+} Transients in FDB Muscle Fibers of the Mouse

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