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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The fast, low affinity, Ca^{2+} dye Mag-Fluo-4 loaded in its AM moiety, in mouse FDB fast-twitch fibers has allowed to study, with high temporal resolution, the decay phase of single or tetanic (350 ms-100 Hz), Ca^{2+} transients (Calderón et al. JMRCM 2009;30). At 23°C, a double exponential function with time constants (always expressed in ms), τ_1=1.4 ± 0.1, τ_2=15.3 ± 1.3 (n=20), and τ_1=23.4 ± 2.5, τ_2=594 ± 102.4 (n=13), respectively, describe the decay phase of single and tetanic transients. These time constants have been associated with Ca^{2+} binding to parvalbumin, and SERCA, respectively. We tested for differential effect of tetanic Ca^{2+} loads and temperature on the putative Ca^{2+} removal mechanisms. Increasing temperature, from 23 to 33°C, reversibly diminishes the single transient amplitude (ΔF/F), by 31%, and increases the base line by 32% (n=25). At 33°C, in 15 out of 20 fibers, the decay of single transients followed a single exponential, τ=4.7 ± 0.7, while in the other 5 fibers, two exponentials provided a better fit, τ_1=0.6 ± 0.1 and τ_2=5.1 ± 0.4; two exponentials τ_1=10.1 ± 1.8 and τ_2=394.9 ± 51.2 (n=13), provided the best fit for tetanic decay. The results show that the two time constants, that describe Ca^{2+} single and tetanic responses decay, are differentially affected by temperature and by tetani imposed Ca^{2+} loads. The fast decay components of single and tetanic responses, represented by τ_1, and associated with parvalbumin Ca^{2+} binding, are affected similarly by Ca^{2+} load (17x increase) or by temperature (2.3x increase for 10°C increase). The slower components of the single and tetanic responses, represented by τ_2, and associated with SERCA operation, are differentially affected by a 10°C temperature raise (3.1x vs 1.5x) and by the tetanic Ca^{2+} load (39x vs 77x) respectively.

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Cellular Mechanisms of Cardiac Depression and Recovery in Endotoxemic Mice

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Sepsis and septic shock are associated with a reversible cardiac dysfunction, that complicates management and worsens prognosis. In patients that survive septic shock, cardiac function recovers spontaneously, through mechanisms that are currently unknown. Here we aimed to identify the intracellular calcium (Ca^{2+}) transporters responsible for cardiac recovery after endotoxemic challenge in mice. Male C57Bl6 mice were administered lipopolysaccharide (LPS, 7 μg/g weight, ip). 12h after LPS administration, cardiomyocyte sarcomere shortening (SS) and Ca^{2+} transients (ΔCai, measured with fura2-AM) were decreased to 53 ± 10% and 78 ± 5% of control, respectively, in association with a decrease in trans-sarcolemmal Ca^{2+} influx (Cainf) through the L-type Ca^{2+} channels (LTCC, to 65 ± 9%) and sarcoplasmic reticulum (SR) Ca^{2+} pump (SERCA) reuptake (88 ± 5%, n > 19 cells from 3 mice for all). SR Ca^{2+} load (CaSR, measured with caffeine) was unchanged, while SR fractional release (FR, as αCai/CaSR) was decreased to 84 ± 6% of control (n's as above). 72h after LPS administration, survival was 40%. In cells isolated from surviving mice 72h after LPS, ΔCa, FR and Cainf were fully recovered, while SERCA showed a supranormal function (138 ± 4% of control, n's as above). SS showed a trend towards a partial depression (to 82 ± 7% of control, p = 0.077, n's as above) at 72h, that persisted up to 6 days after LPS administration. In conclusion, the recovery of cardiac Ca^{2+} handling after LPS challenge is associated with a full recovery of LTCC dysfunction, a supranormal activation of SERCA, despite, possibly, a persistent dysfunction in the contractile mechanisms downstream of the Ca^{2+} transient.

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Conditional Up-Regulation of SERCA2a Exacerbates Ventricular and Atrial Arrhythmias in the Setting of Catecholaminergic Polymorphic Ventricular Tachycardia

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SERCA2a gene transfer is an emerging therapy for treating contractile dysfunction in heart failure. While improving contractile performance, SERCA2a overexpression has been shown to exacerbate arrhythmias, although beneficial effects of SERCA2a up-regulation on cardiac rhythm have also been reported. To examine the role of SERCA2a and the consequences of its acute up-regulation in arrhythmogenesis, we conditionally overexpressed SERCA2a in a genetic mouse model featuring catecholaminergic polymorphic ventricular tachycardia (CPVT) due to loss of calsequestrin 2 (CASQ2). CASQ2 knock-out (KO) mice were crossbred with doxycycline (DOX)-inducible SERCA2a transgenic mice to generate KO-TG mice. In-vivo ECG studies showed that uninduced KO-TG (DOX-) mice developed both ventricular and atrial arrhythmias in response to catecholamine challenge (isoproterenol, ISO or a combination of ISO and caffeine). Induction of SERCA2a (DOX^+) markedly exacerbated both ventricular and atrial arrhythmias in response to ISO. Besides ventricular bigeminy, KO-TG (DOX^+) also displayed frequent bursts of sustained ventricular ectopic beats that were not present in KO-TG (DOX-). Moreover, episodes of atrial rhythm disturbances in KO-TG (DOX^+) mice occurred even under baseline conditions (no ISO). ISO further promoted atrial tachy- and brady-arrhythmias in the KO-TG (DOX^+) mice. Consistent with the in-vivo studies, confocal Ca imaging in both ventricular and atrial myocytes demonstrated that acute SERCA2a overexpression significantly increased the rate of occurrence of diastolic spontaneous and triggered Ca release events. Thus, our results suggest that acute overexpression of SERCA2a exacerbates both ventricular and atrial arrhythmias in settings of CPVT by further elevating diastolic Ca release.

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Fractal-Like Behavior of the Heart-Beat Intervals is Encoded within Intrinsic Complexity of Pacemaker Cells Residing in the Sinoatrial Node and Modulated by Autonomic Input to the Heart

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The heart rate and rhythm are controlled by complex chaotic neural, chemical and hormonal networks which are not strictly regular, but exhibit fluctuations across multiple-time scales. Therefore, it is not surprising that decoding of the ECG in mammals, even under resting conditions, reveals scale-invariant dynamics and beat-interval variability (BIV). Moreover, fractal-like behavior of heart beat intervals, which reveals itself in a power-law dependence of the frequency distribution of its intrinsic regimes, contributes to the complexity of the mammalian heart's rhythm. The traditional explanation for BIV and fractal-like behavior of heart beat intervals is that this result from the balance of sympathetic and parasympathetic autonomic input to the heart. But whether the sinoatrial node (SAN), the heart's primary pacemaker, or pacemaker cells isolated from the SAN exhibit fractal-like behavior of beating intervals in the absence of autonomic input is unknown.

We analyzed beating rhythms: (i) in vivo, when the brain input to the SAN is intact; ii) during autonomic denervation in vivo; iii) in intact isolated (denervated) SAN; and iv) in single pacemaker cells isolated from the SAN. By segregating each component of the brain-pacemaker cascade, we discovered that fractal-like beating interval exhibit in SAN tissue and although the beat interval of single pacemaker cells isolated from the SAN is rhythmic it does not exhibit fractal-like behavior. Therefore, cell-to-cell communication among pacemaker cells within the SAN tissue is required to impart their fractal-like beating interval behavior within the SAN. Autonomic input from the brain to the heart in vivo modulates both the rate and rhythm at which pacemaker cells beat and its fractal-like behavior, but it is not required for isolated SAN fractal-like complexity.

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The Mstn-Cmpt Dl1Abc- Mice. A Mouse Model to Study Muscle Weakness, Fatigue and Soce

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In our mouse model, a naturally occuring 12-bp deletion in the myostatin gene is considered responsible for the compact phenotype (Mstn(Cmpt-dl1Abc)) labeled by a tremendous increase in body weight along with signs of muscle weakness and easier fatigability.

Western blot screenings showed significantly reduced endogenous STIM1 and Orai1 protein levels in the compact mouse muscle samples. As a consequence, we hypothesized that SOCE may be consequently altered. Enzymatically isolated fluo-8 AM loaded FDB fibers from wild type and Mstn(Cmpt-dl1Abc) mice were used. To elicit a massive SR Ca^{2+}-release a RyR1 agonist (4-chloro-meta-cresol) was applied in a Ca^{2+} free medium and in the presence of the SR Ca^{2+} pump inhibitor (thapsigargin). The above cocktail triggered a