

transporter inhibitors (such as probenecid) present in the assay system. Organic-anion transporter inhibitors are often toxic to cells and also interfere with the activities of bioactive compounds to be screened. In conclusion, Cal 520 AM is an improved fluorescent indicator for the measurement of intracellular calcium. The high signal-to-noise ratio and good intracellular retention properties make the Cal 520 AM a robust tool for evaluating GPCR and calcium channel targets as well as for screening their agonists and antagonists.

1578-Pos Board B348

Studying Calcium Signal Reshaping by Buffers Observing the Competition of Two Dyes

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Ca²⁺ signaling is ubiquitous across cell types. Ca²⁺ liberation through inositol 1,4,5-trisphosphate receptors (IP3Rs) is a key component of the Ca²⁺ signaling toolkit. The specificity and universality of intracellular calcium signals rely on the variety of spatio-temporal patterns that the concentration of this ion can display. It has been observed by I. Parker and collaborators that buffers can shape these patterns in different ways depending on their kinetics. In this work we study how these different effects may arise by observing IP3R-mediated Ca²⁺ signals using two dyes simultaneously. To this end we use a multi-spectral confocal microscope and two dyes (Fluo4 and Rhod2) that differ in their binding kinetics and emission spectra. In this way we are able to study the competition between the two dyes probing the effect of their different kinetics on the observed signals.

1579-Pos Board B349

Intercellular Calcium Waves in Vascular Smooth Muscle Cells

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Muscular arteries are able to actively modify their diameter by modulating the tone of the smooth muscle cells (SMCs) located within the arterial wall. The contractile state of the SMCs is regulated by cytosolic calcium transients and propagates as a wave over a significant distance along the vessel.

We studied the intercellular calcium wave propagation in primary cultured SMCs from rat mesenteric arteries. By using photolithography technique, two types of in vitro cells networks were developed: lines and loop patterns. We have recorded and analyzed calcium response and membrane potential variations for each cell, induced by single cell mechanical stimulus. The loop network was more efficient in transmitting the calcium signals from cell to cell. The calcium wave propagation through gap junctions was spatially limited in line patterns. Longer distance in calcium propagation was obtained in the presence of angiotensin II.

This study provides new experimental data supporting the idea that mechanical stimulation evokes a membrane potential depolarization which propagates to neighboring cells. The electrical depolarization is followed by a fast calcium entry that triggers calcium release from intracellular stores. In addition, these results suggest that increasing the expression of the gap junction protein connexin 43 by angiotensin II treatment, facilitates longer distance propagation of calcium waves in primary cultured SMCs.

1580-Pos Board B350

Sarcoplasmic Reticulum Calcium Leak in Normal and Dystrophic Skeletal Muscle

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Under resting conditions, external calcium is known to enter skeletal muscle cells while calcium stored in the sarcoplasmic reticulum (SR) leaks into the cytosol. The nature of the pathways involved in the resting sarcolemmal calcium entry and in the SR calcium leak are still debated but several lines of evidence suggest that an up-regulation of these calcium fluxes occurs in Duchenne Muscular Dystrophy (DMD). We investigated here SR calcium permeation at resting potential and in response to depolarization in voltage-controlled skeletal muscle fibers from control and *mdx* mice, the murine model of DMD. Using the cytosolic calcium dye Fura2, we first demonstrated that the rate of calcium increase induced by CPA (cyclopiazonic acid) inhibition of SR Ca²⁺-ATPases at resting potential is significantly higher in *mdx* fibers suggesting an elevated SR passive calcium leak. However, in these experiments, sarcolemmal calcium influx may contribute to the CPA-induced calcium increase and another series of experiments indicated that CPA-induced SR calcium leak was deeply mod-

ified in the absence of external calcium. Fibers were then loaded with the low affinity calcium dye Fluo5N-AM and dialyzed with 50 mM EGTA to measure intraluminal SR calcium changes. Depolarization pulses evoked voltage-dependent Fluo5N fluorescence decreases followed by a recovery phase which was inhibited by CPA, demonstrating that Fluo5N actually reports intraluminal SR calcium changes. Voltage-dependence and magnitude of depolarization-induced SR calcium depletion were found to be unchanged in *mdx* fibers but the rate of the recovery phase that followed depletion was found to be faster, suggesting a higher SR calcium reuptake capacity in *mdx* fibers. Finally, CPA-induced SR calcium leak at -80 mV was found to be significantly higher in *mdx* fibers. The elevated SR passive calcium leak may participate to the muscle degenerative process in *mdx* muscle.

1581-Pos Board B351

Altered Skeletal Muscle Excitation Contraction Coupling in Dysferlinopathy

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Limb Girdle Muscular Dystrophy Type 2B (LGMD2B) and Miyoshi Myopathy (MM) are caused by mutations in the dysferlin gene, but the role of dysferlin in healthy muscle and the changes that occur when it is mutated or absent are poorly understood. Previous work supported a role for dysferlin in sarcolemmal repair following laser wounding or other in vitro injuries. We study the response of A/J mice, which lack dysferlin, to injury by large-strain lengthening contractions in vivo. We find that dysferlin promotes normal recovery from this physiological injury but is not necessary for sarcolemmal repair. Consistent with this, immunofluorescence microscopic studies of healthy muscle, that we fixed and treated in a hot, mildly acidic solution to expose dysferlin's epitopes, show that dysferlin is primarily in transverse tubules (TT), not the sarcolemma as previously reported. Furthermore, TT are disrupted when skeletal muscle is injured physiologically, and disruption is much more extensive in A/J muscles than in controls. Studies of FDB myofibers in tissue culture also demonstrate the presence of dysferlin in TT. Brief exposure of control myofibers to hypoosmotic solutions damages TT, in a process dependent upon extracellular Ca²⁺. As in vivo, A/J myofibers are more extensively damaged by osmotic shock than controls; they are indistinguishable from controls when shocked in Ca²⁺-free medium, however. Thus Ca²⁺ may promote damage, rather than participate in dysferlin-dependent membrane repair, as previously reported. Our results suggest that the changes in TT following injury in vivo and in vitro are similar, that they require extracellular Ca²⁺, and that they are much more pronounced when dysferlin is absent. We propose that dysferlin is essential for the integrity of the TT of skeletal muscle, in maintaining this integrity during contraction and relaxation, and in repairing damaged TT following injury.

1582-Pos Board B352

Caffeine Treatment and Depolarization Alter the Spatial and Temporal Characteristics of Calcium Sparks on Intact Amphibian Skeletal Muscle

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Calcium sparks were recorded on intact skeletal muscle fibers of the frog using high time resolution confocal microscopy (x-y scan: 30 Hz, line-scan: 60 kHz). Sparks were elicited by 1 mmol/l caffeine or subthreshold depolarization to different membrane potentials (data presented for -60 mV). Both treatments increased the frequency of sparks and altered their morphology. Images were analyzed by custom-made computer programs. Both the amplitude (in $\Delta F/F_0$; 0.49 ± 0.025 vs. 0.29 ± 0.001 ; $n = 22426$ vs. 23714 ; mean $\pm$ SEM, $p < 0.05$) and the full width at half maximum (FWHM, in μm ; parallel with fiber axis: 2.33 ± 0.002 vs. 2.21 ± 0.005 ; perpendicular to fiber axis: 2.07 ± 0.003 vs. 1.88 ± 0.004) of sparks was significantly greater after caffeine treatment than on depolarized cells. On x-y scans 25.8% (caffeine) and 16.4% (depolarization) of detected sparks overlapped with another one on the previous frame. Center of signal mass of overlapping sparks travelled shorter distances between consecutive frames after caffeine treatment than after depolarization (in μm ; 1.80 ± 0.017 vs.

2.95 ± 0.082; component parallel with fiber axis: 1.05 ± 0.015 vs. 1.90 ± 0.057; component perpendicular to fiber axis: 1.21 ± 0.014 vs. 1.85 ± 0.062). Similar differences in amplitude and FWHM values were observed on line-scan images. On these images spark pairs close enough to be detected as single events were more abundant compared to simulated images containing the same number of randomly distributed sparks. Calcium sparks had significantly slower rising phase under both conditions as compared to the control. Complex events corresponding to overlapping sparks with differently located centers of signal mass on consecutive x-y frames were also found on line-scan images.

1583-Pos Board B353

Quantification of the CICR Response to Artificial Ca Sparks in Striated Muscle

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In cardiac muscle physiologic Ca²⁺ release is CICR, but in skeletal muscle the role of CICR remains unsettled. "Artificial Ca²⁺ sparks" of up to 8 μM were produced by 2-photon irradiation of Ca²⁺ cages, applied in the near vicinity of membrane-permeabilized skeletal fibers from frogs and mice, and rabbit atrial and ventricular myocytes, while confocally imaging their Ca²⁺ response with Fluo-4FF. In response to transients as low as 0.28 μM, frog fibers produced waves (n=16) that satisfied the definition of CICR (Endo, PhRev 2009), including inhibition by Mg²⁺. Cardiomyocytes responded similarly, with waves that could propagate fully or partially through the cells. Responses were absent in mouse skeletal fibers (n=51) unless RyR opening was promoted pharmacologically (n=24). The underlying Ca²⁺ flux was calculated from fluorescence images by generalizing the "backward" method (Ríos et al, JGP 1999). In frog fibers, fluxes were found to reach 55 mM/s, which is close to values measured under voltage clamp depolarization. A positive three-way correlation was found among flux, speed and extent of propagation. Accordingly, the responses of cardiomyocytes and pharmacologically conditioned mammalian fibers, both of which had smaller flux, were of lower velocity and sometimes limited propagation range. The quantitative properties of the propagating wave, in all cases, were well described by a simple continuum model of sources and sinks, with a common threshold [Ca²⁺] for channel opening (Kupferman et al, BJ 1997). This description suggests a fundamental commonality of mechanisms. The lower flux and higher threshold [Ca²⁺] in mice are consistent with the notion that CICR is supported by isoform 3 or beta (in the frog), and that isoform 1 *in situ* is less susceptible to CICR. Supported by NIAMS and NHLBI (NIH).

Calcium Signaling Pathways

1584-Pos Board B354

Excitation Coupled Calcium Entry (ECCE) is Enhanced in mdx Myofibers and is Modulated by ROS

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Altered Ca²⁺ homeostasis and elevated ROS have been implicated in the pathogenesis of Duchenne Muscular Dystrophy (DMD). In these studies we investigated the contribution of Excitation Coupled Calcium Entry (ECCE) to the altered Ca²⁺ signaling phenotype and whether this Ca²⁺ influx pathway is redox sensitive. Adult, post necrotic mdx mice (e.g., > 4 months) exhibited significantly enhanced basal Ca²⁺ influx at rest compared to WT as determined by Mn²⁺ quench. Upon depolarization, Ca²⁺ influx (i.e., ECCE) was significantly enhanced in the mdx compared to WT. Scavenging ROS with n-acetylcysteine (NAC) did not alter the basal Ca²⁺ influx in the WT or mdx muscle fibers. NAC, however, significantly reduced ECCE in mdx muscle by approximately 66% with no effect on WT. The non-specific TRP channel inhibitor 4-methyl-4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide (BTP-2) reduced basal Ca²⁺ influx and ECCE in mdx muscle fibers by 51% and 72% respectively. In WT, BTP2 reduced ECCE by 41% and had no effect on basal Ca²⁺ permeability. We conclude that basal Ca²⁺ influx and ECCE

are likely TRP dependent and only ECCE in the mdx can be modulated by ROS scavenging. Funding: LPM - 5F31NR011245-03, CWW- RC2 NR011968, GGR-K01AR051519

1585-Pos Board B355

Effect of Oxidative Stress on Intracellular Ca²⁺ and Na⁺ Concentration in Duchenne Muscular Dystrophy Myotubes

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Duchenne muscular dystrophy (DMD) is an X-linked myopathy leading to progressive muscle weakness that is the most common and devastating type of muscular dystrophy in boys. Oxidative stress has been suggested as a potential upstream contributor to the skeletal muscle damage in DMD. Here we test this hypothesis by comparing the effect of ROS in intracellular concentrations of Ca²⁺ and Na⁺. Intracellular calcium concentration ([Ca²⁺]_i) and intracellular Na⁺ concentration ([Na⁺]_i) were measured using Ca²⁺ and Na⁺ selective microelectrodes in Wt and mdx myotubes. Mdx myotubes showed significantly elevated [Ca²⁺]_i and [Na⁺]_i in comparison to Wt cells. In mdx myotubes [Ca²⁺]_i was 333 ± 35 nM (mean ± SD, n=35) and [Na⁺]_i was 17.5 ± 1.3 mM (n=15) versus 115 ± 10 nM (n=40) and 7.9 ± 0.6 mM, (n=15) in Wt. Ca²⁺ influx at rest measured by Mn²⁺ quenching also revealed increased Ca²⁺ entry in mdx. The effect of ROS on cultured myotubes was tested by exposing myotubes to 10 μM H₂O₂, a concentration that did not alter cell viability. H₂O₂ caused a significant elevation of [Ca²⁺]_i and [Na⁺]_i in both mdx and Wt. However, the relative increase of both ions was much greater in mdx than Wt. Whereas in mdx [Ca²⁺]_i reached 1167 ± 138 nM (n=14) and [Na⁺]_i 23.6 ± 2 mM (n=11) in Wt cells these concentration were 152 ± 11 nM (n=15) and 10.5 ± 0.5 mM (n=14), respectively. In addition, pre-treatment of mdx and Wt myotubes with nonselective TRPC blockers 5 μM BTP2 or 20 μM gadolinium prevented the increase in [Ca²⁺]_i and [Na⁺]_i induced by H₂O₂. These results suggest that mdx myotubes are more susceptible than Wt to elevation of [Ca²⁺]_i and [Na⁺]_i induced by oxidative stress. Moreover, H₂O₂-induced dysregulation of [Ca²⁺]_i and [Na⁺]_i seems primarily mediated by Ca²⁺ entry.

Supported by Grants P01AR47605 (to PDA) and 5K01AR054818 (to CFP)

1586-Pos Board B356

Intracellular Sodium Overload Contributes to Deterioration of Function in Dystrophic Heart

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[Na⁺]_i is found to be elevated in several models of heart failure. This is believed to favor Ca²⁺ extrusion from mitochondria via the mNCX and therefore reduce mitochondrial Ca²⁺ accumulation. As several dehydrogenases of the mitochondrial Krebs cycle are stimulated by Ca²⁺, the defects in cellular sodium homeostasis may induce mitochondrial oxidative stress. Previously we have estimated that resting [Na⁺]_i is significantly elevated in ventricular myocytes isolated from dystrophic (mdx) mice compared to those from wild-type (WT) mice (24.2 ± 3.1 mM, n=13 vs. 14.0 ± 1.7 mM, n=9). Moreover, the mitochondrial matrix was significantly oxidized at resting conditions (35 ± 3.1 %, n=27 vs. 53 ± 5.2 %, of NADH reduction n=10, in mdx and WT respectively). Here maximal oxidation of NADH by FCCP/oligomycin was taken as 0% reduced NADH and maximal reduction by rotenone and β-hydroxy-butyrate was defined as 100% reduced NADH. We suggested that beat-to-beat mechanical activity in dystrophic heart might lead to greater [Na⁺]_i accumulation and oxidation of the mitochondrial matrix. Here we investigated whether elevated [Na⁺]_i affects mitochondrial energetics during excitation-contraction coupling in dystrophic myocytes. We measured NADH autofluorescence in WT and mdx cells under control conditions and during 4Hz stimulation in the presence of isoproterenol. An abrupt increase in workload resulted in significant oxidation of NADH both in mdx and WT cardiomyocytes. However, the extent of oxidation was significantly greater in dystrophic cells (50 ± 9 %, n=10 vs 15 ± 6 %, n=11 decrease in NADH, respectively). When workload was discontinued, the mitochondrial matrix quickly returned back to its base line levels in WT but not in mdx cells. These results support the notion that the elevated [Na⁺]_i contributes to the impaired energetics in dystrophic cardiomyocytes and therefore contributes to the development of dystrophic cardiomyopathy.