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Free Communications 1: Skeletal Muscle

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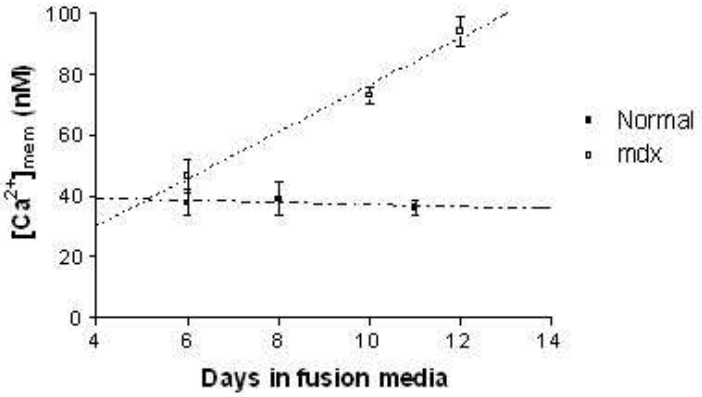
Chair: Gordon Lynch

Near-membrane cytosolic [Ca^{2+}] levels and Ca^{2+} transients measured in myotubes grown from normal and dystrophic (mdx) mice using the Ca^{2+} indicator FFP-18.

A.J. Bakker[1], R. Han[1] and M.D. Grounds[2], [1]School of Biomedical & Chemical Sciences and [2]School of Anatomy & Human Biology, The University of Western Australia, Crawley, WA 6009, Australia.

Abnormal extracellular Ca^{2+} influx has been suggested to be involved in the process of muscle wasting in Duchenne muscular dystrophy. However, studies comparing the resting intracellular Ca^{2+} levels in normal and dystrophic muscle cells from patients with Duchenne muscular dystrophy and *mdx* mice have yielded contradictory findings (Gillis, 1996). Ca^{2+} indicators targeted to the inner sarcolemmal membrane have recently been reported to be more sensitive to sarcolemmal Ca^{2+} influx than standard cytosolic Ca^{2+} indicators such as fura-2 (Bruton *et al.*, 1999). In this study, we measured the resting Ca^{2+} levels and Ca^{2+} transients in myotubes grown from *mdx* and normal mice using the near-membrane Ca^{2+} indicator FFP-18.

Skeletal muscle satellite cells were isolated from the hind limbs of neonatal normal and *mdx* mice that had been killed by decapitation. Myotubes were grown on glass coverslips coated with collagen. The myotubes were loaded with the Ca^{2+} indicator by exposure to FFP-18-AM (3 μM) and 0.0125% Pluronic F-127 for 45 min at room temperature (22-23°C). Ca^{2+} measurements were made with a Cairn spectrophotometer attached to a Nikon inverted microscope equipped for epifluorescence. The myotubes were stimulated by electrical field stimulation (EFS) via two small platinum wires (single 0.2 ms pulse).



Resting near membrane [Ca^{2+}] ($[Ca^{2+}]_{mem}$) levels increased significantly during development in the *mdx* myotubes, (slope; 8.19 ± 1.47, $p<0.0001$). However, no change in $[Ca^{2+}]_{mem}$ was found in normal myotubes during development (slope; -0.40 ± 1.14, $p=0.73$). From the fitted lines, the $[Ca^{2+}]_{mem}$ in 12 days old *mdx* and normal myotubes was estimated at 93 and 36 nM respectively (Figure). Increasing the driving force for Ca^{2+} influx by raising extracellular Ca^{2+} to 18 mM, increased the steady state $[Ca^{2+}]_{mem}$ by 156.1 ± 14.2 % (to ˜ 208 nM) (n=14) in *mdx* myotubes, while in normal myotubes, the $[Ca^{2+}]_{mem}$ increased by only 28.8 ± 7.6 % (to ˜ 49 nM) (n=6), ($p=0.007$, unpaired Student's *t*-test). The half-relaxation time of EFS-induced Ca^{2+} transients was significantly increased in *mdx* (314.5 ± 36.9 ms, n=8) compared to normal myotubes (163.3 ± 28.4 ms, n=6) ($p=0.01$, unpaired t-test), which is consistent with previous studies using standard Ca^{2+} indicators.

The results of this study further support the hypothesis that increased Ca^{2+} influx results in raised intracellular levels in dystrophin-deficent skeletal muscle cells. The use of FFP-18 to measure steady state cytosolic Ca^{2+} in normal and *mdx* myotubes in the presence of raised extracellular Ca^{2+} could provide a more reliable method for detecting the altered Ca^{2+} homeostasis in dystrophic muscle cells.

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Elevated temperature effects on sarcoplasmic reticulum function in mammalian skeletal muscle fibres

C. van der Poel and D.G. Stephenson, School of Zoology, Faculty of Science and Technology, La Trobe University, Victoria 3086, Australia.

Superoxide (O2-) has been shown to be produced by the muscle at elevated temperatures (40-46°C) and to cause marked reversible changes in contractile activation characteristics of mammalian skinned fibres (van der Poel &;Stephenson, 2002). Here we examine the effect of similar temperature treatments on sarcoplasmic reticulum (SR) function in mammalian skeletal muscle. Long-Evans hooded rats were killed by an overdose of halothane in accordance with the procedure approved by La Trobe University Animal Ethics Committee. Extensor digitorum longus (EDL) muscles were dissected out and underwent temperature treatment at 40°C for either 5 or 30 min, 43°C for 30 min and 46°C for 5 min. Single muscle fibres were dissected from EDL muscles after exposure to elevated temperatures, mechanically skinned under paraffin oil and mounted on a force transducer. The endogenous SR Ca2+ content was then estimated by releasing all SR Ca2+ with 30mM caffeine and low Mg2+ (Release Solution), and measuring the area under the force response as an indicator of the amount of SR Ca2+ released. The fibre was then re-loaded with Ca2+ under standard conditions ([Ca2+] 200nM and pH 7.10) for either 30, 60 or 90sec and the SR Ca2+ was subsequently released in the Release solution. The relative area under the force responses was again used as the indicator of the relative amount of Ca2+ in the SR prior to the exposure to the Release solution. In order to determine the extent of Ca2+ leak out of the SR, the preparation was loaded with Ca2+ for 90 sec and then washed for either 30 or 90 sec in a leak solution (pCa = 8, 0.5mM EGTA). The remaining Ca2+ in the SR was then released in the Release solution and the ratio between the areas under the caffeine-induced responses after 90 and 30 sec exposure to leak solution was used to estimate the fraction of SR Ca2+ remaining after 60 sec in the leak solution (Macdonald &;Stephenson, 2001). Results show that after exposure of the EDL muscle to 40°C for 5 or 30 min, 43°C for 30 min and 46°C for 5 min the endogenous amount of Ca2+ in the SR was greatly reduced. This was accompanied by a significant decrease in the rate and ability of the SR to load Ca2+ and by a large increase in the rate of SR Ca2+ leak, which could explain the decrease in both endogenous SR Ca2+ and the rate of SR Ca2+ loading. No significant recovery was observed in the parameters (0-3hrs after temperature treatment). Experiments using 20 µM TBQ (2,5-di(tert-butyl)-1,4-hydroquinone) to block the SR Ca2+ pump and Ruthenium Red (5 µM) to block the RyR/SR Ca2+ release channels indicated that the major route of the Ca2+ leak was through the SR Ca2+ pump. Pre-treatment of the muscles with the superoxide scavenger Tiron (20mM) markedly reduced the temperature-induced changes on the SR function suggesting that the observed temperature effects are influenced by O2- production. The results can explain the earlier observations on isolated muscle preparations exposed to temperatures greater than 35°C, when force production becomes markedly and irreversibly depressed (Lännergren &;Westerblad, 1986). Lännergren, J. &;Westerblad, H. (1986) Journal of Physiology, 390: 285-293. Macdonald, W. A. &;Stephenson, D. G. (2001) Journal of Physiology, 532: 499-508. van der Poel, C. &;Stephenson, D. G. (2002) Journal of Physiology, 544: 765-776.

Effect of low ATP concentrations on action potential-induced Ca^{2+} release in mechanically-skinned EDL fibres of the rat

T. Dutka and G. Lamb, Department of Zoology, La Trobe University, Victoria 3086, Australia.

During vigorous and/or prolonged activity, the average [ATP] within the cytoplasm may decrease from ~7 mM to ~1 mM (Karatzaferi *et al.*, 2001). It may decrease even lower in areas with high ATP utilisation and/or limited diffusion (e.g. triadic junction). Furthermore, ATP facilitates the opening of isolated Ca^{2+} release channels (RyRs) (Laver *et al.*, 2001), but it is currently unclear whether ATP is needed on the RyR for it to be activated by the voltage-sensor (VS) when the VS is activated in a potent and coordinated manner by an action potential (AP). By using adenosine (a competitive weak agonist for the ATP stimulatory site on the RyR) and examining force development of twitch and tetanic force responses, we sought to address whether ATP is crucial for normal AP-mediated Ca^{2+} release.

Male Long-Evans hooded rats were killed under deep anaesthesia (2% v:v halothane) and the extensor digitorum longus (EDL) muscles were excised. Single fibres were mechanically-skinned, connected to a force transducer and immersed in a standard K-HDTA solution (1mM free Mg^{2+}; 8 mM total ATP; 10 mM creatine phosphate (CP) at pH 7.10, containing 50 µM EGTA, pCa 7.0). Individual fibres were then electrically stimulated (75 V cm^{-1}, 2 ms pulse) to produce either twitch or tetanic (50 Hz) force responses at control (8 mM ATP) or at low [ATP] (0.1-2 mM, where ATP was replaced with CP) with or without adenosine present (2 or 4 mM). In parallel experiments, the response of the contractile apparatus to [Ca^{2+}] steps was examined by pre-equilibrating a fibre in a weakly Ca^{2+}-buffered K-HDTA solution (100 µM EGTA) at pCa 7.0 at a given [ATP] and/or adenosine condition, and then rapidly activating it by plunging it into a heavily Ca^{2+}-buffered solution (50 mM CaEGTA/EGTA, pCa 6.0 or 4.4) with the same [ATP] to produce either submaximal or maximal force. These fibres had been Triton X-100 treated so only the contractile apparatus was functional.

Compared to the bracketing control responses (8 mM ATP), the mean twitch peak amplitude was significantly ($P<0.05$) reduced under all low [ATP] conditions (to 71±4%, n=7; 66±3%, n=24; 56±3%, n=51 and 28±4%, n=8, in the presence of 2, 1, 0.5 and 0.1 mM ATP respectively). Peak tetanic force and the rate of tetanic force production was also reduced at low [ATP]. The slowing of the rise in tetanic force at ≤0.5 mM ATP was greater than that explicable by effects of low [ATP] on the rate of force development by the contractile apparatus. Therefore, it appears that the amount of AP-mediated Ca^{2+} release must have been reduced at ≤0.5 mM ATP. The reduction of twitch peak amplitude was exacerbated as the ratio of [adenosine]:[ATP] (mM:mM) was increased (2:8=96±2%, n=3; 2:2=67±4%, n=4; 2:1=41±4%, n=17; 4:1=36±3%, n=7, compared to the absence of adenosine). Since adenosine did not significantly hinder force development of the contractile apparatus, this finding indicates that adenosine competitively interfered with ATP binding to the RyR (Laver *et al.*, 2001), and hence caused reduced Ca^{2+} release. These experiments indicate that ATP must be bound to the stimulatory site on RyRs for the VS to trigger Ca^{2+} release in response to an AP, the normal *in vivo* stimulus.

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Magnesium inhibition of skeletal muscle ryanodine receptors modified by DIDS, ryanodine and ATP

E.R. O'Neill[1], G.D. Lamb[2] and D.R. Laver [1], [1]School of Biomedical Sciences, University of Newcastle, Australia and [2]Department of Zoology, LaTrobe University, Australia.

In skeletal muscle the activity of ryanodine receptor (RyR) calcium release channels in the sarcoplasmic reticulum is regulated by the dihydropyridine receptor (DHPR) voltage sensors in the t-tubule membrane. Ca^{2+}, Mg^{2+} and ATP are potent intracellular regulators of RyRs. The effects of these substances on isolated RyRs are well characterised yet it is not clear how they regulate RyR opening under voltage-sensor control. RyRs are activated by μM cytoplasmic Ca^{2+} and mM ATP while physiological [Mg^{2+}] (~1 mM) in the cytoplasm fully inhibits them. It is proposed that during muscle contraction, DHPRs transiently relieve Mg^{2+} inhibition which then permits activation of RyRs by ATP (Lamb *et al.*, 1991).

Mg^{2+} is thought to inhibit RyRs by binding both to low affinity sites that show little specificity between divalent ions (I-sites) and to high affinity sites for Ca^{2+} (A-sites) thus preventing Ca^{2+} from activating the channel (Laver *et al.*, 1997). However, ATP is known to activate RyRs in the absence of cytoplasmic Ca^{2+} so it is not clear how Mg^{2+} at the A-sites affects channel opening under physiological conditions. Here we investigate the mechanism of Mg^{2+} inhibition in the presence of ATP and two drugs, 4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid (DIDS) and ryanodine, which also activate RyRs in the absence of Ca^{2+}.

RyRs were isolated from rabbit skeletal muscle and incorporated into lipid bilayers using standard techniques (O'Neill *et al.*, 2003). Skeletal muscle was removed from dead rabbits. Cytoplasmic solutions contained 250 mM Cs^+ (230 mM $CsCH_3O_3S$ and 20 mM CsCl) 10 mM TES at pH 7.4. Luminal solutions contained 50 mM Cs (30 mM $CsCH_3O_3S$ and 20 mM CsCl), 10 mM TES, pH 7.4.

DIDS decreased I-site affinity for Mg^{2+} and Ca^{2+} by 10 fold and ryanodine abolished binding completely. Cytoplasmic Mg^{2+} inhibited RyRs via the Ca^{2+} activation site even in the absence of Ca^{2+} indicating that Mg^{2+} inhibition is not merely due to the prevention of Ca^{2+} binding. In the case of ryanodine modified RyRs, monovalent ions (Cs^+) could also activate the channel. RyR activity in the virtual absence of Ca^{2+} (~1 nM) was not due to sensitisation of the channel to Ca^{2+} as previously thought (Du *et al.*, 2001; Masumiya *et al.*, 2001) but was due to Ca^{2+}-independent channel opening by ryanodine. The apparent Mg^{2+} affinity at the A-site was decreased by cytoplasmic Cs^+ and Ca^{2+} as well as by luminal Ca^{2+} in a way which suggests that cytoplasmic Mg^{2+}, Cs^+ and Ca^{2+} compete for a site near the cytoplasmic entrance. Ions at this site may progress to the A-site further into the pore. Binding of these ions at the A-site is in competition with luminal Ca^{2+} and leads to either activation (2 × Cs^+ or Ca^{2+}) or inhibition (Mg^{2+}) of RyRs.

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Nitric oxide alters the rate and sensitivity of sarcoplasmic reticulum calcium uptake in ovine skeletal muscle

J.J. Cottrell[1], R.D. Warner [1], F.R. Dunshea[1], M.B. McDonagh[1] and R. Kuypers[2], [1]Department of Primary Industries, Research and Development Division, 600 Sneydes Rd, Werribee, 3030, Victoria, Australia and [2]Food Science Australia, P.O. Box 3312, Tingalpa, 4173, Queensland, Australia.

Calcium leakage from the sarcoplasmic reticulum (SR) to the cytosol can occur via reduced sarcoplasmic/ endoplasmic reticulum ATPase (SERCA) activity, increased efflux via the Ryanodine receptor (RyR) Ca^{++} channel or SR membrane leakage. The aim of this experiment was to investigate if nitric oxide (NO) influences SR Ca^{++} uptake and release from lamb carcasses after control (none), medium (300V, 14Hz) or high (700V, 14Hz) voltage electrical stimulation (ES) applied for 1 min approximately 5 min post-mortem. From 9 lambs, the SR was isolated from the *Longissimus thoracis et lumborum* (LTL) at the 13^{th} thoracic vertebra approximately 10 min post-mortem. Isolated SR membranes were incubated for 30 min with the 100mM final concentration of the NO donors Diethylamine NONOate (NONO) or Sodium nitroprusside (SNP) at $25^{o}C$ before assay of SR Ca^{++} uptake, release and ATPase activity. Incubation with NONO increased the linear and maximal rates V_{max} of SR Ca^{++} uptake ($P<.05$ and $P<.01$ respectively), without affecting the ATPase activity ($P>.05$). This resulted in an increased coupling ratio ($P<.05$) between V_{max} and ATPase for NONO, indicating greater efficiency of the SERCA pump. The calcium concentration for half maximal uptake ($[Ca^{++}]_{0.5}$) was also increased by NONO, indicating reduced sensitivity of Ca^{++} induced Ca^{++} uptake. Collectively, these data indicate that while NONO increases the rate of Ca^{++} uptake, NO desensitised the SERCA to initiate Ca^{++} uptake. No effect of SNP or ES was observed on SR Ca^{++} uptake ($P>0.05$). Neither NONO nor SNP affected SR Ca^{++} release via the RyR. However, ES resulted in increased SR Ca^{++} efflux following thapsigargin-induced inhibition of the SR ATPase. Due to the low rates of release observed, this was most likely due to membrane damage or increased SR permeability, not opening of RyR. In conclusion, the NO donor NONO influenced the SERCA, reducing its Ca^{++} sensitivity, but increasing its rate of uptake. Reduced sensitivity of Ca^{++} induced Ca^{++} uptake ($[Ca^{++}]_{0.5}$) may increase cytosolic Ca^{++} concentrations due higher Ca^{++} required to induce uptake, likely increasing cytosolic Ca^{++} concentrations and activating Ca^{++} dependent proteases.

Phosphorylation status of calsequestrin does not alter its ability to regulate native ryanodine receptors

N.A. Beard[1], M.G. Casarotto[1], D.R. Laver[2] and A.F. Dulhunty[1], [1]John Curtin School of Medical Research, Australian National University, Canberra, ACT, and [2]School of Biomedical Science, Faculty of Health, University of Newcastle, NSW, Australia.

Depolarisation of the sarcolemma triggers Ca^{2+} release through ryanodine receptor (RyR) calcium release channels in the sarcoplasmic reticulum (SR) of skeletal muscle. The protein responsible for calcium storage within the SR is calsequestrin (CSQ), which is located wholly within the SR lumen. CSQ is tethered to the RyR by two anchoring proteins, triadin and junctin, as well as probably forming a direct physical coupling with the channel itself.

Recent studies have shown that CSQ regulates RyRs via two mechanisms. The first (indirect) interaction is presumably mediated by triadin and junctin, resulting in RyR inhibition, whilst the second interaction is via a direct physical connection between CSQ and the RyR (Szegedi *et al.*, 1999; Herzog *et al.*, 2000; Beard *et al.*, 2000). This second interaction requires dephosphorylated CSQ to modify RyR activity. The role *in vivo* of CSQ phosphorylation is not clear, nor has a definitive phosphorylation mechanism been reported. It is unknown whether the interaction of CSQ with native RyRs (those containing RyR co-proteins, such as triadin and junctin) depends on CSQ dephosphorylation in the same manner as the direct interaction of CSQ with purified RyRs.

To study the effects of altering CSQ's phosphorylation status on its ability to regulate native RyR regulation, rabbit skeletal SR vesicles containing RyRs (isolated from back and leg muscle of New Zealand male rabbits killed by a captive bolt) were incorporated into artificial planar lipid bilayer membranes, which were formed across an aperture with a diameter of 150-200 μM in a delrin cup. The bilayer separates two chambers, *cis* (cytoplasmic) and *trans* (luminal). Solutions contained Ca^{2+} (1 mmol/l), $CsCl_2$ (20 mmol/l), caesium methane sulfonate (250/30 mmol/l; *cis/trans*) and TES (10 mmol/l). CSQ was purified according to Costello *et al.*, (1986). Phosphorylation status of CSQ was determined using ^{31}P NMR, and CSQ was dephosphorylated according to the methods of Cala & Jones (1983).

In a single channel study, RyRs were exposed to 500 mM Cs^+ to dissociate endogenous CSQ (recently shown to successfully dissociate CSQ from bilayer incorporated RyRs; Beard *et al.*, 2002). After subsequent perfusion of the *trans* chamber with 250 mM Cs^+, 20-50 μg of either phosphorylated or dephosphorylated CSQ was added to the *trans* chamber. There was no significant difference between the regulation of the RyR by phosphorylated or dephosphorylated CSQ. Both forms of CSQ significantly inhibited RyR activity.

Unlike the phosphorylation-dependant regulation of purified RyRs by CSQ, altering the phosphorylation status of exogenous CSQ did not alter CSQs ability to inhibit native skeletal RyR activity. In combination with the results of Szegedi *et al.* (1999) and Herzog *et al.* (2000), these data illustrate that CSQ imposes two very different regulatory mechanisms on RyRs, and suggest that phosphorylation-dependant changes *in vivo* do not alter the triadin/junctin mediated regulation of RyRs and SR Ca^{2+} release by CSQ.

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