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Chair: Brett Cromer, David Allen

# Calcium-phosphate precipitation in the sarcoplasmic reticulum reduces action potential-mediated Ca$^{2+}$ release in mammalian skeletal muscle

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Rapid ATP buffering during vigorous activity is predominantly achieved by the enzyme creatine kinase and the substrate creatine phosphate (CrP), which is present at ~40 mM (Allen *et al.*, 1995). As ATP is hydrolysed to ADP and inorganic phosphate (P$_i$), CrP donates its phosphate to the ADP to resynthesize ATP, and the [P$_i$] within the cytoplasm of fast-twitch muscle fibres may reach $\geq$ 30 mM. Evidently P$_i$ can enter the sarcoplasmic reticulum (SR) passively (Posterino & Fryer, 1998), via small conductance chloride channels that conduct P$_i$ (Laver *et al.*, 2001). It has been proposed (Fryer *et al.*, 1995) that once inside the SR, P$_i$ could bind to Ca$^{2+}$ forming a calcium-phosphate (Ca-P) precipitate. We examined whether Ca-P precipitate formed in the SR and whether it reduced normal action potential (AP)-mediated Ca$^{2+}$ release, and hence could contribute to the later stages of metabolic muscle fatigue that result from a failure of Ca$^{2+}$ release (Allen *et al.*, 1995).

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 'control' solution (1mM free Mg$^{2+}$; 8 mM total ATP; 10 mM creatine phosphate (CP) at pH 7.10, containing 75 µM EGTA, pCa 6.9). Individual fibres were then stimulated: 1) electrically (75 V cm$^{-1}$, 20 pulses of 2 ms duration) to produce tetanic (50 Hz) force responses, or 2) by exposure to a 30 mM caffeine-0.05 mM Mg$^{2+}$ solution with 0.5 mM EGTA present, which produced a submaximal longer-lasting force response (e.g. ~10 sec). 30 mM P$_i$ solutions (replacing 23 mM HDTA with 30 mM P$_i$, and adjusting the total [Mg$^{2+}$]) were made similar to the standard K-HDTA solution (with or without 10 mM CrP present). The fibre was exposed to either no P$_i$ (control), 10 or 30 mM P$_i$ for 10 s, then immersed in paraffin oil (1 min), placed back into the same solution (10 s) as before and then transferred back into the oil (1 min). This procedure created a 'closed' system around the fibre and prevented any appreciable net Ca$^{2+}$ uptake or loss by the SR from the weakly Ca$^{2+}$-buffered solution trapped inside the fibre. The fibre was then washed (30 s) in standard solution to remove any P$_i$ in the cytoplasm before stimulating the fibre.

Total SR Ca$^{2+}$ content was ascertained by pre-equilibrating the fibre for 20 s in standard solution with a known [BAPTA] present and then lysing all membranous compartments within the fibre by exposure to an emulsion of Triton-X100 (10% v:v) in paraffin oil (Owen *et al.*, 1998). All experiments were performed at 24 ±1 °C.

After a 2 min exposure to 30 mM P$_i$ (with, n=4, or without, n=6, 10 mM CrP present) the total amount of Ca$^{2+}$ released from the SR by caffeine-low [Mg$^{2+}$] stimulus was significantly ($P<0.05$) reduced by ~20%, and the initial rate of force development slowed (~55%). Peak tetanic (50 Hz) force was also significantly reduced by ~25% and ~45% after 10 and 30 mM P$_i$ exposures respectively, n=4 for 10 mM P$_i$ and n=14 for 30 mM P$_i$). Tetanic force responses produced after 30 mM P$_i$ exposure were nearly identical to those seen in the same fibre following depletion of total SR Ca$^{2+}$ by ~35% (using a tetanic stimulus in the presence of 2 mM BAPTA, the total Ca$^{2+}$ remaining in the SR was 0.75 ± 0.03 mM, n=5). Ca$^{2+}$ content assays revealed that the total amount of Ca$^{2+}$ remaining in the SR was not detectably changed after 30 mM P$_i$ exposure (initially 1.16 ± 0.04 mM, n=9 and 1.16 ± 0.07 mM, n=3 after 30 mM P$_i$ exposure) thus indicating that Ca$^{2+}$ had not leaked out of the SR but instead formed a precipitate with the P$_i$, thereby reducing the amount of available Ca$^{2+}$ for rapid release.

These results suggest that Ca-P precipitation occurring within the SR may contribute to the failure of Ca$^{2+}$ release observed in the later stages of metabolic muscle fatigue. They also demonstrate that a drop in the amount of total SR Ca$^{2+}$ to a level substantially below the normal endogenous level will appreciably reduce tetanic force.

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# Digoxin effects on muscle strength, fatigue and $K^+$ fluxes during exercise in healthy young adults

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The $Na^+,K^+ATPase$ enzyme constrains muscle $K^+$ loss and $Na^+$ gain and is vital for skeletal muscle contractility, but our recent studies have found that maximal $Na^+,K^+ATPase$ activity is depressed with fatigue. We investigated the effects of the specific $Na^+,K^+ATPase$ inhibitor digoxin on muscle strength, fatiguability and performance; and on $K^+$ fluxes across active and inactive muscles during exercise.

Ten active, but not well-trained healthy volunteers (9 M, 1 F), with normal ECG, plasma electrolytes, renal function, and no history of adverse cardiovascular events gave written informed consent. A series of exercise tests were performed after taking digoxin (DIG, 0.25 mg.d$^{-1}$) or a placebo (CON) for 14 d, in a randomised, counterbalanced, cross-over, double blind design study, with trials separated by 4 weeks.

Quadriceps muscle strength (peak torque at 0-360°/s) and fatiguability during 50 maximal contractions (fractional decline in peak torque at 180°/s) were measured on day 13 on a Cybex isokinetic dynamometer. All subjects performed incremental cycle ergometer exercise to measure $VO_{2peak}$ and to determine 33, 67 and 90% $VO_{2peak}$ work rates. Subjects also performed an incremental test using concentric, dynamic finger flexor contractions to determine their peak work rate ($WR_{peak}$). On day 14 subjects completed two invasive trials separated by ~2 h. A finger flexion exercise trial comprised three 1-min bouts, then a final bout to fatigue, at 100% $WR_{peak}$. Two-legged cycling comprised 10 min each at 33% and 67% $VO_{2peak}$, then to fatigue at 90% $VO_{2peak}$. Radial arterial (a) and deep antecubital venous (v) blood was sampled simultaneously at rest, before and during each exercise bout and in recovery, for both exercise trials.

Serum digoxin was 0.7±0.2 nM at day 13 and 0.8±0.2 nM at day 14 (Mean±SD) in the DIG trial, and < 0.4 nM for CON. Muscle peak torque and the fatigue index (CON 0.57±0.10 *vs* DIG 0.54±0.09) were unchanged by digoxin. Time to fatigue during finger flexion exercise was not significantly affected by digoxin (CON 236±211 *vs* DIG 157±118 s, n=9). During finger flexion exercise, each of $[K^+]_a$, $[K^+]_v$ and $[K^+]_{a-v}$ were greater with exercise in CON (by 0.37±0.21, 1.29±0.84 and -0.89±0.69 mM), and similarly with DIG (by 0.34±0.36, 1.12±0.87 and -0.69±0.69 mM). The unchanged $[K^+]_{a-v}$ suggests unaltered $K^+$ release from contracting muscles with DIG. Time to fatigue during leg cycling exercise was not significantly affected by digoxin (CON 254±125 *vs* DIG 262±156 s). During leg exercise, each of $[K^+]_a$, $[K^+]_v$ and $[K^+]_{a-v}$ were greater with exercise than at rest in CON (by 2.51±0.83, 1.22±0.52 and 1.29±0.68 mM), but none were modified by DIG (by 2.62±0.57, 1.18±0.73 and 1.43±0.78 mM). The unchanged $[K^+]_{a-v}$ suggests unaltered $K^+$ uptake by inactive muscles with DIG.

In summary, DIG at therapeutic levels did not adversely affect muscle performance, $[K^+]$ or $K^+$ fluxes during exercise in healthy young adults. Whether this reflects inadequate digitalization, a safety tolerance to small reductions in functional $Na^+,K^+ATPase$, or limited adverse effects of digitalization when muscle $Na^+,K^+ATPase$ is normal (i.e. high) is unclear.

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# The peak tetanic force-$[K^+]_o$ relationship in mouse fast- and slow-twitch muscle: modulation with $[Na^+]_o$ or $[Ca^{2+}]_o$

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Potassium ($K^+$) is frequently postulated to cause skeletal muscle fatigue. Indeed, the trans-sarcolemmal $K^+$ gradient falls during high-intensity exercise (Sejersted & Sjøgaard, 2000) and experimentally raising extracellular $[K^+]$, ($[K^+]_o$) causes depolarisation and reduces force in non-fatigued muscle (Cairns *et al.*, 1997, 1998). However, large elevations of $[K^+]_o$ are necessary to cause a severe reduction of force (Cairns *et al.*, 1997). The aim of the present study was to extend our understanding of the role of $K^+$ in fatigue, by testing for interactive effects between raised $[K^+]_o$ and other ionic changes that occur during intense exercise (Cairns *et al.*, 1998; Sejersted & Sjøgaard, 2000), i.e., diminished trans-sarcolemmal sodium ($Na^+$) and calcium ($Ca^{2+}$) gradients.

Isometric contractions were evoked by supramaximal electric field stimulation (parallel plate electrodes) in isolated slow-twitch soleus (SOL) or fast-twitch extensor digitorum longus (EDL) muscles of mice. Muscles were bathed in control Krebs solution (4 mM $K^+$, 147 mM $Na^+$, 1.3 mM $Ca^{2+}$, 128 mM $Cl^-$) at 25°C. With raised $K^+$ solutions NaCl was replaced with KCl, with lowered $Na^+$ solutions NaCl was replaced with N-methyl-D-glucamine, and with altered $Ca^{2+}$ solutions $Ca^{2+}$ was replaced with $Mg^{2+}$, or $CaCl_2$ was added. Maximum tetanic force was achieved at 125 Hz in SOL and 200 Hz in EDL. Fatigue was induced with repeated tetanic stimulation at 125 Hz for 500 ms, every 1 s, for 100 s.

When $[K^+]_o$ was raised from 4 to 7 mM fatigue was exacerbated, and when $[K^+]_o$ was lowered to 2 mM the fatigue was slowed in SOL. The relative force at 100 s of stimulation (mean value) was 50% initial at 2 mM $K^+$, 40% at 4 mM $K^+$, and 23% at 7 mM $K^+$. The relationship between peak tetanic force and $[K^+]_o$ (8-12 mM) was established in non-fatigued muscles. At raised $[K^+]_o$ (i) increasing the stimulation pulse strength (20 to 26 V) increased force in SOL but not EDL, (ii) increasing the stimulation pulse duration (0.1 to 0.15 to 0.25 ms) progressively restored force, but to a greater extent in SOL than EDL, and (iii) stimulating with transverse wire rather than parallel plate electrodes resulted in a greater force loss, especially in SOL. When $[K^+]_o$ was raised to 8 mM and $[Na^+]_o$ lowered to 100 mM, synergistic depressive effects occurred on peak tetanic force in both SOL and EDL, i.e., the peak tetanic force-$[K^+]_o$ relationship shifted leftwards towards lower $[K^+]_o$. Force could then be partially restored by lowering the stimulation frequency but only in SOL. Raising the $[Ca^{2+}]_o$ (1.3 to 2.5 to 10 mM) shifted the peak tetanic force-$[K^+]_o$ relationship in SOL rightwards towards higher $[K^+]_o$. Conversely, lowering $[Ca^{2+}]_o$ shifted the relationship leftwards, e.g., at 8 mM $K^+$ the peak force was 77% initial at 1.3 mM $Ca^{2+}$ and 52% initial at 0.5 mM $Ca^{2+}$.

In summary, moderate changes of $[K^+]_o$ clearly influence the rate of fatigue in SOL which implicates $K^+$ in the fatigue process. The peak tetanic force-$[K^+]_o$ relationship in non-fatigued muscle depended on the stimulation pulse parameters and stimulation electrode type, and more so in SOL than EDL. Muscles are more susceptible to $K^+$-induced force depression at slightly lowered $[Na^+]_o$ and slightly lowered $[Ca^{2+}]_o$ which is a likely physiological scenario. Thus, when such ionic shifts occur simultaneously during exercise, they are likely to act together to impair muscle force production, i.e., cause fatigue.

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# The effect of dithiothreitol (DTT) application on isolated mouse muscle fatigued at 37°C

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We have previously shown that muscle fatigue at 37°C *in vitro* is associated with a reduction in calcium sensitivity and that this process could be prevented by the antioxidant Tiron (Moopanar & Allen, 2005). Previous studies have also found that application of the reducing agent dithiothreitol (DTT) to the rat diaphragm improves recovery post-fatigue (Diaz *et al.*, 1998). The aim of the current study was to determine whether DTT could reverse the effect of temperature- and fatigue-induced myofibrillar desensitization.

Single muscle fibres were isolated from the foot of balb-C mice and were attached to a force transducer. The temperature in the muscle chamber was raised to 37°C prior to each experiment. Fibres were microinjected with indo-1 to measured intracellular calcium ($[Ca^{2+}]_i$), and were stimulated at a range of frequencies (20, 30, 50, 70 and 100 Hz and 100 Hz in the presence of 10 mM caffeine) to establish myofibrillar sensitivity to calcium. The preparation was then fatigued and sensitivity was immediately reassessed. Finally, the muscle preparation was treated with DTT (0.5 mM) for two minutes and myofibrillar sensitivity was again tested.

The $Ca_{50}$, which is the level of $[Ca^{2+}]_i$ that produces half maximum force and a measure of $Ca^{2+}$-sensitivity, was initially found to be $649 \pm 40$ nM (n=18). This value was increased post fatigue to $872 \pm 40$ nM (n=9). There was no change to the $Ca_{50}$ in the absence of fatiguing stimuli. Application of DTT to the fatigued muscle caused the $Ca^{2+}$- sensitivity to return to prefatigue values ($683 \pm 40$ nM (n=6)). In order to determine whether the decline in muscle function was due to a change in maximum calcium activated force ($F_{max}$), fibres were stimulated at 100 Hz in the presence of caffeine (10 mM). There was no significant change in $F_{max}$.

These results indicate that the process of myofibrillar desensitization at 37°C requires repeated stimulation to occur. In addition, we show that the desensitization can be reversed by DTT. This suggests that a protein involved in calcium sensitivity has critical S-H groups which can be oxidized to form disulphide bonds (S-S) with loss of $Ca^{2+}$-sensitivity. This reaction can be reversed by the reducing agent DTT.

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# Cytoplasmic ATP-sensing CBS domains regulate gating of skeletal muscle ClC-1 chloride channels

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ClC proteins are a family of chloride channels and transporters that are found in a wide variety of prokaryotic and eukaryotic cell-types. The mammalian voltage-gated chloride channel ClC-1 is important for controlling the electrical excitability of skeletal muscle. Reduced excitability of muscle cells during metabolic stress can protect cells from metabolic exhaustion and is thought to be a major factor in fatigue. Here we identify a novel mechanism linking excitability to metabolic state by showing that ClC-1 channels are modulated by ATP. The high concentration of ATP in resting muscle effectively inhibits ClC-1 activity by shifting the voltage-gating to more positive potentials. ADP and AMP had similar effects to ATP but IMP had no effect, indicating that the inhibition of ClC-1 would only be relieved under anaerobic conditions such as intense muscle activity or ischaemia, when depleted ATP accumulates as IMP. The resulting increase in ClC-1 activity under these conditions would reduce muscle excitability, thus contributing to fatigue. We show further that the modulation by ATP is mediated by cystathionine-β-synthase-related (CBS) domains in the cytoplasmic C-terminus of ClC-1. This defines a function for these domains as gating-modulatory domains sensitive to intracellular ligands, such as nucleotides, a function that is likely to be conserved in other ClC proteins.

# Modelling diffusive $O_2$ supply to isolated muscle preparations

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The sole source of oxygen ($O_2$) to an isolated muscle preparation is by diffusion from the muscle surface. A.V. Hill (1928) derived equations that described the spatial and temporal dependencies of intramuscular $O_2$ partial pressure ($PO_2$) for muscles of various shapes and showed solutions for frog muscles. The purpose of the current study was to use Hill's diffusion equation for cylindrical muscles to assess the adequacy of diffusive $O_2$ supply into commonly-used mammalian cardiac and skeletal muscles during typical experimental contraction protocols. The diffusion equation was solved numerically to give (1) the maximum $O_2$ diffusion distances during steady-state activity at various contraction duty cycles and temperatures and (2) the time, in more severe contraction protocols, before central anoxia would develop during the rest-to-work transition. The effects of incorporating myoglobin-facilitated $O_2$ diffusion were also assessed.

The analysis was performed for soleus, extensor digitorum longus (EDL) and cardiac papillary muscles from the rat and mouse and for frog sartorius muscle using published metabolic data. The results indicated that for all the preparations considered, it would be difficult to ensure adequate $O_2$ supply using whole muscles; adequate $O_2$ supply can only be ensured over a reasonable range of duty cycles by using preparations with radii substantially smaller than those of whole muscles. Reducing experimental temperature is an effective strategy for enhancing $O_2$ supply to skeletal muscle. However, diffusive $O_2$ supply to isolated papillary muscles is not greatly affected by temperature because increasing temperature has opposite effects on active and resting metabolic rates of cardiac muscle. Taking account of $O_2$ supply from myoglobin had only minimal effects on oxygenation under typical isolated muscle conditions.

Hill, A.V. (1928) *Proceedings of the Royal Society of London Series B: Biological Sciences* 104, 39-96.