AuPS/ASB Meeting - Adelaide 2010

Symposium: Skeletal muscle: the coupling of excitation to contraction

Monday 29th November 2010 - Hickinbotham Hall - 11:00

Chair: Nicole Beard

Voltage-dependent and -independent Ca^{2+} entry into skeletal muscle during excitation-contraction coupling

B.S. Launikonis, School of Biomedical Sciences, The University of Queensland, Brisbane, QLD 4072, Australia.

Excitation-contraction coupling (EC coupling) is the process that links the activation of the surface membrane of a muscle to the force response produced. This process is reliant on a rapid and large change in cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_{cyto}$) due to release activated from the internal Ca^{2+} store, the sarcoplasmic reticulum (SR). The change in $[Ca^{2+}]_{cyto}$ regulates the movement of the contractile proteins in the muscle to produce force. Also, during EC coupling there can be an input of Ca^{2+} across the surface membrane, or from the invagination of this membrane, the tubular (t-) system. In skeletal muscle, this influx of Ca^{2+} is not strictly required for a contracture to occur, but its importance may be in the longer-term regulation of function or may indeed have more subtle, immediate consequences that are not clear from present measurements. Several pathways can be responsible for an influx of Ca^{2+} into skeletal muscle fibres during EC coupling. These can be voltage-dependent, including L-type Ca^{2+} current, action potential-activated Ca^{2+} current (APACC) and excitation-coupled Ca^{2+} entry (ECCE); or voltage-independent, including store-operated Ca^{2+} entry (SOCE), stretch-activated Ca^{2+} entry or otherwise activated to enter through transient receptor protein (TRP) channels. It should be noted that the conditions that prevail under bouts of EC coupling are the conditions that activate voltage-dependent and –independent Ca^{2+} entry during EC coupling in skeletal muscle (Allen *et al*. 2005; Launikonis *et al.*, 2010).

The nature of these voltage-dependent and -independent pathways in skeletal muscle have been examined by a number of groups using either fully differentiated fibres or myotubes. A problem that does arise here is that properties derived on myotubes are often assumed to be very similar to that in the fully differentiated muscle. There are significant differences in myotube and adult fibre physiology and membrane ultrastructure, making extrapolation of results derived on one cell to other not a simple matter. The examination of Ca^{2+} currents in myotubes may have been necessary in some instances because conventional electrophysiological methods are difficult or do not have the sensitivity to measure such tiny Ca^{2+} currents in adult fibres. Furthermore, the use of pharmacological agents commonly used to block Ca^{2+} entry pathways are, unfortunately, non-specific in most cases. A combination of these factors may have lead to a misrepresentation of certain pathways existing in myotubes or adult fibres.

In adult, mammalian skeletal muscle fibres it has been possible to image Ca^{2+} in the lumen of the t-system using a low-affinity Ca^{2+}-sensitive fluorescent dye. The dye is trapped in the t-system of skinned fibres, where the surface membrane has been removed by microdissection. With this preparation, Ca^{2+} movement across the t-system membrane is visualized as a net change in the Ca^{2+}-dependent fluorescence signal emitted from the t-system lumen. This can be monitored in conjunction with a spectrally separate Ca^{2+}-sensitive dye in the cytoplasm to monitor changes in $[Ca^{2+}]_{cyto}$, which will be predominately due to fluxes across the SR membrane. This preparation has allowed the identification and characterization of SOCE during voltage-independent Ca^{2+} release and also APACC, which has been identified and observed to occur during single and trains of action potentials in adult mammalian skeletal muscle fibres.

Allen DG, Whitehead NP, Yeung EW. (2005) *Journal of Physiology*, **567**: 723-735.
Launikonis BS, Murphy RM, Edwards JN. (2010) *Pflügers Archiv*, **460**: 813-823.

Coupling and uncoupling of the DHPRs and Ca^{2+} release channels in skeletal muscle fibres

T.L. Dutka, Department of Zoology, La Trobe University, VIC 3086, Australia.

This study focused on the importance of transverse tubular (T)-system potential on excitation-contraction (E-C) coupling and the consequences of disrupted dihydropyridine receptor (DHPR)-ryanodine receptor (RyR) coupling on sarcoplasmic reticulum (SR) Ca^{2+} handling. Male Long-Evans hooded rats were killed by anaesthetic overdose (4 % v:v isoflurane) and EDL muscles swiftly excised and immersed in paraffin oil at resting length. Individual fibres were mechanically skinned, connected to a force transducer (stretched to 120 %) and immersed in a standard K-HDTA-based solution. All solutions contained as follows (in mM); 1 free Mg^{2+}, 8 ATP; 10 creatine phosphate, 55, 66 or 126 K^+, at pH 7.1, and were equilibrated to room temperature (~23°C). Single fibres were electrically stimulated (75 V cm^{-1}, 1 ms pulse) to produce twitch or tetanic (50 and 100 Hz) force responses. Additionally, paired pulses with differing intervals (0-50 ms) were applied to determine the repriming period of sodium channels in the T-system membrane (Dutka & Lamb, 2007a).

The importance of T-system membrane potential. Partial long-lasting depolarization of the T-system membrane (achieved by lowering the cytoplasmic [K^+]) reduces tetanic force by impairing AP repriming and preventing the generation of APs in quick succession thus reducing DHPR-mediated Ca^{2+} release through RyR1. This reduced muscle excitability was not due to DHPR inactivation because lowering the frequency of stimulation partly ameliorated the effect. Furthermore, when fibres were chronically partially depolarized, PCr/CK ATP regeneration system was not optimal and slowed the repriming period of T-system Na^+ channels. Addition of Phospho(enol) pyruvate (PEP) hastened AP repriming and hence, improved T-system excitability (Dutka & Lamb, 2007b) implying Na^+/K^+-ATPases function better when ATP is produced glycolytically in the triad junction instead of by PCr/CK elsewhere.

The importance of strict DHPR-RyR1 coupling. Disruption of the interaction between DHPRs and RyR1 appears to cause an irreversible Ca^{2+}-leak from the SR through RyR1. When DHPR are in there *in situ* state they have been shown to suppress RyRs activity (*i.e.* Ca^{2+} spark) at rest in cultured mammalian muscle (Zhou *et al.*, 2006). Similarly, Weiss *et al.*, (2004) showed that a mutation in the DHPR cytoplasmic III-IV loop of alpha (1S) subunit (R1086H) greatly enhanced RyR1 sensitivity to activation by voltage/caffeine, indicating that DHPRs had a negative allosteric modulatory effect on RyR1. Furthermore, high Ca^{2+}-induced uncoupling of DHPRs from RyRs has also been shown to cause a similar irreversible SR Ca^{2+} leak in mechanically-skinned fibres (Lamb & Cellini, 1999). In the experiments described here, immediate application of S-nitrosoglutathione $GSNO_{imm}$ (a reactive oxygen and nitrogen species) to the fibres reduced twitch and tetanic force responses (15 and 10 % respectively) even though it caused an ~0.1 pCa unit increase in contractile Ca^{2+}-sensitivity. These reductions to AP-mediated force responses were due to impaired DHPR-RyR coupling and concomitantly $GSNO_{imm}$ treatment also caused Ca^{2+} leakage through RyRs, which was not reversible with DTT. The Ca^{2+} leak through RyRs was substantially blocked by raising the free [Mg^{2+}] from 1 to 10 mM. The irreversible Ca^{2+} leak caused by DHPR uncoupling observed by others (Lamb & Cellini, 1999; Weiss *et al*., 2004; Zhou *et al*., 2006) is strikingly similar to that observed here caused by exposure to $GSNO_{imm}$, which might help explain the ROS-mediated loss of signal transduction observed during prolonged low frequency fatigue (Bruton *et al*., 2008).

Bruton JD, Place N, Yamada T, Silva JP, Andrade FH, Dahlstedt AJ, Zhang SJ, Katz A, Larsson NG & Westerblad H. (2008). *Journal of Physiology* **586,** 175-184.

Dutka TL & Lamb GD. (2007a). *American Journal of Physiology. Cell Physiology* **292,** C2112-2121.

Dutka TL & Lamb GD. (2007b). *American Journal of Physiology. Cell Physiology* **293,** C967-977.

Weiss RG, O'Connell KM, Flucher BE, Allen PD, Grabner M & Dirksen RT. (2004). *American Journal of Physiology. Cell Physiology* **287,** C1094-1102.

Zhou J, Yi J, Royer L, Launikonis BS, Gonzalez A, Garcia J & Rios E. (2006). *American Journal of Physiology. Cell Physiology* **290,** C539-553.

One is enough: RyR1 allele-specific gene silencing in mouse models of central core disease (CCD) and malignant hyperthermia (MH)

R.T. Dirksen, R.E. Loy, J.D. Lueck and M. Mostajo-Radji, University of Rochester, Department of Pharmacology and Physiology, Rochester, NY 14642, USA. (Introduced by Angela Dulhunty)

Central Core Disease (CCD) and Malignant Hyperthermia (MH) are linked to single amino acid substitutions in the skeletal muscle Ca^{2+} release channel, the type 1 ryanodine receptor (RyR1). We focus on two autosomal dominant (AD) RyR1 mutations, Y522S (YS) and I4898T (IT), which cause MH and CCD, respectively. The AD mode of inheritance and data indicating knock-out of one RyR1 allele is well-tolerated in mice led us to hypothesize that allele-specific gene silencing (ASGS) of the mutant allele would rescue RyR1 functional defects in skeletal muscle cells from YS and IT knock-in mice.

We evaluated the functional consequences of allele-specific silencing in YS and IT muscle cells using short interfering RNAs (siRNAs). To screen potential siRNAs for relative knockdown efficacy and allele specificity, we generated cDNAs encoding fusion proteins derived from wild type (WT) (Venus-Exons-3XFLAG) and either YS or IT mutation-containing (Cherry-Exons-3XHA) exons. Simultaneous transfection of these cDNAs and siRNAs into HEK293 cells and subsequent evaluation of mRNA (semi-quantitative RT-PCR) and protein levels (fluorescence microscopy and western blotting) was used to determine knockdown efficacy and allele-specificity prior to functional rescue experiments. Myotubes derived from heterozygous YS mice (YS/+) exhibit ~4-fold increase in caffeine sensitivity (EC50 values were 0.5mM and 2.3mM for YS/+ and WT, respectively). Treatment of YS/+ myotubes with a YS-selective siRNA, normalized caffeine sensitivity (EC50 = 2.5mM) without decreasing peak caffeine-induced release. Similarly, YS-selective siRNA treatment rescued the increased voltage sensitivity of Ca^{2+} release in YS/+ myotubes determined in perforated-patch clamp experiments (VF1/2: WT = -18mV, YS/+ scrambled = -35mV, YS/+ YS-selective = -18mV). These results indicate that ASGS represents a promising approach for normalization of RyR1 function in MH and CCD. Similar functional rescue experiments in adult skeletal muscle fibres are currently underway.