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Voltage-Dependent Stochastic Gating Models of TRIC-B Channels

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TRIC-A and TRIC-B are two, related, trimeric intracellular cation channels present in sarcoplasmic/endoplasmic reticulum (SR) and are thought to provide counter-current for SR Ca^{2+}-release. TRIC-B knockout mice die immediately after birth demonstrating the importance of this isoform [Yazawa et al., 2007, *Nature*, **448**, 78-82]. To study the distinct single-channel gating behaviour of TRIC-B, we incorporated skeletal muscle light SR from TRIC-A knockout mice into artificial membranes under voltage-clamp conditions in symmetrical 210 mM K-PIPES, pH 7.2. We developed Markov models of TRIC-B gating, with up to 4 distinct sub-conductance states (S_1-S_4), using both QuB [Qin F., 2004, *Biophys J.,* **86**(3), 1488-501] and our own software. Our models incorporate different connectivity schemes to account for the intrinsic variability in gating that was observed between different channels. Despite the variability, some obvious trends emerged. TRIC-B activity was higher at positive than at negative holding potentials. At positive potentials, the majority of channels exhibited long bursts of openings where predominant gating transitions were between the full open state and S_1, the largest sub-conductance state. Some channels, however, gated preferentially in sub-states S_3 and S_4, only visiting the full open state briefly. At negative potentials, channel activity consisted primarily of brief transitions between sub-conductance states. Closed lifetime distributions at positive potentials comprised of fast components ($\tau \approx 1$ ms), corresponding to brief transitions from the full open state, as well as slower components corresponding to inter-burst intervals. At negative potentials, inter-burst intervals were orders of magnitude longer demonstrating that the frequency of channel opening is heavily dependent on voltage. It will be important to develop comprehensive models of TRIC-B channel gating in order to fully understand the role of this important ion-channel in intracellular Ca^{2+}-release.

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Different Modes of Interaction between Junctin and Ryanodine Receptors

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The sarcoplasmic reticulum (SR) Ca^{2+} store in skeletal and cardiac muscle is important in regulating Ca^{2+} release and the strength of contraction, through key proteins including the ryanodine receptor (RyR), calsequestrin (CSQ) and intrinsic membrane co-proteins, junctin and triadin, which link CSQ to RyRs. Junctin contributes to SR Ca^{2+} release, store size, CSQ2 depolymerisation and activates RyR1 and RyR2 in lipid bilayers when added to the luminal solution (1). Two regions of junctin's luminal domain interact with luminal loops on RyR2 (2), indicating that junctin modulates RyR2 from within the SR lumen. Similar experiments have not been done with RyR1 and cytoplasmic interactions between junctin and RyRs have not been fully explored in RyR1 or RyR2.

To explore these interactions, we isolated full length junctin (FLjun) from skeletal muscle, expressed the C-terminal domain of junctin (Cjun) in E. coli and synthesised the Njun peptide corresponding to cytoplasmic N-terminal domain. Deletion constructs of cytoplasmic and luminal domains of RyR1 were expressed in HEK293 cells. Affinity chromatography and co-immunoprecipition confirmed that the FLjun binds to RyR1 and RyR2 and revealed that Njun binds to cytoplasmic regions of RyR1, while Cjun binds to two luminal loops on RyR1, one between TM5 and TM6 (aa4583-4626), and the second in a construct containing the pore helix and adjacent luminal residues (aa4861-4910). Results from lipid bilayers (Li, Mirza, Beard, Dulhunty abstract) indicate that junctin regulates RyR1 and RyR2 via cytoplasmic and luminal interactions and, unexpectedly, the cytoplasmic interaction is functionally dominant. Njun binding to the cytoplasmic domain of RyR1 must underly this action. We predict that Njun will also bind to the cytoplasmic domain of RyR2.

1.Wei L et al. (2009) Int J Biochem Cell Bio 41, 2214.
2.Altschafl et al. (2011) J Physiol 589, 6063.

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A Cytoplasmic Interaction between Junctin and RyRs with Major Consequences for RyR1 and RyR2 Activity In Vitro

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Junctin is an intrinsic sarcoplasmic reticulum (SR) membrane protein in cardiac and skeletal muscle. The bulk of the protein is within the lumen of the SR where it binds to ryanodine receptor (RyR) calcium release channels and regulates Ca^{2+} release from the SR. We have previously reported that junctin added to the luminal solution increases RyR1 activity in lipid bilayers [1].

The contributions of the luminal and cytoplasmic domains of junctin to RyR activation have not previously been explored, although it is assumed that activation depends on interactions between their luminal domains. To explore this further, we expressed junctin's C-terminal domain (Cjun), synthesised a cytoplasmic N-terminal domain peptide (Njun) and isolated full length junctin (FLjun) from skeletal muscle. Consistent with previous findings [1,2], FLjun in luminal solutions activated purified RyR1 and RyR2. We predicted that luminal Cjun would similarly activate RyRs if the proteins interact only via their luminal domains. Unexpectedly, (a) luminal Cjun strongly inhibited RyR1 and RyR2 and (b) cytoplasmic Njun activated channels to a greater degree than luminal FLjun and also bound to cytoplasmic fragments of RyR1 [3]. Neither luminal Njun, nor scrambled Njun in cytoplasmic solution influenced channel activity. To explore cytoplasmic Njun and luminal Cjun effects further, we added both domains sequentially to channels. Excess activation by Njun was reduced to FLjun levels by adding Cjun, while significant inhibition by Cjun added first was reduced by Njun, it remained significantly different from the activation by FLjun. Therefore cytoplasmic interactions between junctin and RyRs determines the overall effect of junctin on channel activity.

1. Wei et al. (2009) *Int J Biochem Cell Biol***41;**2214.
2. Gyorke et al. (2004) *Biophys J* **86**:2121.
3. Mirza et al. (2013) Biophysics abstract.

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Human Aging and Expression of Proteins Interacting with the Ryanodine Receptor in Skeletal Muscle

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Sarcopenia is characterised by reduced muscle mass and reduced force that is only partially attributable to muscle atrophy. Fiber type distribution changes with aging (fast twitch decreases, slow twitch increases). Excitation-contraction coupling (ECC) may be impaired by an uncoupling of the dihydropyridine receptor (DHPR) and the ryanodine receptor (RyR1), possibly due to decreased expression of the DHPR α_{1s} subunit (Delbono et al., J Membr Biol. 1995;148:211-22). The DHPR β_{1a} subunit, which may contribute to ECC (Rebbeck et al., Biophys J. 2011;100:922-30), increases (Taylor et al., Aging cell 2009;8:584-94). The 12KDa FK506 binding protein (FKBP12) stabilizes RyR1 and its dissociation may contribute to decreased ECC (Andersson et al., Cell Metab.2011; 14:196-207). Previous studies have used animal models; here we investigate the expression of these proteins in aging human muscle.

Human muscle samples were obtained from 42 male and female donors (age 40-90) undergoing knee (vastus medialis) and hip (gluteus minimus or gluteus medius) replacements. Results of fiber type distribution in muscle homogenates from female subjects shows fast twitch fibers decreasing and slow twitch increasing with age. Preliminary data shows that levels of FKBP12 relative to RyR1, determined in microsomal vesicles using western blot and densitometry decrease with age. Expression levels of the DHPR α_{1s} and β_{1a} subunits relative to that of actin determined in muscle homogenates using western blot and densitometry are being analysed. We find that β_{1a} subunit is mostly in the homogenate and not associated with membrane fractions. The preliminary data indicates that the levels of several of the proteins that associate with RyR1 and modulate its activity change with age and could affect the release of Ca^{2+} during ECC and muscle function in human sarcopenia.

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Carboxyl-Terminal Domain of DHPR β1A is Essential for DHPR Tetrad Formation

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Lack of expression of DHPR-β1a subunit in skeletal muscle cells severely compromises the DHPRs-RyR1 stereospecific association that results in the assembly of ordered DHPR tetrads arrays. This, in turn results in the disruption of the EC-coupling DHPR-RyR signal. Previously, using expression of β1a subunits with progressive truncations of the carboxyl-terminal (C-term) domain in β1-null myotubes we found that aa residues 488-512 of β1a were critical to the restoration of the EC-coupling signal. Here we explore the effect of C-term truncations on DHPR tetrad formation and whether the ability of different truncated β1a to restore EC-coupling correlate with their ability to restore DHPR

tetrad array. cDNA constructs carrying wt-β1a and C-term truncated clones were transiently expressed in mouse β1-null myotubes and tested for their ability to restore depolarization-induce Ca^{2+} release. Cells were then fixed and freeze-fractured to assess DHPR tetrad arrangement. Our data show that expression of wt-β1a fully restores EC-coupling, as well as normal DHPR tetrads arrays. Construct β14, lacking 14 aa of the C-terminal tail of β1a, still supports normal EC-coupling and also shows normal arrangement of tetrads. On the other hand, β14 clone containing several Leu/Ala mutations (β14L/A) that prevent EC-coupling failed to restore tetrad arrays. Immunofluorescence staining confirmed that all clones were expressed and targeted to the plasmalemma. These results suggest that 1) C-term tail of β1a plays an essential role in permitting and/or maintaining the precise positioning of four DHPRs relative to the four RyR subunits; 2) DHPR positioning in tetrads is essential to EC-coupling and 3) the molecular structure (aa sequence) of the C-term tail is relevant for the role of β1a in the DHPR-RyR structural and functional relationship.

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Interactions between Dihydropyridine β1A Subunit and Ryanodine Receptor Isoforms

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Skeletal excitation contraction (EC) coupling requires a physical interaction between the L-type voltage gated dihydropyridine receptor (DHPR) in the transverse tubule membrane and the type 1 ryanodine receptor (RyR1) in the sarcoplasmic reticulum membrane. The C-terminus of the DHPR β_{1a} subunit influences EC coupling in skeletal myotubes (Beurg et al., Biophys J. 1999;77:2953-67, Sheridan et al., Biophys J. 2003;84:220-37, Sheridan et al., Biophys J. 2004;87:929-42). This may be through a direct interaction with RyR1, as we identified a hydrophobic interaction with L^{496}, L^{500} and W^{503} in the last 35 residues of β_{1a} that increases RyR1 channel activity in phospholipid bilayers (Rebbeck et al., Biophys J. 2011;100;922-30, Karunasekara et al., FASEB J. 2012). Additionally, the K^{3495}KKRR_ _R^{3502} motif in a RyR1 fragment (M^{3201}-W^{3661}) pulls down β_{1a} and facilitates EC coupling (Cheng et al., PNAS USA. 2005;102:19225-30). Our preliminary data indicate that substitution of these 6 basic residues with glutamines, abolishes the effect of β_{1a} on the full length RyR1. We also show that β_{1a} increases RyR2 activity in a similar manner to RyR1 except for significantly less activation of RyR2 by 10nM β_{1a} (10 and 100nM β_{1a} subunit increased RyR2 activity by 1.8- and 2.8-fold, in contrast to 2.6- and 2.8-fold with RyR1). Curiously, this reduced activation of RyR2 by 10nM β_{1a} is similar to lesser activation of the embryonic alternative spliced (ASI(-)) RyR1 isoform by 10nM β_{1a}, that lacks residues A^{3481}-Q^{3485}, compared with activation of adult (ASI(+)) RyR1 isoform by 10nM β_{1a}. Notably, as rabbit RyR2 lacks 4 of the 5 ASI residues. We conclude that β_{1a} may bind to a hydrophobic pocket conserved in the RyR1 and RyR2 and that this region is influenced by the presence of the alternatively spiced ASI residues and the polybasic K^{3495}-R^{3502} motif.

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3D Structural Illumination Microscopy of the Skeletal Muscle Excitation-Contraction Coupling Macromolecular Complex

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To study the structural organization of protein components associated with the membrane compartments involved in skeletal muscle excitation-contraction coupling, we stained enzymatically dissociated mouse FDB fibers with commercially available specific antibodies. Imaging was based on 3 dimensional structured illumination microscopy (3D-SIM, Zeiss Elyra system using a 63x/1.4 Plan-Apochromat lens and an EMCCD camera) or conventional confocal laser scanning microscopy for comparison (Zeiss LSM700). We investigated the localization pattern and distribution of RyR, Cav1.1 and of other protein components involved in excitation-contraction coupling. As expected, imaging of FBD fibers stained with anti -RyR Ab by conventional confocal microscopy revealed highly ordered band-line structures regularly distributed along the sarcomeres which strongly overlapped with Cav1.1. Analysis of the same FDB preparation at higher resolution (Dx-y 110-130 nm and Dz 280-340 nm) by 3D-SIM revealed a more distinct pattern of distribution. RyRs form 100 nm clusters, which are regularly separated and distributed along the longitudinal axis of the fiber. Anti-Cav 1.1 Ab stained clusters having a sarcomeric distribution co-localizing with RyRs. Cav 1.1 Ab also stained structures adjacent to but not overlapping with RyRs. These results suggest the power of the 3D-SIM approach to gain further insight into the structural organization of sarcotubular membranes in normal and diseased condition.

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Super-Resolution Localization and Distribution of Proteins within the Mammalian Couplon

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We have used 3D dSTORM to characterize the distribution and localization of the ryanodine receptor (RyR), the L-type calcium channel (Cav1.2) and the sodium-calcium exchanger (NCX) within the couplon of the rat cardiomyocyte. The images have a resolution of 20nm in X and Y and 50 nm in Z, and cover areas of up to 1200 μm^2 in XY with depths of up to 700nm in Z. In the case of RyR, hundreds of individual clusters could be identified and characterized. The clusters varied greatly in both size and structure; their internal structure showing little evidence of the checkerboard arrangement that has been thought to be predominant. RyR clusters were identified both in the transverse and axial tubules and in some cases could be seen to be separated by a gap typical of the width of a t-tubule. In general, the clusters of Cav1.2 were smaller and far denser than their RyR counterparts, with their centers appearing to be tightly packed. NCX was much more widely distributed than either RyR or Cav1.2 and formed a dense carpet along the cell surface with little clustering or identifiable detail. While small isolated clusters of NCX were present in the tubular system, there were other regions where the labeling was more widespread and the clusters were poorly defined.

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TRIC-B Channels Exhibit Labile Gating Properties; Evidence from TRIC-A Knockout Mice

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Trimeric intracellular cation channels (TRIC-A and TRIC-B) are located in the sarcoplasmic/endoplasmic reticulum (SR/ER) of most cells. Identifying the distinct biophysical properties of TRIC-A and TRIC-B is difficult because both channels are present in most tissues, yet this is crucial for delineating their individual physiological roles. Skeletal muscle SR vesicles (LSR) from TRIC-A knockout mice were incorporated into artificial membranes under voltage-clamp conditions as previously described [Pitt et al., 2010, *Biophys. J.* **99**, 417-426] and single-channel recordings of native TRIC-B were obtained in symmetrical solutions of 210 mM K-PIPES, pH 7.2. The maximum single-channel conductance of TRIC-B was 197 ± 2 pS (n=32; SEM). TRIC-B channels always exhibited sub-conductance gating states and while these were of a variable nature, the predominant sub-conductance levels were found at 156 ± 3 pS (n=17; SEM), 125 ± 2 pS (n=19; SEM), 96 ± 2 pS (n=19; SEM) and 62 ± 2 pS (n=27; SEM). TRIC-B channel gating was voltage-dependent and channels were inhibited at negative holding potentials. For example, the probability of dwelling in the full open channel level was 0.0478 ± 0.0194 at +30 mV but only 0.0010 ± 0.0008 at −30 mV (n=6; SEM; $^*p<0.05$). Application of 300 mM KCl to the cytosolic channel side produced a parallel shift in the current-voltage relationship and a shift in the reversal potential to approximately −20 mV indicating that TRIC-B is not permeable to anions. Our study demonstrates that the single-channel properties of TRIC-B channels are exceptionally labile. This intrinsic variability may be important for enabling flexible physiological regulation of monovalent cation fluxes across the SR membrane.

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Cardiac Ca^{2+} and Free Radical Disturbances in Mice with Arthritis

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Rheumatoid arthritis (RA) is a common inflammatory disease that afflicts ~1% of the population and is more common in women than in men. Cardiovascular disease is the leading cause of premature mortality in patients with RA. Still,