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# Alternative splicing of RyR1 alters the efficacy of skeletal EC coupling

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ABSTRACT

Alternative splicing of ASI residues (Ala$^{3481}$–Gln$^{3485}$) in the skeletal muscle ryanodine receptor (RyR1) is developmentally regulated: the residues are present in adult ASI(+)RyR1, but absent in the juvenile ASI(−)RyR1 which is over-expressed in adult myotonic dystrophy type 1 (DM1). Although this splicing switch may influence RyR1 function in developing muscle and DM1, little is known about the properties of the splice variants. We examined excitation-contraction (EC) coupling and the structure and interactions of the ASI domain (Thr$^{3471}$–Gly$^{3500}$) in the splice variants. Depolarisation-dependent $Ca^{2+}$ release was enhanced by >50% in myotubes expressing ASI(−)RyR1 compared with ASI(+)RyR1, although DHPR L-type currents and SR $Ca^{2+}$ content were unaltered, while ASI(−)RyR1 channel function was actually depressed. The effect on EC coupling did not depend on changes in ASI domain secondary structure. Probing RyR1 function with peptides possessing the ASI domain sequence indicated that the domain contributes to an inhibitory module in RyR1. The action of the peptide depended on a sequence of basic residues and their alignment in an α-helix adjacent to the ASI splice site. This is the first evidence that the ASI residues contribute to an inhibitory module in RyR1 that influences EC coupling. Implications for development and DM1 are discussed.

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## 1. Introduction

The ryanodine receptor (RyR) $Ca^{2+}$ release channel contains two developmentally regulated splice regions, ASI and ASII [1]. The ASI residues (Ala$^{3481}$–Gln$^{3485}$) are lacking in the juvenile RyR1 isoform (ASI(−)RyR1), but present in the adult isoform (ASI(+)RyR1). Little is known about the properties of these splice variants, although it is attractive to consider that the isoforms are tailored to differential excitation-contraction (EC) coupling requirements in embryonic and adult muscle since the physical interaction between the dihydropyridine receptor (DHPR) and RyR1 that supports skeletal type EC coupling is established late in development [2]. The type 3 RyR (RyR3) is strongly expressed in neonatal skeletal muscle and supports EC coupling through $Ca^{2+}$-activated $Ca^{2+}$ release [3,4]. The juvenile RyR1 splice variant is also expressed but its contribution to neonatal EC coupling has not been assessed since the characteristics of EC coupling in the presence of ASI(−)RyR1 are not known. Therefore we examined EC coupling in mytotubes expressing either ASI(−)RyR1 or ASI(+)RyR1.

Understanding the effects of the variable splicing is also important for understanding the myopathy in myotonic dystrophy type 1 (DM1), since the juvenile ASI(−)RyR1 splice variant is preferentially expressed in adults suffering from this disorder [5]. DM1 is caused by a CTG expansion in the 3′ non-coding region of the myotonic dystrophy protein kinase (DMPK) gene, which leads to excess skeletal muscle excitability (myotonia) and muscle degeneration (myopathy). The myotonia is attributed to alternative splicing of ClC-1 mRNA [6–8] leading to loss of chloride channel function [9,10]. The myopathy is less well understood and attributed to a variety of causes including changes in EC coupling [11,12]. The impact of the juvenile RyR1 splice variant on EC coupling in DM1 has not been evaluated, once again because EC coupling mediated by ASI(+) and ASI(−) has not previously been carefully compared.

It seemed likely that the properties of EC coupling may be influenced by alternative splicing of the ASI region because the greater ASI region (Thr$^{3471}$–Gly$^{3500}$) is a significant regulatory domain. This is indicated by observations that changes in the ASI region influence RyR1 open probability [5] and that the ASI region: (1) contributes to an inhibitory module within RyR1 [14] and (2) binds to the DHPR

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β subunit [13]. The DHPR β subunit binds to a sequence of basic residues adjacent to the variably spliced residues and mutation of these basic residues results in depression of EC coupling [13]. This change in EC coupling has been attributed to disruption of DHPR β subunit binding to RyR1 [13], but could equally well be due to the mutation itself altering RyR1 activity during EC coupling. Indeed 4-chloro-*m*-cresol (4-CMC) responses shown in [13] suggest that mutation of basic β-binding residues drastically alters RyR1 responses to this ligand. Therefore, in addition to assessing the effect of the variable splicing on EC coupling and the secondary structure of the ASI domain, we have examined the role of the basic β-binding residues in determining structure of the ASI region and in regulating RyR1 activity. The role of these basic residues in regulating RyR1 activity was examined using the peptide probe approach used in previous studies that identified the ASI inhibitory module in RyR1 [14].

## 2. Methods

### 2.1. Preparation and nuclear cDNA injections of dyspedic myotubes

Primary cultures and nuclear cDNA injections of myotubes from RyR1-null (dyspedic) mice were performed as previously described [5]. Co-injection of a cDNA encoding cherry red fluorescent protein (0.005 μg/μl; [15]) was used to identify injected myotubes with excitation (540 nm) using a monochrometer-based illumination system (TILL Photonics, Pleasanton, CA, USA).

### 2.2. Intracellular $Ca^{2+}$ measurements in myotubes

Myotubes were loaded for 25 min at room temperature with 5 μM fura-FF AM (TEFLABS, Austin, TX, USA), a low to moderate affinity ($K_d$ = 5.5 μM) $Ca^{2+}$ dye, in a Ringer's solution containing: 146 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH7.4. Fura-FF loaded myotubes were alternately excited at 340 and 380 nm (510 nm emission) every 350 ms (8 ms exposure per wavelength and 4 × 4 binning) using a monochrometer-based illumination system and captured using a high speed, digital QE CCD camera (TILL Photonics, Pleasanton, CA, USA). RyR1-mediated $Ca^{2+}$ release was evoked using a 45-s application of a maximal concentration (500 μM) of 4-choro-*m*-cresol (4-cmc) and then reversed during a 60-s wash with control Ringer's. Total store content was assessed during a 120-s application of a 0 $Ca^{2+}$ total store $Ca^{2+}$ release cocktail (ICE) containing: 0.01 mM ionomycin, 0.03 mM cyclopiazonic acid (CPA), and 2 mM EGTA. Maximal fura-FF $Ca^{2+}$ responses were evoked by subsequent application of a 10-mM $Ca^{2+}$ Ringer's solution in the continued presence of 0.01 mM ionomycin to ensure that fura-FF was not saturated during prior 4-cmc or ICE applications.

### 2.3. Voltage clamp measurements

Voltage-gated L-type $Ca^{2+}$ currents (L-currents) and intracellular $Ca^{2+}$ transients were simultaneously recorded from ASI(+)RyR1- or ASI(−)RyR1-expressing myotubes using the whole-cell patch clamp technique as described previously [16]. For these experiments, the external solution contained: 145 mM TEA–Cl, 10 mM $CaCl_2$, and 10 mM HEPES (pH 7.4), and the pipette internal solution contained: 145 mM Cs-aspartate, 0.1 mM EGTA, 1.2 mM $MgCl_2$, 0.2 mM $K_5$-Fluo-4, 5 mM Mg-ATP, and 10 mM HEPES (pH7.4). Cell capacitance was determined by integration of the capacity transient resulting from a +10 mV pulse applied from the holding potential and was used to normalise $Ca^{2+}$ currents (pA/pF) from different myotubes. Fits to the voltage-dependence of L-current density and intracellular $Ca^{2+}$ transients were performed using equations described previously [16]. The maximal rate of voltage-gated SR $Ca^{2+}$ release was approximated from the peak of the first derivative of the fluo-4 fluorescence trace ($\delta F/\delta t$) elicited by a depolarisation to +60 mV. The rate of change in fluo-4 fluorescence is an approximation of the maximal rate of $Ca^{2+}$ release in myotubes because these cells exhibit a negligible $Ca^{2+}$ removal flux [17].

### 2.4. Synthesis of domain peptides

Domain peptides were synthesised as described previously [14]. Stock solutions (5 mM) were prepared in $H_2O$ and frozen in 20 μl aliquots.

RyR1 domain peptides

– ASI(+): $^{3471}$TADSKSKMAKAGDAQ SGGSDQERTKKKRRG$^{3500}$

– ASI(-): $^{3471}$TADSKSKMAK----------SGGSDQERTKKKRRG$^{3500}$

– ASI(mutant): $^{3471}$TADSKSKMAK----------SGGSDQERTAAKLRG$^{3500}$

– ASI(short): $^{3493}$RTKKKRRG$^{3500}$

– DP4: $^{2422}$LIQAGKGEALRIRAILRSLVPLDDLVGIISLPLQIP $^{2477}$

DHPR domain peptide

– Peptide ***A*** : $^{671}$TSAQKAKAEERKRRKMSRGL$^{690}$

### 2.5. Nuclear magnetic resonance (NMR), circular dichroism (CD) and structural modelling

These structural techniques have been described previously [18]. Peptides (2 mM) in 10% $D_2O$/90% $H_2O$ adjusted to pH 5.0 (HCl or NaOH) for NMR spectroscopy. Peptides were diluted to 50 mM at pH 5.0 for CD at 5 °C on a Jobin Yvon CD6 Dichrograph (Cedex, France) using a cell path of 1 mm. Ten spectra were collected per sample, averaged and subjected to a smoothing function. Structural models were constructed from NMR experiments, then energy minimised using the program Discover (Accelrys software Insight II), first by steepest descent and then by conjugate gradient until the maximum derivative was <0.001 kcal/A.

### 2.6. [$^3$H]ryanodine binding and $Ca^{2+}$ release

[$^3$H]ryanodine binding [14] to SR vesicles was assessed at 37 °C in 100 mM KCl, 20 mM PIPES pH 6.8, 15 nM [$^3$H]ryanodine, 1 mM EGTA, 200 μM AEBSF(4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) with appropriate [$Ca^{2+}$]. $Ca^{2+}$ release was monitored at 710 nm with the $Ca^{2+}$ indicator, antipyrylazoIII. SR vesicles were added to 2 ml of solution containing: 100 mM $KH_2PO_4$ (pH7), 4 mM $MgCl_2$; 1 mM $Na_2ATP$; 0.5 mM antipyrylazo III.

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