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Allosterically coupled calcium and magnesium binding sites are unmasked by ryanodine receptor chimeras

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Abstract

We studied cation regulation of wild-type ryanodine receptor type 1 ($_{WT}$RyR1), type 3 ($_{WT}$RyR3), and RyR3/RyR1 chimeras (Ch) expressed in 1B5 dyspedic myotubes. Using [^{3}H]ryanodine binding to sarcoplasmic reticulum (SR) membranes, Ca^{2+} titrations with $_{WT}$RyR3 and three chimeras show biphasic activation that is allosterically coupled to an attenuated inhibition relative to $_{WT}$RyR1. Chimeras show biphasic Mg^{2+} inhibition profiles at 3 and 10 μM Ca^{2+}, no observable inhibition at 20 μM Ca^{2+} and monophasic inhibition at 100 μM Ca^{2+}. Ca^{2+} imaging of intact myotubes expressing Ch-4 exhibit caffeine-induced Ca^{2+} transients with inhibition kinetics that are significantly slower than those expressing $_{WT}$RyR1 or $_{WT}$RyR3. Four new aspects of RyR regulation are evident: (1) high affinity (H) activation and low affinity (L) inhibition sites are allosterically coupled, (2) Ca^{2+} facilitates removal of the inherent Mg^{2+} block, (3) $_{WT}$RyR3 exhibits reduced cooperativity between H activation sites when compared to $_{WT}$RyR1, and (4) uncoupling of these sites in Ch-4 results in decreased rates of inactivation of caffeine-induced Ca^{2+} transients.

Keywords: Ryanodine receptors; Calcium-induced calcium release; Channel regulation; Regulation of calcium signaling; Skeletal muscle

Three isoforms of wild-type ryanodine receptors ($_{WT}$RyR1, 2 and 3) are expressed in specialized regions of endoplasmic/sarcoplasmic reticulum (ER/SR) in most mammalian cells where they function as Ca^{2+} release channels that produce local and global Ca^{2+} signals [1,2]. Fluctuating physiological cation concentrations, especially Ca^{2+} and Mg^{2+}, tightly regulate the activity of all three $_{WT}$RyR isoforms [3–9]. Cytoplasmic Ca^{2+} ranging from nM to μM enhances the open probability of $_{WT}$RyRs, whereas >100 μM Ca^{2+} or Mg^{2+} depresses channel activity [5,10] and [11]. The "bell shaped" regulation of $_{WT}$RyR by Ca^{2+} is thought to be responsible for physiological and pathophysiological Ca^{2+}-induced Ca^{2+} release (CICR) phenomena observed in many cell types, including muscle and neurons [1,2,12,13] and [14].

$_{WT}$RyR isoforms preferentially bind the plant alkaloid ryanodine with nM affinity when in the open state [3,6] and [7]. Ca^{2+} and Mg^{2+} titrations in [^{3}H]ryanodine binding and Ca^{2+} release experiments have revealed Hill coefficients >1, indicating coordinated regulation of CICR channels by multiple cation binding sites [15]. Mg^{2+} inhibition studied with channels reconstituted in lipid bilayer membranes [16] and Ca^{2+} release from SR membrane vesicles [4] and [11] suggest dual mechanisms of Mg^{2+} inhibition through competition with Ca^{2+} for high affinity (H) activation sites and binding at low affinity (L) cation inhibition sites. At physiological concentrations, free Mg^{2+} (1–2 mM) is likely to occupy both H and L sites, providing a basal level of $_{WT}$RyR inhibition that must be overcome for EC-coupling to occur [17,18]. Studies have suggested that the physical coupling between $_{WT}$RyR and the dihydropyridine receptor (DHPR) may remove the Mg^{2+} block

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during EC-coupling [18]; whereas, other authors have suggested oxidation of $_{WT}$RyR sulfhydryl groups may override Mg^{2+} inhibition [19]. Functional overlap of Ca^{2+} and Mg^{2+} interactions at H and L regulation sites have confounded cation regulation studies of $_{WT}$RyR1 and resulted in conclusions partially derived from extrapolation. A system that permits more direct analysis of cytoplasmic Ca^{2+} and Mg^{2+} regulation would greatly facilitate mechanistic interpretations of RyR cation regulation in health and disease.

RyR3/RyR1 chimeras were designed to identify regions of $_{WT}$RyR1 directly associated with the DHPR during EC-coupling [20,21]. In this report, [^{3}H]ryanodine binding experiments on $_{WT}$RyR1, $_{WT}$RyR3, and a subset of these chimeras (Ch-4, $_{WT}$RyR$_1$ 1681–3770; Ch-17, 1681–2217; Ch-21, 1924–2446) reveal a biphasic cytoplasmic Ca^{2+} activation profile, suggesting variable cooperativity between H activation sites, revealing coupled but separated interactions at H activation and L inhibition sites. This study provides direct insight into Ca^{2+} and Mg^{2+} regulation, suggests new aspects of $_{WT}$RyR function, and establishes the chimeras as models for future studies of $_{WT}$RyRs and cation regulation.

Materials and methods

Chimeric RyR1/RyR3 constructs. Specific primers were designed for PCR amplification of the selected fragments using $_{WT}$RyR1 as a template. Amplified fragments from $_{WT}$RyR1 encoding aa 1681–2217 (Ch-17), 1924–2446 (Ch-21) and 1681–3770 (Ch-4) were inserted, in frame, into the endogenous restriction site(s) of HSV-RyR3 plasmid as described previously [20]. All chimeric constructs were cloned into the HSV-1 amplicon vector and packaged using a helper virus-free packaging system [22].

Cell culture, infection, and membrane preparation. 1B5 myoblasts were cultured and differentiated into myotubes as described previously [20] and [23]. Plates with differentiated myotubes were infected with virion containing wild-type and RyR1/RyR3 chimeric cDNA for 2 h and membrane extracts were prepared 36 h after infection. Myotubes were homogenized and membrane fractions obtained by differential centrifugation as described previously [24].

[^{3}H]Ryanodine binding assay. High affinity binding of [^{3}H]ryanodine ([^{3}H]Ry; 56 Ci/mmol; New England Nuclear, Boston, MA) to membranes (10–50 μg/ml protein) was performed in the presence of 250 mM KCl, 20 mM Hepes, pH 7.4, and 5 nM [^{3}H]Ry [6]. Free Ca^{2+} and Mg^{2+} concentrations buffered with EGTA were determined using the Bound And Determined software [25]. The binding reaction was equilibrated at 37 °C for 3 h. Non-specific binding was assessed in the presence of 5 μM unlabeled ryanodine. Bound ligand was separated from free by filtration through Whatman GF/B glass fiber filters using a Brandel cell harvester (Gaithersburg, MD), washed with ice-cold buffer, placed into 5 ml scintillation cocktail (ScintiVerse; Fisher Scientific), and radioactivity counted.

Equations for binding analysis. Curve fitting was generated by Microcal™ Origin® Version 6.0 using the equations:

Activation:

$$\text{(a) } y = \frac{(B_{max})(x^n)}{k^n + x^n} \qquad \text{(b) } y = \frac{(B_{max_1})(x^{n_1})}{k_1^{n_1} + x^{n_1}} + \frac{(B_{max_2})(x^{n_2})}{k_2^{n_2} + x^{n_2}}$$

where, B_{max} = maximum bound, $k = EC_{50}$ and n = Hill coefficient

Inhibition:

$$\text{(c) } y = \frac{A_1 - A_2}{1 + (\frac{x}{xo})^p} + A_2 \qquad \text{(d) } y = A_2 + \frac{(A_1 - A_2)f}{1 + 10^{(x - \log xo_1)}} + \frac{(A_1 - A_2)(1-f)}{1 + 10^{(x - \log xo_2)}}$$

where A1 = top asymptote, A2 = bottom asymptote, xo = IC50, f = fraction, and p = power.

Ca^{2+} imaging. Calcium imaging was performed during the stable phase of transduced protein expression in the myotubes 36–48 h post-infection as previously described [20]. Ca^{2+} release was induced with 20 s perfusion of 20 mM caffeine. Monophasic exponential decay curves were fitted with GraphPad Prism® Version 4.0 according to following function: $y = Span \cdot e^{(-k \cdot X)} + Plateau$ were k = rate constant.

Results and discussion

To examine cation regulation, both $_{WT}$RyRs and RyR3/RyR1 chimeras were expressed in dyspedic 1B5 myotubes, a myogenic cell line devoid of all $_{WT}$RyR isoforms, but containing the accessory proteins necessary for normal $_{WT}$RyR function [26]. Regulation of $_{WT}$RyRs and chimeras in membrane fractions was assessed using [^{3}H]Ry binding to probe cation regulation of channel conformation permitting direct analysis of cytoplasmic Ca^{2+} and Mg^{2+} regulation of RyR conformation [6] without confounding influences of varying luminal Ca^{2+} [27].

Ca^{2+} regulation

Ca^{2+} activation of $_{WT}$RyR1 and $_{WT}$RyR3 determined by [^{3}H]Ry binding (Fig. 1A) indicated monophasic activation of $_{WT}$RyR1 ($EC_{50} = 0.54 \pm 0.05$ μM) and biphasic activation of $_{WT}$RyR3 ($EC_{50(1)}$ and $EC_{50(2)} = 0.36 \pm 0.06$ μM and 20.0 ± 5.78 μM) with a plateau from 4–8 μM (inset). Similar analyses of Ch-21, -17 and -4 revealed accentuated biphasic Ca^{2+} responses, with plateaus of 20–100 μM, 10–100 μM, and 3–100 μM, respectively (Fig. 1B). Ch-21, -17 and -4 exhibited $EC_{50(1)}$ values of 0.90 ± 0.19 μM, 0.39 ± 0.09 μM and 0.18 ± 0.03 μM, and $EC_{50(2)}$ values of 397 ± 190 μM, 510 ± 174 μM, and 761 ± 192 μM, respectively (Table 1).

The biphasic profiles suggest $_{WT}$RyR3 and chimeras possess reduced cooperativity between H activation sites compared to $_{WT}$RyR1. The Ca^{2+} dependence of $_{WT}$RyR3 has been previously published based on immunoprecipitated protein and the resulting data fitted using a single-site model indicating monophasic activation of $_{WT}$RyR3 by Ca^{2+} [28] and [29]. Two significant methodological differences distinguish the present study. First, the use of SR membranes from $_{WT}$RyR3-expressing 1B5 myotubes preserves known interactions with lumenal proteins such as calsequestrin [26] that are known to contribute to cation regulation of $_{WT}$RyR1 and $_{WT}$RyR2 [27] and [30]. Second, the extremely broad titrations in previous studies with immunopurified $_{WT}$RyR3 consisted of 2–4 data points covering several log range of Ca^{2+} concentrations and would have missed the biphasic activation of $_{WT}$RyR3 by Ca^{2+} if present after immunopurification. The present study is the first report of a distinctly biphasic activation for $_{WT}$RyR3 and may result from an altered conformation relative to $_{WT}$RyR1 that is exaggerated in the chimeras. A slight variation in protein conformation would explain the deviation in chimeric Ca^{2+} activation from that of