Published in final edited form as:

Biochem Biophys Res Commun. 2008 February 22; 366(4): 988–993. doi:10.1016/j.bbrc.2007.12.058.

Allosterically Coupled Calcium and Magnesium Binding Sites are Unmasked by Ryanodine Receptor Chimeras

Andrew A. Voss^{a,c}, Paul D. Allen^b, Isaac N. Pessah^a, and Claudio F. Perez^b

^a Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, One Shields Avenue, Davis, CA 95616

^b Department of Anesthesia Perioperative and Pain Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115

Abstract

We studied cation regulation of wild type ryanodine receptor type 1 (w_T RyR1), type 3 (w_T 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 w_T RyR3 and three chimeras show biphasic activation that is allosterically coupled to attenuated inhibition relative to w_T RyR1. Chimeras show biphasic Mg^{2+} inhibition profiles at 3 and 10 μ M Ca^{2+} , no observable inhibition at 20 μ M Ca^{2+} and monophasic inhibition at 100 μ M 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 w_T RyR1 or w_T 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) w_T RyR3 exhibits reduced cooperativity between H activation sites when compared to w_T RyR1, and 4) uncoupling of these sites in Ch-4 results in decreased rates of inactivation of caffeine-induced Ca transients.

Keywords

ryanodine receptors; calcium-induced calcium release; channel regulation

Three isoforms of wild type ryanodine receptors (w_T 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] and [2]. Fluctuating physiological cation concentrations, especially Ca^{2+} and Mg^{2+} , tightly regulate all three w_T RyR isoforms [3], [4], [5], [6], [7], [8] and [9]. Cytoplasmic Ca^{2+} ranging from nM to μ M enhances the open probability of w_T RyRs, whereas >100 μ M Ca^{2+} or Mg^{2+} depresses channel activity [5], [10] and [11]. The “bell shaped” regulation of w_T 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].

Correspondence: Isaac N. Pessah, Email: E-mail: inpessah@ucdavis.edu; Phone: (530) 752-6696; Fax: (530) 752-4698.

^cCurrent Address: Department of Physiology, David Geffen School of Medicine, University of California, Los Angeles, CA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 [^3H]ryanodine binding and Ca^{2+} release experiments have revealed Hill coefficients >1 , indicating multiple, 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+} (1–2mM) 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] and [18]. Studies have suggested that the physical coupling between WT-RyR and the dihydropyridine receptor (DHPR) may remove the Mg^{2+} block during EC-coupling [18]; whereas, other authors have suggested oxidation of WT-RyR sulfhydryl groups may override Mg^{2+} inhibition [19]. Functional overlap in 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 about 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] and [21]. In this report, [^3H]ryanodine binding experiments on WT-RyR1, WT-RyR3, and a subset of these chimeras (Ch-4: WT-RyR1 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].

[^3H]Ryanodine binding assay

High affinity binding of [^3H]ryanodine ([^3H]Ry; 56 Ci/mmol; New England Nuclear, Boston, MA) to membranes (10–50 $\mu\text{g}/\text{ml}$ protein) was performed in the presence of 250mM KCl, 20mM Hepes pH 7.4 and 5nM [^3H]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 3h. 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:

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

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

Inhibition:

$$\text{c) } y = \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p} + A_2 \quad \text{d) } y = A_2 + \frac{(A_1 - A_2)f}{1 + 10^{(x - \log x_{01})}} + \frac{(A_1 - A_2)(1 - f)}{1 + 10^{(x - \log x_{02})}}$$

where A_1 = top asymptote, A_2 = bottom asymptote, x_0 = IC_{50} , f = fraction and p = power

Ca²⁺ imaging

Calcium imaging was performed during the stable phase of transduced protein expression in the myotubes 36–48h post-infection as previously described [20]. Ca²⁺ release was induced with 20s perfusion of 20mM caffeine. Monophasic exponential decay curves were fitted with GraphPad Prism® Version 4.0 according to following function: $y = \text{Span} \cdot e^{(-k \cdot X)} + \text{Plateau}$ where k = rate constant.

Results and Discussion

To examine cation regulation, both W_T RyRs and RyR3/RyR1 chimeras were expressed in dyspedic 1B5 myotubes, a cell line void of all W_T RyR isoforms, but containing the accessory proteins necessary for normal W_T RyR function [26]. Regulation of W_T RyRs and chimeras in membrane fractions was assessed using [³H]Ry binding to probe cation regulation of channel conformation permitting direct analysis of cytoplasmic Ca²⁺ and Mg²⁺ regulation of RyR conformation [6] without confounding influences of varying luminal Ca²⁺ [27].

Ca²⁺ regulation

Ca²⁺ activation of W_T RyR1 and W_T RyR3 determined by [³H]Ry binding (Fig. 1A) indicated monophasic activation of W_T RyR1 (EC_{50} = $0.54 \pm 0.05 \mu\text{M}$) and biphasic activation of W_T RyR3 ($EC_{50(1)}$ and $EC_{50(2)}$ = $0.36 \pm 0.06 \mu\text{M}$ and $20.0 \pm 5.78 \mu\text{M}$) with a plateau from 4 – 8 μM (inset). Similar analyses of Ch-21, -17 and -4 revealed accentuated biphasic Ca²⁺ 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 $0.90 \pm 0.19 \mu\text{M}$, $0.39 \pm 0.09 \mu\text{M}$ and $0.18 \pm 0.03 \mu\text{M}$, and $EC_{50(2)}$ values of $397 \pm 190 \mu\text{M}$, $510 \pm 174 \mu\text{M}$, and $761 \pm 192 \mu\text{M}$, respectively (Table 1).

The biphasic profiles suggest W_T RyR3 and chimeras possess reduced cooperativity between H activation sites compared to W_T RyR1. The Ca²⁺ dependence of W_T RyR3 has been previously published based on immunoprecipitated protein and the resulting data fitted using a single-site model indicating monophasic activation of W_T RyR3 by Ca²⁺ [28] and [29]. Two significant methodological differences distinguish the present study. First, the use of SR membranes

from w_T RyR3-expressing 1B5 myotubes preserves known interactions with luminal proteins such as calsequestrin [26] that are known to contribute to cation regulation of w_T RyR1 and w_T RyR2 [27] and [30]. Second, the extremely broad titrations in previous studies with immunopurified w_T RyR3 consisted of 2–4 data points covering several log range of Ca^{2+} concentrations and would have missed the biphasic activation of w_T RyR3 by Ca^{2+} if present after immunopurification. The present study is the first report of a distinctly biphasic activation for w_T RyR3 and may result from an altered conformation relative to w_T 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 w_T RyR1 and 3, an unpredictable response based on primary sequence alone. It is also noteworthy that previously published whole cell Ca^{2+} imaging experiments indicate these chimeras expressed in 1B5 cells are functional, with all three constructs exhibiting calcium-induced Ca^{2+} release (CICR) and Ch-4 and -21 additionally engaging skeletal-type EC-coupling [20]. Interestingly, a recent [3H]Ry-binding study with membranes isolated from rat ventricular muscle showed a similar biphasic Ca^{2+} activation curve for RyR2, which was reverted to a monophasic binding profile in the presence of Mg^{2+} [31]. Collectively these results support a mechanism by which coordinated Ca^{2+} activation sites regulate RyR conformations that bind ryanodine with high affinity.

[3H]Ry binding analysis of Ca^{2+} inhibition through L sites revealed an attenuated response that correlates to H site cooperativity (Fig. 1C & D). Compared to w_T RyR1, multiple aspects of Ca^{2+} inhibition were shifted in w_T RyR3 and chimeric constructs. First, the onset of inhibition is attenuated in w_T RyR3 and Ch-21 to ~ 1 mM and in Ch-17 and -4 to ~ 3 –4 mM vs. w_T RyR1 at 100 μ M. Additionally, the extent of inhibition observed in the chimeras is shifted, where Ch-21, -17, and -4 were respectively 84%, 60% and 39% of the near complete inhibition observed in w_T RyR1 and w_T RyR3. Finally, as summarized in Table 2, the IC_{50} values suggested attenuated inhibition compared to w_T RyR1 (0.84 ± 0.08 mM) in w_T RyR3 (3.08 ± 0.88 mM), Ch-21 (4.11 ± 0.27 mM), Ch-17 (5.09 ± 4.13 mM) and Ch-4 (5.86 ± 1.18 mM) (Table 2). The extent of attenuated Ca^{2+} inhibition through L sites appeared related to H site cooperativity, in which channels with the greatest reduction in H site cooperativity exhibit the largest attenuation in L site mediated inhibition. These data strongly suggests H and L sites are allosterically coupled.

The observations presented heretofore illustrate a model of RyR regulation by Ca^{2+} in which the binding of at least one H site by Ca^{2+} induced a plateau in [3H]Ry binding. This plateau was undetected in w_T RyR1 due to cooperative binding of Ca^{2+} to at least one additional H site, but was observed in w_T RyR3 and further exacerbated in chimeras where there was a reduction in cooperativity between H sites. Binding of Ca^{2+} to the additional H site(s) induced a conformational transition to a state of maximal ryanodine binding. Further increases of Ca^{2+} occupied L sites and decreased [3H]Ry binding. This data indicates H and L sites are allosterically coupled, as the onset of L site mediated inhibition did not occur until complete H site occupation, and complete inhibition through L sites required near-complete cooperativity between H sites.

Mg²⁺ inhibition

In light of Ca^{2+} regulation through coupled but separated H and L sites, we examined with [3H]Ry the role of Mg^{2+} in Ch-4 cation regulation (Fig. 2; Table 3), with curve statistics summarized in Table 3. In the presence of 3 and 10 μ M Ca^{2+} , Mg^{2+} produced biphasic responses with $IC_{50(1)}$ values of 0.40 ± 0.13 mM and 0.64 ± 0.09 mM, respectively. The $IC_{50(2)}$ values for Mg^{2+} at 3 and 10 μ M Ca^{2+} were 5.71 ± 0.31 mM and 4.18 ± 0.16 mM, respectively. At 100 μ M Ca^{2+} , the first phase of Mg^{2+} inhibition was not detected and only an incomplete ($\sim 40\%$ of maximum), monophasic inhibition ($IC_{50} = 4.06 \pm 0.40$ mM) was observed. The biphasic Mg^{2+} profiles at 3 and 10 μ M Ca^{2+} likely represent competition with Ca^{2+} for H sites in the

first phase and binding to L sites in the second. Competition by Mg^{2+} for H sites is supported by a shifting first phase for Mg^{2+} inhibition at 3 and 10 μM Ca^{2+} that is not observed at 100 μM Ca^{2+} . Binding of Mg^{2+} to L sites is described by a monophasic inhibition at 100 μM Ca^{2+} ($IC_{50}=4-6 mM$), a range that corresponds to the observed Ca^{2+} inhibition through L sites ($5.86 \pm 1.18 mM$) in Fig. 1D. These results provide more direct biochemical evidence in support of the dual mechanism of Mg^{2+} inhibition originally proposed by Meissner and Laver based on experiments with w_T RyR [4], [16] and [32].

An intriguing finding was that Mg^{2+} titrations in the presence of 20 μM Ca^{2+} produced no observable inhibition in [3H]Ry binding experiments. Loss of Mg^{2+} inhibition would be possible through competition at H sites and a conformational state at the Ca^{2+} biphasic plateau, which reduces the affinity of Mg^{2+} at L sites relative to conformations at low (3 and 10 μM) and high (100 μM) Ca^{2+} . Alternatively, Mg^{2+} binding could impart an activating effect on H sites, as suggested for rat RyR2 [31]. This further supports allosteric coupling between H and L sites and suggests Ca^{2+} may contribute along with DHPR [18] and oxidation [19] to relieving the inherent physiological block provided by Mg^{2+} .

Physiological relevance of the observations presented in this work depend on the idea that chimeric characteristics unmasked by the reduced H site cooperativity are inherent to w_T RyR, but are difficult to observe in wild type (especially w_T RyR1) due to overlapping interactions at H and L sites. For w_T RyR3 however, the present results would predict unique functional effects at Ca^{2+} concentrations corresponding to the activation plateau (4–8 μM) shown in Fig. 1A. Indeed, at 5–10 μM Ca^{2+} , w_T RyR3 was shown to exhibit increased subconductance behavior relative to w_T RyR1 [28] and [33]. This correlation is additionally supported by increased subconductance behavior of brain w_T RyRs at specific Ca^{2+} and ATP concentrations [34].

Ca^{2+} release from SR stores

To extend the [3H]Ry binding results, we examined the effect of altered Ch-4 cation regulation on Ca^{2+} release in transduced 1B5 myotubes. In these cells, transduced Ch-4 was shown to engage skeletal-type EC-coupling and respond to caffeine stimulation [20] and [24]. We therefore compared the kinetics associated with caffeine-induced Ca^{2+} release from myotubes expressing w_T RyR1, w_T RyR3, and Ch-4. Since Ch-4 cation inhibition is attenuated at basal levels of intracellular Ca^{2+} or Mg^{2+} due to incomplete occupation of H sites, we predict a prolonged Ca^{2+} response following a caffeine-induced Ca^{2+} transient for myotubes expressing Ch-4 compared to those expressing w_T RyR1 or w_T RyR3. Figure 3 shows the average Ca^{2+} transients induced by 20 mM caffeine in 1B5 myotubes expressing each construct and loaded with Fluo-4. Upon caffeine application a fast phase of Ca^{2+} release and subsequent slower phase of cytoplasmic Ca^{2+} removal was followed by a fast Ca^{2+} removal phase after caffeine withdrawal. Whereas all constructs showed seemingly identical activation kinetics for caffeine-induced Ca^{2+} release, Ch-4-expressing cells presented a noticeable reduction in the slow Ca^{2+} removal phase and hence prolonged Ca^{2+} response when compared to cells expressing either w_T RyR1 or w_T RyR3. Analysis of the slow Ca^{2+} removal phase using a monophasic exponential decay reveals that the rate constant (k) for Ch-4 expressing myotubes is nearly 2.5 fold slower than those measured for w_T RyR1 or w_T RyR3 myotubes (Fig. 3). Since saturating concentrations of caffeine were used to induce Ca^{2+} release, the differences in k values observed in this study were most likely associated to the differences in Ca^{2+} and Mg^{2+} regulation of Ch-4 and not to potential differences in sensitivity to the agonist between the three constructs.

Conclusion

The results with RyR1/RyR3 chimeras presented here provide novel insights into the allosteric mechanisms by which Ca^{2+} and Mg^{2+} regulate RyRs, especially interactions between H and L cation binding sites. This allosteric coupling underscores the importance of considering the contribution of Mg^{2+} in addition to Ca^{2+} when assessing RyR function, as physiological levels of Mg^{2+} likely occupy wT RyR L sites and may influence Ca^{2+} activation through H sites. Furthermore, these results suggest that observed differences between wT RyRs and/or chimeric constructs should be interpreted with awareness of specific channel responsiveness to and the concentrations of Ca^{2+} and Mg^{2+} . This is made apparent by variable responses to the same cation concentrations among wild type and chimeric RyRs. The contribution of RyR effectors such as FKBP, ATP, DHPR and oxidation in the context of the cation allosteric regulation presented here is yet to be determined. Additionally, the association of subconductance behavior to Ca^{2+} concentrations in the activation plateau needs to be further examined. This work provides new insights into wT RyR regulation and function, and establishes RyR chimeras, particularly Ch-4, as a model for future studies of RyR cation regulation and subconductance behavior.

Acknowledgments

This work was supported by 1RO1 AR43140-10A1 (PDA and INP) and Training Grant T20 ES07059 (AAV) from the National Institutes of Health.

References

1. Berridge MJ. Calcium microdomains: organization and function. *Cell Calcium* 2006;40:405–12. [PubMed: 17030366]
2. Zalk R, Lehnart SE, Marks AR. Modulation of the ryanodine receptor and intracellular calcium. *m. Annu Rev Biochem* 2007;76:367–85. [PubMed: 17506640]
3. Pessah IN, Waterhouse AL, Casida JE. The calcium-ryanodine receptor complex of skeletal and cardiac muscle. *Biochem Biophys Res Commun* 1985;128:449–56. [PubMed: 3985981]
4. Meissner G, Darling E, Eveleth J. Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+} , Mg^{2+} , and adenine nucleotides. *Biochemistry* 1986;25:236–44. [PubMed: 3754147]
5. Smith JS, Coronado R, Meissner G. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by Ca^{2+} and ATP and modulation by Mg^{2+} . *J Gen Physiol* 1986;88:573–88. [PubMed: 2431098]
6. Pessah IN, Stambuk RA, Casida JE. Ca^{2+} -activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg^{2+} , caffeine, and adenine nucleotides. *Mol Pharmacol* 1987;31:232–8. [PubMed: 2436032]
7. Chu A, Diaz-Munoz M, Hawkes MJ, Brush K, Hamilton SL. Ryanodine as a probe for the functional state of the skeletal muscle sarcoplasmic reticulum calcium release channel. *Mol Pharmacol* 1990;37:735–41. [PubMed: 1692609]
8. Murayama T, Ogawa Y. Purification and characterization of two ryanodine-binding protein isoforms from sarcoplasmic reticulum of bullfrog skeletal muscle. *J Biochem (Tokyo)* 1992;112:514–22. [PubMed: 1337084]
9. Murayama T, Ogawa Y. Similar Ca^{2+} dependences of $[3\text{H}]$ ryanodine binding to α - and β -ryanodine receptors purified from bullfrog skeletal muscle in an isotonic medium. *FEBS Lett* 1996;380:267–71. [PubMed: 8601438]
10. Bezprozvanny I, Watras J, Ehrlich BE. Bell-shaped calcium-response curves of $\text{Ins}(1,4,5)\text{P}_3$ - and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature* 1991;351:751–4. [PubMed: 1648178]
11. Zimanyi I, Pessah IN. Comparison of $[3\text{H}]$ ryanodine receptors and Ca^{++} release from rat cardiac and rabbit skeletal muscle sarcoplasmic reticulum. *J Pharmacol Exp Ther* 1991;256:938–46. [PubMed: 1848635]

12. Thibault O, Gant JC, Landfield PW. Expansion of the calcium hypothesis of brain aging and Alzheimer's disease: minding the store. *Aging Cell* 2007;6:307–17. [PubMed: 17465978]
13. Sobie EA, Song LS, Lederer WJ. Restitution of Ca^{2+} release and vulnerability to arrhythmias. *J Cardiovasc Electrophysiol* 2006;17(Suppl 1):S64–S70. [PubMed: 16686684]
14. Rossi AE, Dirksen RT. Sarcoplasmic reticulum: the dynamic calcium governor of muscle. *Muscle Nerve* 2006;33:715–31. [PubMed: 16477617]
15. Meissner G. Regulation of mammalian ryanodine receptors. *Front Biosci* 2002;7:d2072–80. [PubMed: 12438018]
16. Laver DR, Baynes TM, Dulhunty AF. Magnesium inhibition of ryanodine-receptor calcium channels: evidence for two independent mechanisms. *J Membr Biol* 1997;156:213–29. [PubMed: 9096063]
17. Donoso P, Hidalgo C. pH-sensitive calcium release in triads from frog skeletal muscle. Rapid filtration studies. *J Biol Chem* 1993;268:25432–8. [PubMed: 8244976]
18. Lamb GD, Stephenson DG. Effect of Mg^{2+} on the control of Ca^{2+} release in skeletal muscle fibres of the toad. *J Physiol* 1991;434:507–28. [PubMed: 1708823]
19. Donoso P, Aracena P, Hidalgo C. Sulfhydryl oxidation overrides Mg^{2+} inhibition of calcium-induced calcium release in skeletal muscle triads. *Biophys J* 2000;79:279–86. [PubMed: 10866954]
20. Perez CF, Voss A, Pessah IN, Allen PD. RyR1/RyR3 Chimeras Reveal that Multiple Domains of RyR1 Are Involved in Skeletal-Type E-C Coupling. *Biophys J* 2003;84:2655–63. [PubMed: 12668474]
21. Sheridan DC, Takekura H, Franzini-Armstrong C, Beam KG, Allen PD, Perez CF. Bidirectional signaling between calcium channels of skeletal muscle requires multiple direct and indirect interactions. *Proc Natl Acad Sci U S A* 2006;103:19760–5. [PubMed: 17172444]
22. Wang Y, Fraefel C, Protasi F, Moore RA, Fessenden JD, Pessah IN, DiFrancesco A, Breakefield X, Allen PD. HSV-1 amplicon vectors are a highly efficient gene delivery system for skeletal muscle myoblasts and myotubes. *Am J Physiol Cell Physiol* 2000;278:C619–26. [PubMed: 10712251]
23. Protasi F, Paolini C, Nakai J, Beam KG, Franzini-Armstrong C, Allen PD. Multiple regions of RyR1 mediate functional and structural interactions with $\alpha(1\text{S})$ -dihydropyridine receptors in skeletal muscle. *Biophys J* 2002;83:3230–44. [PubMed: 12496092]
24. Perez CF, Mukherjee S, Allen PD. Amino acids 1–1,680 of ryanodine receptor type 1 hold critical determinants of skeletal type for excitation-contraction coupling. Role of divergence domain D2. *J Biol Chem* 2003;278:39644–52. [PubMed: 12900411]
25. Brooks SP, Storey KB. Bound and determined: a computer program for making buffers of defined ion concentrations. *Anal Biochem* 1992;201:119–26. [PubMed: 1621949]
26. Moore RA, Nguyen H, Galceran J, Pessah IN, Allen PD. A transgenic myogenic cell line lacking ryanodine receptor protein for homologous expression studies: reconstitution of RyR1 protein and function. *J Cell Biol* 1998;140:843–51. [PubMed: 9472036]
27. Laver DR. Ca^{2+} stores regulate ryanodine receptor Ca^{2+} release channels via luminal and cytosolic Ca^{2+} sites. *Clin Exp Pharmacol Physiol* 2007;34:889–96. [PubMed: 17645636]
28. Murayama T, Oba T, Katayama E, Oyamada H, Oguchi K, Kobayashi M, Otsuka K, Ogawa Y. Further characterization of the type 3 ryanodine receptor (RyR3) purified from rabbit diaphragm. *J Biol Chem* 1999;274:17297–308. [PubMed: 10358090]
29. Murayama T, Ogawa Y. Characterization of type 3 ryanodine receptor (RyR3) of sarcoplasmic reticulum from rabbit skeletal muscles. *J Biol Chem* 1997;272:24030–7. [PubMed: 9295356]
30. Gyorke S, Terentyev D. Modulation of ryanodine receptor by luminal calcium and accessory proteins in health and cardiac disease. *Cardiovasc Res*. 2007
31. Chugun A, Sato O, Takeshima H, Ogawa Y. Mg^{2+} activates the ryanodine receptor type 2 (RyR2) at intermediate Ca^{2+} concentrations. *Am J Physiol Cell Physiol* 2007;292:C535–44. [PubMed: 16971497]
32. Meissner G. Ryanodine receptor/ Ca^{2+} release channels and their regulation by endogenous effectors. *Annu Rev Physiol* 1994;56:485–508. [PubMed: 7516645]
33. Fessenden JD, Wang Y, Moore RA, Chen SR, Allen PD, Pessah IN. Divergent functional properties of ryanodine receptor types 1 and 3 expressed in a myogenic cell line. *Biophys J* 2000;79:2509–25. [PubMed: 11053126]

34. Bull R, Marengo JJ, Finkelstein JP, Behrens MI, Alvarez O. SH oxidation coordinates subunits of rat brain ryanodine receptor channels activated by calcium and ATP. *Am J Physiol Cell Physiol* 2003;285:C119–28. [PubMed: 12637263]

Fig. 1.

Ca^{2+} regulation of wild type and chimeric RyR3/RyR1 expressed in dyspedic 1B5 myotubes, determined by $[^3\text{H}]\text{Ry}$ binding (see METHODS). (A) Activation of RyR1 ($\square$) and RyR3 ($\blacksquare$) curve fit with Hill and biphasic Hill equations, respectively. (B) Activation of Ch-4 ($\blacktriangle$), Ch-17 ($\diamond$) and Ch-21 (∇) curve fit with a biphasic Hill equation. (C) Inhibition of RyR1 ($\square$) and RyR3 ($\blacksquare$) curve fit with a monophasic equation. (D) Inhibition of Ch-4 ($\blacktriangle$), Ch-17 ($\diamond$) and Ch-21 (∇) with a biphasic equation. Data points are the mean values, error bars are $\pm$ standard deviations. Activation profiles for wT RyR1 , wT RyR3 and Ch-4 were the combined result of 2–4 experiments performed in duplicate or triplicate from multiple 1B5 membrane preparations.

Fig. 2. Mg^{2+} inhibition at variable Ca^{2+} of Ch-4 expressed in dyspedic 1B5 myotubes, determined by ^3H -ryanodine binding (see METHODS). In the presence of 3 ($\blacksquare$) and 10 μM ($\square$) Ca^{2+} curve fit with a biphasic equation. In the presence of 20 μM Ca^{2+} ($\blacktriangle$) fit linearly ($m = -0.0032 \pm 0.0029$ and $b = 0.611 \pm 0.015$) and at 100 μM Ca^{2+} ($\blacklozenge$) curve fit with a monophasic equation. Data points are the mean value for at least two experiments, error bars are $\pm$ standard deviations.

Fig 3. Caffeine-induced Ca^{2+} release kinetics of 1B5 myotubes expressing Ch-4, $_{WT}RyR1$ or $_{WT}RyR3$. Average Ca^{2+} transients, normalized by the peak amplitude, are presented for each construct (Mean $\pm$ SE). For clarity trend line for Ch-4 is displayed without SE. Rate constant were calculated using only the decay portion of each Ca^{2+} transient.

Table 1
Curve fit statistics for [³H]Ry binding analyses of Ca²⁺ activation

Construct	EC ₅₀₍₁₎ (μM)	EC ₅₀₍₂₎ (μM)	Hill Coeff. 1	Hill Coeff. 2	r ²
wtRyR1	0.54 ± 0.05	N/A	2.42 ± 0.48	N/A	0.982
wtRyR3	0.36 ± 0.06	20.0 ± 5.78	3.26 ± 1.75	1.29 ± 0.40	0.985
Ch-4	0.18 ± 0.03	761 ± 192	1.04 ± 0.17	1.78 ± 0.65	0.986
Ch-17	0.39 ± 0.09	510 ± 174	1.20 ± 0.33	3.08 ± 2.90	0.961
Ch-21	0.90 ± 0.19	397 ± 190	1.03 ± 0.19	3.06 ± 4.32	0.985

Table 2Curve fit statistics for [³H]Ry binding analyses of Ca²⁺ inhibition

Construct	IC ₅₀ (mM)	r ²
WT-RyR1	0.84 ± 0.08	0.997
WT-RyR3	3.08 ± 0.88	0.999
Ch-4	5.86 ± 1.18	0.985
Ch-17	5.09 ± 4.13	0.999
Ch-21	4.11 ± 0.27	0.994

Table 3Curve fit statistics for [³H]Ry binding analyses of Ch-4 Mg²⁺ inhibition at variable Ca²⁺

[Ca ²⁺]	IC ₅₀₍₁₎ (mM)	IC ₅₀₍₂₎ (mM)	r ²
3 μM	0.40 ± 0.13	5.71 ± 0.31	0.964
10 μM	0.64 ± 0.09	4.18 ± 0.16	0.998
20 μM	No Inhibition		
100 μM	4.06 ± 0.40	N/A	0.881