Characterization of a calcium-regulation domain of the skeletal-muscle ryanodine receptor

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A negatively charged region of the N-terminal portion of the skeletal ryanodine receptor (RyR), located between residues 1872–1923, is involved in Ca²⁺-dependent regulation of the Ca²⁺-release channel. This region is divergent between the skeletal (RyR1) and cardiac (RyR2) isoforms of the channel, and is known as D3. Ca²⁺ exerts important regulatory functions on the RyR, being involved in both activation and inactivation functions of the channel, i.e. the effects occurring at micromolar and millimolar Ca²⁺ concentrations respectively. To characterize the role of D3 in the Ca²⁺-dependent regulation of the Ca²⁺-release channel, we studied the functional consequences of deleting the D3 region from RyR1 (Δ D3-RyR1) using a heterologous expression system, [³H]ryanodine binding assays and single-channel recordings in lipid bilayers. Deletion of the D3 region selectively

affected Ca²⁺-dependent regulation of RyR1, but did not alter [³H]ryanodine binding or the effect of other modulators on the RyR. Compared with full-length RyR1 (wt-RyR1), the Ca²⁺-dependence curve of Δ D3-RyR1 is broader, reflecting increased sensitivity to Ca²⁺ activation and decreased sensitivity to Ca²⁺ inactivation. In addition, Δ D3-RyR1 was more resistant to inhibition by Mg²⁺. Comparison of the effect of caffeine on wt-RyR1 and Δ D3-RyR1 suggested that D3 is an important region of RyR that participates in Ca²⁺-dependent activation and inactivation of the Ca²⁺-release channel.

Key words: excitation–contraction coupling, lipid bilayer reconstitution, ryanodine binding, sarcoplasmic reticulum.

INTRODUCTION

The sarcoplasmic reticulum Ca²⁺-release channels, also known as ryanodine receptors (RyRs), are an integral component of excitation–contraction coupling. In response to a depolarization signal at the cell-surface membrane, the RyR channels open and release Ca²⁺ into the cytosol, resulting in myocyte contraction. The RyR is a homotetramer with a subunit mass of approx. 560 kDa [1], and each subunit (approx. 5000 amino acid residues) consists of two domains. The C-terminal fifth of the protein includes four to twelve transmembrane segments, and forms the pore of the Ca²⁺-release channel [2,3]. The larger N-terminal portion is hydrophilic, is believed to be intracytoplasmic, and forms the junctional ‘foot’ that is in close apposition to the surface-membrane L-type Ca²⁺ channels/dihydropyridine receptors [1,4].

Cytoplasmic Ca²⁺ activates and inactivates Ca²⁺-release channels, presumably by binding to high- and low-affinity Ca²⁺-binding sites respectively [5]. Skeletal and cardiac muscle express different isoforms of the Ca²⁺-release channel, namely RyR1 and RyR2 respectively [4]. Whereas both channel isoforms are activated by nanomolar to micromolar concentrations of Ca²⁺, RyR2 is significantly more resistant to Ca²⁺-dependent inactivation at millimolar Ca²⁺ concentrations than RyR1 [6,7]. The high-affinity Ca²⁺-binding site (Ca²⁺-sensor) is believed to reside in the C-terminal portion of the protein (Glu⁴⁰³²), but the exact

location of the inactivation domain (low-affinity Ca²⁺-binding site) has not yet been determined [3,8]. Both isoforms are remarkably similar in primary structure, except for three regions of divergence referred to as D1, D2 and D3 [9]. The cytoplasmic D3 region of RyR1 is rich in negatively charged residues (amino acids 1872–1923) compared with RyR2 (residues 1852–1890), and had been hypothesized to form a low-affinity Ca²⁺-binding site [10]. In single-channel experiments, the D3 region of RyR1 has recently been shown to participate in the Ca²⁺-dependent regulation of the Ca²⁺-release channel [11]. Compared with the full-length recombinant RyR1, deletion of the D3 region resulted not only in a rightward shift of the Ca²⁺-inactivation curve, but also (surprisingly) in a leftward shift in the Ca²⁺-activation curve, suggesting a role for D3 in Ca²⁺-dependent activation of RyR1. However, [³H]ryanodine binding studies by Du and MacLennan [12] failed to identify a role for the D3 region in the Ca²⁺-dependent inactivation function of RyR.

In addition to Ca²⁺, the RyR is activated by caffeine and ATP, inhibited by Mg²⁺ and Ruthenium Red, and modulated by ryanodine (reviewed in [13]). At low concentrations (< 70 μ M), ryanodine binds with high affinity to the RyR and ‘locks’ it in a subconductance state, whereas higher concentrations (> 200 μ M) of ryanodine irreversibly block channel activity [14,15]. The plant alkaloid appears to bind to a conformationally sensitive portion of the channel, because activators of Ca²⁺-release channels (caffeine, ATP, micromolar [Ca²⁺])

Abbreviations used: CHO, Chinese hamster ovary cells; IpTx_a, imperatoxin A; p[CH₂]ppA, adenosine 5′-[β , γ -methylene]triphosphate; RyR1/RyR2, skeletal and cardiac isoforms of ryanodine receptor respectively; Δ D3-RyR1, RyR1 with the D3 region deleted; wt-RyR1, full-length RyR1.

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increase ryanodine binding, whereas inhibitors of Ca^{2+} -release channels (Ruthenium Red, Mg^{2+} , millimolar $[\text{Ca}^{2+}]$) reduce binding [13]. Thus, under well-defined conditions of ligand and receptor concentration, the $[\text{^3H}]$ ryanodine binding assay may be used as an index of the activity of a large population of RyRs.

In order to characterize the role of the negatively charged region of divergence D3 in the function of RyR1, we generated a RyR mutant that specifically lacks the D3 region ($\Delta\text{D3-RyR1}$). After stable expression of $\Delta\text{D3-RyR1}$ in Chinese hamster ovary (CHO) cells, the functional properties of this mutant were studied with $[\text{^3H}]$ ryanodine binding and single-channel experiments in the absence or presence of relevant physiological modulators. Our results suggest that the D3 region of RyR1 exerts a negative feedback on Ca^{2+} -dependent activation of the Ca^{2+} -release channel. In addition, the D3 region appears to participate in the Ca^{2+} - and Mg^{2+} -related inhibition of RyR1.

MATERIALS AND METHODS

Construction of RyR cDNA mutants

The entire cDNA for RyR1 was cloned into the eukaryotic expression vector pRRS11, with transcription under the control of the simian virus 40 promoter [2]. The expression vector includes a neomycin-resistance gene, allowing selection of stable clones. The generation of the deletion mutants $\Delta\text{D2-RyR1}$ and $\Delta\text{D3-RyR1}$ has been described in detail previously [11,16].

Expression of RyR in CHO cells

CHO cells were grown in Ham's F12 medium (Gibco BRL, Gaithersburg, MD, U.S.A.) enriched with 10% (v/v) fetal-bovine serum and supplemented with penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) in a 5% CO_2 incubator at 37 °C. Cells were harvested using a calcium-free PBS solution (containing, in mM: NaCl 136.8, KCl 2.68, Na_2HPO_4 8.1, KH_2PO_4 1.47 and EDTA 0.357). Full-length and mutant RyR proteins were heterologously expressed in CHO cells using liposome-based methods (LipofectAMINE[™]; Gibco BRL). Following transfection (48 h), cells were exposed to 0.5 mg/ml G418 (Calbiochem, La Jolla, CA, U.S.A.) and selected for stable transfectant clones. Stable CHO cell lines expressing full-length RyR1 (wt-RyR1) and the deletion mutants were generated by serial dilution and subculturing of single-cell clones expressing the respective proteins in the presence of G418.

Membrane vesicle preparation

CHO cells stably expressing the different RyR mutants were harvested, and microsomal membrane vesicles were prepared as described previously [3]. Briefly, following harvesting, cells were resuspended in a hypotonic lysis solution [10 mM Hepes/1 mM EGTA (pH 7.4)] containing protease inhibitors [aprotinin (1 mg/ml), benzamidin (1 mM), leupeptin (1 mM), Pefabloc (0.5 mM) and pepstatin (1 mM)] and subjected to nitrogen cavitation on ice at 500 lb/in² (3.4×10^6 Pa) for 20 min. Cells were then Dounce-homogenized (10 strokes) and resuspended in an equal volume of restoration buffer [500 mM sucrose/10 mM Hepes/1 mM EDTA (pH 7.4)]. This was followed by another round of Dounce homogenization (15 strokes) and centrifugation at 10000 *g* for 10 min. Microsomal membrane vesicles were obtained by centrifugation of the supernatant at 100000 *g* for 20 min at 4 °C, with subsequent resuspension of the pellet in a buffer containing 250 mM sucrose and 10 mM Hepes, pH 7.2. Expression of RyR proteins was assayed by immunoblots, using specific RyR antibodies as described previously [11].

Reconstitution of RyR channels in the planar lipid bilayer and analysis of single-channel data

A mixture of phosphatidylethanolamine/phosphatidylserine/cholesterol (6:6:1, by wt), dissolved in n-decane at a concentration of 40 mg/ml, was used to form lipid-bilayer membranes across an ≈ 200 μm aperture separating the *cis*-side from the *trans*-side. Membrane vesicles containing RyR channels were added to the *cis*-solution and allowed to fuse with the bilayer under a concentration gradient of 200 to 50 mM caesium gluconate (*cis/trans*). In addition, both *cis* and *trans* solutions included 1 mM CaCl_2 , 1 mM Cs_2EGTA and 10 mM Hepes, pH 7.4. The free ionic Ca^{2+} concentration in the presence of 1 mM EGTA was measured to be 26 μM . Channel fusion was facilitated by applying positive voltage and the addition of Ca^{2+} in the *cis* solution to a final free concentration of 220 μM . Channel recordings were performed under symmetrical conditions of 200 mM caesium gluconate. Single-channel data were measured using an Axopatch 200A amplifier patch-clamp unit in combination with a Digidata 1200 A/D-to-D/A conversion board (Axon Instruments, Foster City, CA, U.S.A.) and a Pentium II Computer. The bilayer membrane was either held at a particular test potential and channel activity recorded, or kept at 0 mV and pulsed to different test potentials, 0.5–1 s in duration. The currents were sampled at 0.2 ms/point, and filtered through an eight-pole Bessel filter at 1 kHz. The analysis of single-channel data was performed using the pClamp7 program and Sigma plot software.

$[\text{^3H}]$ Ryanodine binding assays

Measurements of $[\text{^3H}]$ ryanodine binding were performed as described previously [17,18]. In brief, to obtain saturation binding curves, microsomal preparations of CHO cells expressing wt-RyR1, $\Delta\text{D2-RyR1}$ or $\Delta\text{D3-RyR1}$ were incubated for 90 min at 36 °C with 1–40 nM $[\text{^3H}]$ ryanodine in medium containing 1 M KCl, 20 mM sodium-Hepes, pH 7.2 and 10 μM CaCl_2 . This high KCl concentration (1 M) was used to optimize binding for saturation curves only, since high-salt concentrations have a stimulatory effect on $[\text{^3H}]$ ryanodine binding. This concentration, however, would not be useful to assay the effects of various modulators on channel function because the channel would be near-maximally activated, and therefore a lower KCl concentration (0.2 M) was used to study RyR modulation (see below). The protein concentration of the membrane vesicles in the final reaction mixture (0.1 ml) was kept in the range of 0.3 to 0.6 mg/ml. Non-specific binding, amounting to 20–40% of the total binding, was determined in the presence of 10 μM unlabelled ryanodine, and has been subtracted from the data presented. K_d (affinity constant of the $[\text{^3H}]$ ryanodine–RyR complex) and B_{max} (maximal density of binding sites) values were obtained from saturation binding curves, were shown to follow Michaelis–Menten kinetics, and were fitted to a one-site model with the computer program Origin 6.0 (Microcal Inc., Northampton, MA, U.S.A.). Unless otherwise indicated, the effect of various modulators was assayed in the presence of 7 nM $[\text{^3H}]$ ryanodine in 0.2 M KCl, 20 mM Hepes, pH 7.2, 1 mM EGTA and various amounts of CaCl_2 to set free $[\text{Ca}^{2+}]$ in the range of 10 nM to 30 mM. The amount of free Ca^{2+} in the reaction mixture was calculated using the stability constants of Fabiato and Fabiato [19]. Reaction samples, run in duplicates, were filtered on to Whatman GF/C glass-fibre filters and washed twice with 5 ml of room-temperature water on a Brandel M-24R harvester (Gaithersburg, MD, U.S.A.). The data are presented as means $\pm$ S.E.M., with $n = 3$ or 4 for each experiment. Comparison between data groups was assessed using an unpaired

Student's *t* test, with *P* values < 0.05 considered to be statistically significant.

RESULTS

[³H]Ryanodine binding to expressed RyR

CHO cells were transfected with cDNAs encoding wt-RyR1 or ΔD3-RyR1 and, following selection with G418, single stable clones of CHO cells that permanently express full-length RyR1 (wt-RyR1) or ΔD3-RyR1 were isolated. Successful stable expression of wt-RyR1 and ΔD3-RyR1 in CHO cells was confirmed by Western-blot analysis, immunocytochemistry, caffeine-induced Ca²⁺-release assays in single CHO cells and PCR analysis of genomic DNA ([11], and results not shown for ΔD2-RyR1).

To study the functional characteristics of wt-RyR1 and ΔD3-RyR1, microsomal membrane vesicles were isolated from CHO cells stably expressing the respective recombinant RyR proteins. Figure 1(A) shows equilibrium binding curves of [³H]ryanodine to microsomal vesicles from CHO cells expressing wt-RyR1 and ΔD3-RyR1. Similar to native RyR [17,18], [³H]ryanodine binding to wt-RyR1 and ΔD3-RyR1 was saturable, with *K_d* values of

5.74 nM and 6.97 nM and *B_{max}* measurements of 0.84 and 0.83 pmol/mg of protein respectively, similar for both groups of channels (Figure 1B). This indicates that deletion of D3 from RyR1 neither hampered the expression of the RyR mutant nor affected its affinity for [³H]ryanodine.

To test for the functional effect of ryanodine on single wt-RyR1 and ΔD3-RyR1 channels, we reconstituted vesicles from CHO cells expressing either protein in planar lipid bilayers. To facilitate identification of the Ca²⁺-release channel, we used Cs⁺ as the current-carrying ion in the recording solution. The use of Cs⁺ as current carrier allows buffering of the free [Ca²⁺] to any desired level. In addition, Cs⁺ eliminates most of the K⁺-channel activities present in the microsomal membrane vesicles. Incorporation of membrane vesicles expressing wt-RyR1 and ΔD3-RyR1 in lipid bilayers resulted in the appearance of single-channel activity with fast transitions between closed and open states (Figure 1C, upper traces). In the presence of 50 μM ryanodine, both wt-RyR1 and ΔD3-RyR1 single channels exhibited the characteristic long-lived subconductance states that constitute the signature effect of ryanodine (Figure 1C, lower traces). Taken together, these data suggest that expressed RyR1 channels behave similarly to native RyR1 channels with respect

Figure 1 Characterization of ryanodine binding to wt-RyR1 and ΔD3-RyR1

(A) Kinetics of association of ryanodine with wt-RyR1 and ΔD3-RyR1. [³H]Ryanodine (0–20 nM) was incubated for 90 min at 36 °C with microsomal membrane vesicles expressing wt-RyR1 (open circles) or ΔD3-RyR1 (filled circles). The incubation medium consisted of 1 M KCl, 10 mM Hepes and 10 μM free Ca²⁺, pH 7.2. Non-specific binding (in the presence of 10 mM unlabelled ryanodine) has been subtracted from the data in all Figures. The equilibrium binding is saturable and > 90% binding is seen at 10 nM ryanodine for both wt-RyR1 and ΔD3-RyR1. The data presented are representative of three separate experiments. (B) Representative Scatchard analysis of [³H]ryanodine binding to wt-RyR1 and ΔD3-RyR1 were obtained by plotting 'bound/free' against 'bound' data. The *K_d* and *B_{max}* values were obtained by linear fitting of the data points. (C) Representative single-channel recordings of wt-RyR1 and ΔD3-RyR1 at 220 μM free [Ca²⁺] in the absence (upper traces) or presence (lower traces) of ryanodine (Ry; 50 μM). Data were recorded at +50 mV test potential for both channels with 200 mM caesium gluconate as the current carrier. wt-RyR1 and ΔD3-RyR1 channels exhibit rapid transitions between closed (C) and open states (O), with transient subconductance states (S) noted for both channels. Ryanodine locks the channels in long open subconductance states.

Figure 2 Effects of RyR modulators on wt-RyR1 and ΔD3-RyR1

[³H]Ryanodine (7 nM) was incubated with both vesicles at 36 °C for 90 min in 200 mM KCl, 10 mM Hepes and 10 μM free Ca²⁺ in the presence of 100 nM IpTx_a, 3 mM p[CH₂]ppA (AMP-PCP) and 1 μM Ruthenium Red (RuRed). Results are reported as percentages of binding compared with [³H]ryanodine binding at pCa 5 (100%) (A). Single-channel data were recorded at +50 mV, as described in the Materials and methods section. The traces demonstrating the effects of caffeine, IpTx_a and RuRed on wt-RyR1 and ΔD3-RyR1 single channels were acquired from continuous experiments with the channels shown in the control traces (B). A higher concentration of IpTx_a (300 nM) was used in the bilayer experiments for faster equilibration of the toxin with the channel. SR, sarcoplasmic reticulum.

to ryanodine binding, and that deletion of the D3 region of RyR1 does not alter the binding of ryanodine or its effect on the Ca²⁺-release channel.

Effect of RyR modulators

To characterize further the consequences of deleting the D3 region of RyR1, we tested the effects of various RyR modulators on [³H]ryanodine binding to native skeletal and cardiac sarcoplasmic-reticulum vesicles, as well as vesicles of CHO cells expressing wt-RyR1 and ΔD3-RyR1 (Figure 2A). Imperatoxin A (IpTx_a) is a short (33-amino-acid) peptide toxin isolated from the venom of the scorpion *Pandinus imperator*, which activates

RyR1 with high affinity and specificity [20]. In binding assays at pCa 5, 100 nM IpTx_a activated native RyR1 (250 ± 15%), wt-RyR1 (217 ± 15%) and ΔD3-RyR1 (189 ± 10%), but not native RyR2 (110 ± 10%). Similarly, the non-hydrolysable ATP analogue, adenosine 5'-[β,γ-methylene]triphosphate (p[CH₂]ppA; 3 mM), significantly activated, whereas Ruthenium Red (1 μM) inhibited, [³H]ryanodine binding to all four vesicles tested. The effects of these modulators on single-channel function of wt-RyR1 and ΔD3-RyR1 were comparable with the binding data (Figure 2B).

Inward rectification of the ΔD3-RyR1 channel

Examination of the current–voltage (or *I*–*V*) relationship revealed that the ΔD3-RyR1 channel exhibits inward rectification in its *I*–*V* relationship (Figure 3). Step pulses of negative and positive voltages of equal amplitude were applied to a single ΔD3-RyR1 Ca²⁺-release channel, and inward and outward currents were recorded under symmetrical ionic condition of 200 mM caesium gluconate (Figure 3A). The *I*–*V* curve for ΔD3-RyR1 is linear over voltage ranges of –80 to 0 mV and 0 mV to +80 mV. However, the inward conductance of this Ca²⁺-release channel (372 ± 18 pS) is larger than the outward conductance (315 ± 17 pS) (Figure 3B). No such rectification was noted in the *I*–*V* relationship of wt-RyR1 [21], suggesting that deletion of the D3 region from activated RyR1 may alter the intrinsic ion-conduction properties of the Ca²⁺-release channel.

In summary, except for the reduced conductance at positive voltages, ΔD3-RyR1 displays normal single-channel properties and conventional pharmacological profiles; deletion of the D3 region from RyR1, therefore, does not appear to lead to a generalized disruption of the channel's structure and function.

Ca²⁺-dependent regulation of wt-RyR1 and ΔD3-RyR1

Similar to native RyR1 [13], both wt-RyR1 and ΔD3-RyR1 exhibited a bell-shaped [³H]ryanodine binding curve in response to Ca²⁺ (Figure 4). Both channels displayed an increase in [³H]ryanodine binding (Ca²⁺-dependent activation) between pCa 8 and pCa 4; higher Ca²⁺ concentrations then gradually decreased [³H]ryanodine binding (inactivation component). However, both the activation and inactivation components of ΔD3-RyR1 were skewed compared with wt-RyR1. At pCa 7, [³H]ryanodine binding to wt-RyR1 was only 6.7% of the maximum, as compared with 26% for ΔD3-RyR1. At pCa 3, binding to wt-RyR1 decreased to 21% of maximum, in agreement with most studies that have used native channels, whereas binding to ΔD3-RyR1 was still at 59% of the maximum. At pCa 2, both wt-RyR1 and ΔD3-RyR1 displayed equal and near-complete inhibition. As a result of the increased sensitivity of ΔD3-RyR1 to activation by Ca²⁺, the EC₅₀ value ([Ca²⁺] that produces half-maximal activation) was significantly lower for ΔD3-RyR1 than for wt-RyR1 (0.59 as compared with 2.6 μM). In contrast, the decreased sensitivity to Ca²⁺-dependent inactivation of ΔD3-RyR1 yielded an IC₅₀ value ([Ca²⁺] that produces half-maximal inhibition) that was considerably higher for ΔD3-RyR1 than for wt-RyR1 (1.44 compared with 0.46 mM).

To determine whether the altered Ca²⁺-dependent regulation of ΔD3-RyR1 is specific to D3 deletion, or whether it may result from conformational changes imparted by deletion of 52 residues from its 'foot' region, we examined the effect of D2 deletion from RyR1 on the Ca²⁺-dependence of [³H]ryanodine binding to the Ca²⁺-release channel. D2 is another region of divergence in the primary structure between RyR1 and RyR2, and is comparable in size with D3 [16]. A stable CHO cell line expressing ΔD2-RyR1 (lacking amino acid residues 1303–1406) was es-

Figure 3 Inward rectification of the $\Delta D3$ -RyR1 Ca^{2+} -release channel

Single-channel currents through the $\Delta D3$ -RyR1 channel were measured in a symmetrical solution of 200 mM caesium gluconate. The single channel was subjected to sequential step pulses of negative and positive voltages of equal magnitude, ranging from -80 mV to $+80$ mV (A). Recorded episodes with no channel openings were subtracted from the representative traces, thus eliminating the capacitive transients. Single-channel currents are plotted as a function of test potential (B). Data points represent unitary amplitudes of full-conductance openings only. Subconductance states were occasionally detected in both $\Delta D3$ -RyR1 and wt-RyR1 channels. The I - V relationship for $\Delta D3$ -RyR1 is linear between -80 mV and 0 mV with a conductance of 372 ± 18 pS. The linear I - V relationship between 0 and $+80$ mV, however, displays a reduced conductance of 315 ± 17 pS, reflecting inward rectification of the $\Delta D3$ -RyR1 channel. The data points represent averages from multiple experiments ($n = 3-15$).

Figure 4 Ca^{2+} -dependence of $[\text{H}]\text{ryanodine}$ binding to wt-RyR1 and $\Delta D3$ -RyR1

Plot of $[\text{H}]\text{ryanodine}$ binding for wt-RyR1 (circles), $\Delta D3$ -RyR1 (squares) and $\Delta D2$ -RyR1 (triangles) as a function of $[\text{Ca}^{2+}]$. The final $[\text{H}]\text{ryanodine}$ concentration was 7 nM and experiments were performed as described in the Materials and methods section. The biphasic Ca^{2+} -dependence of $[\text{H}]\text{ryanodine}$ binding gives rise to a bell-shaped curve for all RyR1 phenotypes. Whereas $\Delta D3$ -RyR1 exhibits a broader Ca^{2+} -dependence curve compared with wt-RyR1, the binding curve for $\Delta D2$ -RyR1 is very similar to that of wt-RyR1. The plots are representative of at least four experiments with each phenotype.

Table 1 Ca^{2+} -dependence of $[\text{H}]\text{ryanodine}$ binding to recombinant RyR1 vesicles in the presence and absence of caffeine

The affinity constants and slope values were obtained as described in the Materials and methods section. Values are shown as the means $\pm$ S.E.M. for at least three experiments under each separate condition. *, $P < 0.05$ compared with wt-RyR1 without caffeine.

	Without caffeine			20 mM caffeine	
	wt-RyR1	$\Delta D3$ -RyR1	$\Delta D2$ -RyR1	wt-RyR1	$\Delta D3$ -RyR1
EC_{50} (μM)	2.61 ± 0.32	$0.59 \pm 0.20^*$	3.03 ± 0.39	$0.097 \pm 0.04^*$	$0.051 \pm 0.03^*$
Slope	1.32 ± 0.13	$0.72 \pm 0.16^*$	$0.76 \pm 0.06^*$	$0.58 \pm 0.14^*$	$0.45 \pm 0.12^*$
IC_{50} (mM)	0.46 ± 0.11	$1.44 \pm 0.26^*$	0.38 ± 0.18	$1.36 \pm 0.07^*$	$1.66 \pm 0.16^*$
Slope	1.85 ± 0.44	$1.04 \pm 0.21^*$	2.14 ± 0.91	$0.91 \pm 0.04^*$	$0.73 \pm 0.05^*$

established and confirmed using immunocytochemistry, immunoblotting, fluorimetric measurements *in vivo* of caffeine-induced Ca^{2+} -release and PCR analysis of genomic DNA (results not shown). The Ca^{2+} -dependence of the $[\text{H}]\text{ryanodine}$ binding curve of microsomal vesicles expressing $\Delta D2$ -RyR1 (EC_{50} $3.03 \mu\text{M}$; IC_{50} 0.38 mM) was similar to that of wt-RyR1, but significantly different from that of $\Delta D3$ -RyR1 (Table 1 and Figure 4). The slope of the activation curve of $\Delta D3$ -RyR1 is significantly less than that of wt-RyR1, probably reflecting a

Figure 5 Caffeine effect on $[^3\text{H}]$ ryanodine binding to wt-RyR1 and $\Delta\text{D3-RyR1}$

The effect of 20 mM caffeine on the Ca^{2+} -dependence of $[^3\text{H}]$ ryanodine binding to wt-RyR1 (top panel) and $\Delta\text{D3-RyR1}$ (middle panel) as compared with control (no caffeine). Microsomal membrane vesicles (0.4–0.6 mg/ml) were incubated in the presence of 20 mM caffeine and various concentrations of Ca^{2+} , as described in the text. Bottom panel: direct comparison of caffeine effect on wt-RyR1 and $\Delta\text{D3-RyR1}$. Ca^{2+} -dependence curves, in the presence of caffeine, of wt-RyR1 (open circles in the top panel) and $\Delta\text{D3-RyR1}$ (open squares in the middle panel) are plotted on one chart.

negative effect of the deletion on the co-operativity of the Ca^{2+} -activation site. However, unlike $\Delta\text{D2-RyR1}$, $\Delta\text{D3-RyR1}$ exhibits a much lower EC_{50} value compared with wt-RyR1, resulting in a leftward shift of the Ca^{2+} -activation curve. The inactivation component slopes for wt-RyR1 and $\Delta\text{D2-RyR1}$ are similar (Hill coefficients of approx. 2), reflecting a co-operative effect of elevated Ca^{2+} on the inactivation process. In contrast, $\Delta\text{D3-RyR1}$ displays a slope of approx. 1, suggesting a loss of Ca^{2+} co-operativity in the inactivation component of the Ca^{2+} -dependence

Figure 6 Mg^{2+} effect on $[^3\text{H}]$ ryanodine binding to wt-RyR1 and $\Delta\text{D3-RyR1}$

Dose–response curve of the inhibitory effect of Mg^{2+} on $[^3\text{H}]$ ryanodine binding to membrane vesicles of wt-RyR1 and $\Delta\text{D3-RyR1}$. Protein concentration of the vesicles was maintained between 0.4–0.6 mg/ml. Care was taken to maintain $\text{p}[\text{CH}_2]\text{ppA}$ concentration at 3 mM and pCa 5 in the face of changing $[\text{Mg}^{2+}]$. $[^3\text{H}]$ ryanodine binding of 100% reflects binding at pCa 5 in the presence of $\text{p}[\text{CH}_2]\text{ppA}$ (3 mM) and in the absence of Mg^{2+} .

curve. These results, in addition to the intact response of $\Delta\text{D3-RyR1}$ to modulators in single-channel and $[^3\text{H}]$ ryanodine binding assays (Figure 2), strongly suggest that the altered Ca^{2+} -dependent regulation of $\Delta\text{D3-RyR1}$ reflects a specific effect as a result of D3 deletion from RyR1.

Characterization of the role of D3 in the Ca^{2+} -dependent modulation of RyR1

Despite the fact that $\Delta\text{D3-RyR1}$ shows alteration on both limbs of the Ca^{2+} -dependence of the $[^3\text{H}]$ ryanodine binding curve, a potential scenario is that D3 selectively modifies one component of Ca^{2+} -dependent regulation only, affecting secondarily the other Ca^{2+} -dependent process. To probe the role of the D3 region in Ca^{2+} -dependent activation, $[^3\text{H}]$ ryanodine binding measurements were performed in the presence of caffeine. Caffeine is known to activate RyRs by increasing the affinity of the Ca^{2+} -activation site(s) for Ca^{2+} [22,23]. Caffeine (20 mM) shifted the Ca^{2+} -activation curves of both wt-RyR1 (Figure 5, top panel) and $\Delta\text{D3-RyR1}$ (Figure 5, middle panel) to the left. The extent of leftward shift is apparently greater for wt-RyR1, because the basal Ca^{2+} -activation curve for $\Delta\text{D3-RyR1}$ was already shifted to the left compared with wt-RyR1 (Figure 4). However, the EC_{50} values in the presence of 20 mM caffeine were not significantly different between wt-RyR1 and $\Delta\text{D3-RyR1}$, suggesting similar sensitivities to Ca^{2+} activation in response to caffeine (Table 1). On the other hand, caffeine produced a rightward shift in the Ca^{2+} -dependent inactivation curve of wt-RyR1, but no such shift was noted for $\Delta\text{D3-RyR1}$. Consequently, the Ca^{2+} -activation and -inactivation curves for both channels in the presence of caffeine were almost superimposed (Figure 5, bottom panel). Differences in activation and inactivation slopes between wt-RyR1 and $\Delta\text{D3-RyR1}$ were abolished in the presence of caffeine, since the slope values of wt-RyR1 decreased. A similar effect of caffeine on RyR1 has been described before [23]. In summary, the caffeine effect eliminated apparent differences in the Ca^{2+} -dependent regulation curves between $\Delta\text{D3-RyR1}$ and wt-RyR1.

Comparison of the effect of Mg^{2+} on wt-RyR1 and $\Delta D3$ -RyR1

Mg^{2+} has been postulated as an ineffective emulator of Ca^{2+} at the high-affinity Ca^{2+} -binding site, decreasing Ca^{2+} -dependent activation, and as an effective substitute for Ca^{2+} at the inactivation site, potentiating Ca^{2+} -dependent inactivation [5,24]. In our single-channel experiments conducted at $220 \mu M$ $[Ca^{2+}]$, $\Delta D3$ -RyR1 was significantly more resistant to inhibition by Mg^{2+} than was wt-RyR1 [11]. In order to characterize the effect of Mg^{2+} on $[^3H]$ ryanodine binding to wt-RyR1 and $\Delta D3$ -RyR1, Ca^{2+} and $p[CH_2]ppA$ concentrations were adjusted along with varying the $[Mg^{2+}]$ to maintain pCa 5 and the $p[CH_2]ppA$ concentration at ≈ 3 mM. The Mg^{2+} -dependent inhibition of $[^3H]$ ryanodine binding to $\Delta D3$ -RyR1 and wt-RyR1 is shown in Figure 6. At 3 mM Mg^{2+} , the $[^3H]$ ryanodine binding of wt-RyR1 was $55 \pm 4\%$ of the maximum (determined at 0 mM Mg^{2+}), as measured against $91 \pm 3\%$ for $\Delta D3$ -RyR1. At 10 mM Mg^{2+} , values were $12 \pm 7\%$ and $56 \pm 6\%$ respectively. The IC_{50} for wt-RyR1 was 3.24 ± 0.38 mM, and that for $\Delta D3$ -RyR1 was 11.40 ± 0.90 mM, indicating a decreased propensity of $\Delta D3$ -RyR1 to Mg^{2+} -dependent inactivation.

DISCUSSION

Ca^{2+} -dependent regulation of the Ca^{2+} -release channel plays an integral role in RyR function, namely in regulating Ca^{2+} -induced Ca^{2+} release and excitation-contraction coupling. In single-channel experiments, $\Delta D3$ -RyR1 displayed increased sensitivity to low concentrations of Ca^{2+} , and was more resistant to inactivation by elevated cytosolic $[Ca^{2+}]$ [11]. In this study, we used $[^3H]$ ryanodine binding and single-channel measurements to characterize the functional properties of $\Delta D3$ -RyR1, and to assess the role of D3 in Ca^{2+} -dependent regulation of RyR1. $[^3H]$ Ryanodine binding experiments performed with microsomal vesicles of CHO cells expressing $\Delta D3$ -RyR1 and wt-RyR1 revealed a single binding site virtually identical in affinity and maximal density in both populations of vesicles (Figure 1). The saturation curves for both channels were obtained at pCa 5, i.e. the standard $[Ca^{2+}]$ used to establish the K_d and B_{max} values in binding assays. In addition, the pharmacological profile of $\Delta D3$ -RyR1, including the characteristic subconductance state induced by ryanodine, and the modulation of channel activity by well-known ligands of RyRs, were all observed in $\Delta D3$ -RyR1. Taken together, the results suggest that the D3 region is not essential for RyR1 expression, and that its deletion is not accompanied by a generalized disruption of the protein's structure that blunts the response of RyR1 to modulators. Instead, a more clearly defined role for D3 as a Ca^{2+} -regulatory domain of RyR1 emerged, both from Ca^{2+} -dependent $[^3H]$ ryanodine binding curves and from the effect of modulators that affect Ca^{2+} -activation and -inactivation domains. Ca^{2+} -dependence of $[^3H]$ ryanodine binding curves of wt-RyR1 and $\Delta D3$ -RyR1 was compared side-by-side to assess any potential influence of D3 (or, more precisely, the lack thereof) on Ca^{2+} -activation and -inactivation domains. The mere fact that both of these processes were still present in $\Delta D3$ -RyR1 suggests that D3 is not indispensable in terms of generating the typical bell-shaped Ca^{2+} curve, and that its role instead may be circumscribed to feedback on Ca^{2+} -binding domains residing somewhere else on the channel protein. In agreement with this hypothesis, recent reports have identified Ca^{2+} -activation [8] and -inactivation [15,25] domains in the C-terminal end of RyR1, close to the pore-forming region of the channel and downstream of the D3 region. On the other hand, deletion of large segments of the N-terminal segments of RyR1 and RyR2 result in functional channels that are modulated by ryanodine, but which are not inactivated when confronted by an elevated cytosolic

$[Ca^{2+}]$ [3,11]. This suggests that domains in the 'foot' region of RyR (including possibly D3) interact with domains close to the pore region, and modulate channel function. It also raises the possibility that the activation and inactivation domains are not absolutely mutually exclusive, and that there may be an allosteric interaction between these two domains. The observed outward conductance changes of $\Delta D3$ -RyR1 lend further credence to this hypothesis. $\Delta D3$ -RyR1 exhibits inward rectification at positive voltages, suggesting that the removal of 38 net negative charges from the foot region of RyR1 as a result of the deletion of D3 may allosterically alter channel conduction near the pore region. Thus, in our proposed role for D3 as a regulatory region, the basal response of $\Delta D3$ -RyR1 to $[Ca^{2+}]$ and its response to modulators that selectively act on Ca^{2+} activation or inactivation domains are expected to be altered, but not abolished.

In Ca^{2+} -dependence $[^3H]$ ryanodine binding assays, the Ca^{2+} -activation curve of $\Delta D3$ -RyR1 was shifted to the left and the inactivation curve was shifted to the right (Figure 4). The overall effect of deleting D3 was the formation of a RyR1 mutant with both a higher sensitivity to activation by Ca^{2+} and a higher resistance to inactivation by Ca^{2+} . An inherent problem with mutagenesis studies is the difficulty in accurately assigning changes in function to allosteric and local intrinsic modifications that affect the protein structure. However, we have performed critical control experiments to ensure that the deletion of the glutamate-rich sequence of D3 was not accompanied by a generalized disruption of the channel structure: First, protein expression and proper subcellular localization of stable RyR mutants expressed in CHO cells was determined using Western blots and confocal microscopy respectively. In addition, DNA sequencing was carried out in the regions targeted for mutation for $\Delta D3$ -RyR1 and $\Delta D2$ -RyR1. Secondly, we assessed the functional properties of the RyR mutants by monitoring their responsiveness *in vivo* to caffeine-induced Ca^{2+} release using Fura-2 fluorescence analysis [11]. $[^3H]$ Ryanodine binding studies and single-channel measurements in lipid bilayers were also performed. These combined assays constitute a very stringent test of the functional integrity of the mutant channel, because only correctly assembled tetrameric RyRs are capable of producing functional channels. We found that $\Delta D3$ -RyR1 had responses to modulators (Ruthenium Red, IpTx_a and $p[CH_2]ppA$, see Figure 2) similar to those of wt-RyR1, but which differed significantly in terms of Ca^{2+} -dependent regulation and Mg^{2+} inhibition. Thirdly, to test whether this difference was merely due to deletion of an N-terminal segment ($\approx 1\%$ of the total size) of RyR1, we generated a deletion mutant of another 'foot' divergent region (D2; amino acids 1303–1406) and expressed it in CHO cells as an independent control for $\Delta D3$ -RyR1. Unlike $\Delta D3$ -RyR1, $\Delta D2$ -RyR1 behaved similarly to wt-RyR1 in terms of its Ca^{2+} -dependent regulation, suggesting that RyR1 can withstand a deletion of 104 residues (D2) from the 'foot', without apparent changes in sensitivity or response to classical modulators. In addition, single-channel experiments with $\Delta D3$ -RyR1 provided similar results to the binding data [11], indicating that the exquisite interaction of Ca^{2+} with its regulatory domains is specifically perturbed in this mutant, giving rise to abnormal gating of RyR1. We therefore tested whether the altered Ca^{2+} -dependent regulation arising from D3 deletion resulted from modified channel responses to Ca^{2+} activation, inactivation, or both.

To investigate the functional role of D3 in the Ca^{2+} -dependent regulation of RyR, we studied the effect of caffeine on $[^3H]$ ryanodine binding at different $[Ca^{2+}]$. Caffeine is known to increase the affinity of the Ca^{2+} -activation domain for Ca^{2+} [22,23], an effect which presumably arises from interaction of

caffeine with the N-terminal domain of RyR [26]. We found that the basal activation curve (in the absence of caffeine) of $\Delta D3$ -RyR1 was more sensitive to Ca^{2+} than that of wt-RyR1 (Figures 5A and 5B). Caffeine shifted the Ca^{2+} -activation curve of both wt-RyR1 and $\Delta D3$ -RyR1 to the left. These results support the notion that the D3 region exerts an inhibitory effect on the Ca^{2+} -activation domain, and that caffeine is able to increase RyR1 channel activity, irrespective of the D3 region. Given that, in the presence of caffeine, both $\Delta D3$ -RyR1 and wt-RyR1 show an equivalent level of activation by Ca^{2+} (Figure 5, bottom panel), the inhibitory effect of the D3 region must have been overridden by the caffeine effect.

In addition to the effect on the Ca^{2+} -activation domain, the absence of the D3 region had effects on the Ca^{2+} -inactivation curve (Figure 4A). In the absence of caffeine, inactivation of RyR1 at pCa 3 was more pronounced in wt-RyR1 than in $\Delta D3$ -RyR1. Intracellular Mg^{2+} is another physiological ligand that regulates the function of the RyR channel [27]. At low $[Ca^{2+}]$, Mg^{2+} is believed to exert its inhibitory effect by competing with Ca^{2+} at the high-affinity Ca^{2+} -activation domain. At high $[Ca^{2+}]$, Mg^{2+} is thought to mimic the effect of Ca^{2+} by binding to the low-affinity Ca^{2+} -inactivation domain, which does not distinguish Ca^{2+} from Mg^{2+} [5,23,24]. Deletion of the D3 region of RyR1 resulted in functional Ca^{2+} -release channels that were resistant to Mg^{2+} inhibition [11], and RyR2, which displays fewer net negative charges in its D3 region compared with RyR1 (8 compared with 38), also exhibited less efficient inhibition by Mg^{2+} [28]. To characterize further that response, and the role of the D3 region in the Mg^{2+} -dependent inhibition of RyR1, a dose-response curve of Mg^{2+} inhibition of $[^3H]$ ryanodine binding to wt-RyR1 and $\Delta D3$ -RyR1 was obtained at pCa 5. The binding data are consistent with single-channel results, and suggest that the D3 region is a potential site involved in the Mg^{2+} -dependent inhibition of RyR. The shift to the right in Ca^{2+} -dependent inactivation of $\Delta D3$ -RyR1 and our Mg^{2+} data support a role for D3 in Ca^{2+} -dependent inactivation of the Ca^{2+} -release channel. The D3 region might be a Ca^{2+} -inactivation site, or it might allosterically modulate a Ca^{2+} -inactivation site at elevated Ca^{2+} and/or Mg^{2+} concentrations.

Two possible schemes can be proposed for the Ca^{2+} -dependent activation and inactivation processes of the RyR channel. A simplified model, C (closed) $\rightarrow O$ (open) $\rightarrow I$ (inactivated), assumes that the channel activation and inactivation follow a sequential order, and that the affinities of activation (EC_{50}) and inactivation (IC_{50}) do not depend on each other. This model predicts that manipulations affecting the activation process will not affect the inactivation process, or vice versa. Our data for $\Delta D3$ -RyR1 and the effect of caffeine on wt-RyR1 and $\Delta D3$ -RyR1 do not support this linear activation scheme. In its simplest form, an alternative model:

assumes that the activation and inactivation processes of the channel are coupled allosterically, i.e. that the channel can be inactivated from both the closed (I_1) and open states (I_2), but its tendency to enter the I_2 state is greater as a consequence of its coupling to the O state. Such models have been widely used to describe the gating mechanisms of the voltage-dependent Ca^{2+} channels [29,30], K^+ channels (reviewed in [31]) and the depolarization-induced movement of the dihydropyridine receptor/voltage sensor [32]. With this allosteric model, it is possible to simulate the caffeine-mediated shift of activation and

inactivation phases of the $[^3H]$ ryanodine binding curve, simply by changing the $C \rightarrow O$ transition step or the allosteric coupling constant. Similarly, the steady-state distribution of the probability of the channel being open (P_o) as measured against the Ca^{2+} concentration and $[^3H]$ ryanodine binding as measured against the Ca^{2+} concentration of the $\Delta D3$ -RyR1 channel can be predicted by assuming that deletion of the D3 region affects the $C \rightarrow O$ transition step or the allosteric coupling constant. In addition to the allosteric model, other kinetic gating models may also be relevant to the function of RyR. Quantitative modelling of RyR channel function requires not only measurement of steady-state P_o versus $[Ca^{2+}]$, but also more systematic measurement with kinetic determinations, i.e. rate of activation and inactivation in response to rapid changes in $[Ca^{2+}]$. Future experiments with step changes in Ca^{2+} concentration and kinetic measurements will help delineate the molecular mechanisms of RyR function.

In summary, we found that expression of $\Delta D3$ -RyR1, a mutant protein that lacks 52 amino acids encompassing a cluster of negatively charged residues in the 'foot' region of RyR1 ($^{1872}TEEEEEEEEEEEEEEEEEEEKEEDEEEEEKEDAEKEEEEAPEGEKEDLE^{1923}$), result in Ca^{2+} -release channels with pharmacological profile and single-channel activities similar to those of wt-RyR1. Remarkable differences, however, were found in the characteristic bell-shaped Ca^{2+} -dependent $[^3H]$ -ryanodine binding curve. Our data suggest that the D3 region of RyR1 is a regulatory domain of the Ca^{2+} -release channel that may modulate Ca^{2+} -dependent regulation and Mg^{2+} -dependent inhibition of the Ca^{2+} -release channel through close interaction with the Ca^{2+} -activation and -inactivation domains of RyR. Future studies with site-directed mutagenesis aimed at neutralizing negatively charged residues (individual or clusters) within D3 will help to delineate further the function of D3 in the Ca^{2+} -dependent regulation of the Ca^{2+} -release channel.

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