Evidence for a role of C-terminal amino acid residues in skeletal muscle Ca^{2+} release channel (ryanodine receptor) function

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Abstract The effects of deleting 1, 3 and 15 amino acid residues from the highly conserved C-terminus of the tetrameric skeletal muscle ryanodine receptor (RyR) complex were determined. Immunoblot analysis indicated similar expression levels in HEK293 cells for full-length and mutant proteins. Full-length and RyR lacking the last amino acid showed [^{3}H]ryanodine binding and single channel activities typical of native receptors. Deletion of 3 amino acids resulted in decreased activities, whereas deletion of 15 amino acids yielded an inactive RyR. These results suggest that the most 15 C-terminal amino acids are important for the expression of a functional RyR complex.

Key words: Ryanodine receptor; Ca^{2+} release channel; Skeletal muscle; Excitation-contraction coupling

1. Introduction

The process of skeletal muscle contraction and relaxation involves rapid Ca^{2+} release from the sarcoplasmic reticulum (SR) by the Ca^{2+} release channel (ryanodine receptor, RyR) complex comprised of four 560-kDa (RyR polypeptide) and four 12-kDa (FK506-binding protein) subunits [1,2]. Analysis of the deduced amino acid sequence of rabbit skeletal muscle RyR polypeptide has suggested a large cytoplasmic N-terminal 'foot' region, a membrane-spanning pore region, and a short (~100 amino acid residues) cytoplasmic C-terminus [3,4]. Studies with site directed antibodies have confirmed that the N and C termini of skeletal muscle RyR are cytoplasmically located [5,6] and moreover have shown that the C-terminus is folded in an antibody-inaccessible conformation in the native receptor [6]. Sequence analysis of eight members of the RyR family has shown that the cytoplasmic C-terminal region is highly conserved [3,4,7–12] (Fig. 1). There are ten identical amino acids among the last fifteen residues, including the next to last one. Two members of the RyR family (Drosophila, lobster) contain six additional C-terminal amino acids. These findings imply that C-terminal amino acids have a critical role in RyR function.

In the present study, three C-terminal deletion forms of the skeletal muscle RyR missing 1, 3 and 15 amino acids (Δ1, Δ3 and Δ15 RyR, respectively) were prepared and expressed in human embryonic kidney (HEK293) cells. Expression of functional tetrameric RyRs was assessed by sedimentation, [^{3}H]ryanodine binding and single channel measurements. Our results suggest that the most 15 C-terminal amino acid residues are important for the expression of a functional skeletal muscle RyR complex.

2. Materials and methods

2.1. Materials

Taq-polymerase, restriction endonucleases, other DNA modifying enzymes, and Pefabloc SC (a protease inhibitor) were purchased from Boehringer Mannheim. [^{3}H]Ryanodine was obtained from Dupont NEN, unlabeled ryanodine and horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG antibodies from Calbiochem (La Jolla, CA), phospholipids from Avanti Polar Lipids (Birmingham, AL), and HEK293 cells from the Tissue Culture Facility of Lineberger Cancer Center at University of North Carolina. Expression vector pCMV5 was generously provided by Dr. David Russel (University of Texas Southwestern Medical Center, Dallas, TX). All other chemicals were of analytical grade.

2.2. Construction of full-length and deletion mutant RyR cDNAs

A full-length rabbit skeletal cDNA was prepared from three overlapping clones that were constructed from cDNA fragments in pBluescript vector using standard procedures. PBSL (*Hin*dIII/*Xho*I, 117 bp–6596 bp) was obtained by preparing rabbit skeletal muscle mRNA and using the reverse transcription polymerase chain reaction with primers to the published rabbit skeletal muscle cDNA sequence [4]. PBSM (*Xho*I/*Eco*RI, 6597–11 766) was generously provided by Dr. Paul D. Allen (Brigham and Women's Hospital, Boston, MA). PBSR (*Eco*RI/*Hin*dIII, 11 767–15 252) was obtained by screening a rabbit skeletal muscle cDNA library (Stratagene, La Jolla, CA) using an affinity-purified polyclonal antibody raised against the purified rat skeletal muscle RyR [13]. To construct the full-length cDNA, two subclones were initially prepared, using PBSL (*Hin*dIII/Xho), PBSM (*Xho*I/*Bam*HI, 6597–11 113) and pCMV5 (Hind III/*Bam*HI) for preparing pCMV5L+M, and pCMV5 (*Eco*RI/*Hin*dIII) and PBSR (*Eco*RI/*Hin*dIII) for preparing pCMV5R. Full-length RyR cDNA was obtained by the ligation of four restriction site fragments: *Cla*I/*Xho*I from pCMV5L+M, *Xho*I/*Eco*RI from PBSM, *Eco*RI/*Xba*I from pCMV5R, and *Cla*I/*Xba*I from pCMV5 vector. C-Terminal deletions were made by polymerase chain reaction amplification of 804 C-terminal bases of PBSR (*Cla*I/TGA), using three primers that resulted in the loss of three, nine, and 45 bases of the C-terminal sequence encoding the skeletal muscle RyR. The amplified fragments were inserted into pBluescript vector (*Cla*I/*Sma*I) to generate three new subclones: Δ1PBS, Δ3PBS, and Δ15PBS. These were used to construct D1pCMV5R, D3pCMV5R and D15pCMV5R, and subsequently the final deletion mutant RyR cDNAs.

2.3. Expression of full-length and mutant RyRs

RyR cDNAs cloned into pCMV5 were transiently expressed in HEK293 cells using the Lipofectamine (GIBCO BRL, Grand Island, NY) method according to the manufacturer's instructions. Cells were maintained in DMEM-H containing 10% fetal bovine serum and 20 mM HEPES, pH 7.3 at 37°C in 5% CO_2 and plated the day before transfection. For each 10 cm tissue culture dish, 8 μg DNA was used

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Abbreviations: RyR, ryanodine receptor; SR, sarcoplasmic reticulum; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; HEK, human embryonic cells

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at a DNA/Lipofectamine ratio of 1/3 to 1/5. Cells were harvested 42–46 h after transfection

2.4. Preparation of membrane fraction

Cells were grown on 10 cm tissue culture dishes, washed twice with 4 ml ice-cold PBS containing 5 mM EDTA and protease inhibitors (0.2 mM Pefabloc, 100 nM aprotinin, 50 μM leupeptin, 1 μM pepstatin, and 1 mM benzamidine), and harvested in the same solution by removal from plates by scraping. Cells were collected by centrifugation at 3500 rpm for 10 min in a RC3B centrifuge, resuspended in the above solution without EDTA, and pelleted again. A membrane fraction was prepared as described [14] with some modification. Briefly, cell homogenates were centrifuged for 1 h at 35 000 rpm in a Beckman Ti50 rotor, and pellets were resuspended in a buffer containing 25 mM Tris-HEPES, pH 7.4, 0.3 M sucrose, 0.15 M KCl, 20 μM $CaCl_2$ and above protease inhibitors, and stored at −80°C.

2.5. Electrophoresis and Western blotting

Proteins were denatured for 5 min at 95–100°C and separated on 3–12% SDS polyacrylamide gels [15]. Proteins were transferred to Immobilon-P membranes at 4°C at 400 mA for 1–3 h followed by 1 A for 14–16 h. Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk and 0.1% Tween 20 in PBS, and incubated for 3 h at room temperature with a monoclonal antibody specific for skeletal muscle RyR (RyRD110, unpublished studies) in PBS containing 1% non-fat dry milk and 0.1% Tween 20. After washing, the bound antibody was detected with horseradish peroxidase-conjugated anti-mouse IgG using 3,3-diaminobenzidine and H_2O_2.

2.6. Isolation and reconstitution of expressed RyRs

RyRs from two culture dishes were solubilized for 10 min at room temperature in 1.5 ml of a buffer containing 5 mg/ml phosphatidylcholine and 1.45% CHAPS and isolated by rate density centrifugation [16]. To detect RyRs on the gradients, solubilized RyRs were labeled with 20 nM [^{3}H]ryanodine for 1 h at room temperature in the absence and presence of 20 μM unlabeled ryanodine. For Western blot analysis, RyRs were sedimented by centrifuging gradient fractions for 20 h at 45 000 rpm in a Beckman Ti75 rotor at 0°C. For single channel measurements, pooled RyR gradient peak fractions were reconstituted into proteoliposomes by removal of CHAPS by dialysis [17].

2.7. [^{3}H]Ryanodine binding

Membranes of 1/5 culture dish were incubated with [^{3}H]ryanodine at room temperature in 100 μl of a buffer containing 20 mM Tris-HEPES, pH 7.4, 0.6 M KCl, 0.15 M sucrose, 5 mM AMP, 310 μM Ca^{2+}, 200 μM EGTA (110 μM free Ca^{2+}), 0.2 mM Pefabloc, and 10 μM leupeptin. Non-specific binding was determined using a 1000-fold excess of unlabeled ryanodine. After 4 h, aliquots of the samples were diluted with 20 volumes of ice-cold water and placed on Whatman GF/B filters preincubated with 2% polyethyleneimine in water. Filters were washed with 3×5 ml ice-cold 0.1 M KCl, 1 mM KPIPES, pH 7.0. The radioactivity remaining with the filters was determined by liquid scintillation counting to obtain bound [^{3}H]ryanodine.

2.8. Single channel recordings

Single channel measurements were performed by incorporating expressed RyR channels in Mueller-Rudin-type lipid bilayers [18]. Proteoliposomes containing the expressed RyRs were added to the cis chamber of a bilayer apparatus and fused in the presence of an osmotic gradient (350 mM cis KCl/20 mM *trans*-KCl in 10 mM KHEPES, pH 7.3) with planar bilayers containing a 4:1 mixture of bovine brain phosphatidylethanolamine and phosphatidylcholine (50 mg of total phospholipid/ml of *n*-decane). After appearance of channel activity, further fusion of proteoliposomes was prevented by increasing *trans*-[KCl] to 0.35 M. The trans side of the bilayer was defined as ground. Electrical signals were filtered at 4 kHz, digitized at 20 kHz, and analyzed as described [18].

Rabbit Skeletal Muscle(RyR1)	PAGDCFRKQ YEDQLS
Rabbit Cardiac Muscle(RyR2)	PAGDCFRKQ YEDQLN
Rabbit Brain(RyR3)	PAGDCFRKQ YEDQLG
Frog Skeletal Muscle(RyR1)	PAGDCFRKT YEDQLG
Frog Skeletal Muscle(RyR3)	PAGDCFRKQ YEDQLG
Chicken Brain(RyR3)	PAGDCFRKQ YEDQLG
Drosophila	PVGDCFRKQ YEDELSGGGGGG
Lobster Skeletal Muscle	PVGDCFRKQ YEEELSGGGSAS
Consensus	**P-GDCFRK- YE--L-**

Fig. 1. Aligment of C-terminal amino acids of ryanodine receptors.

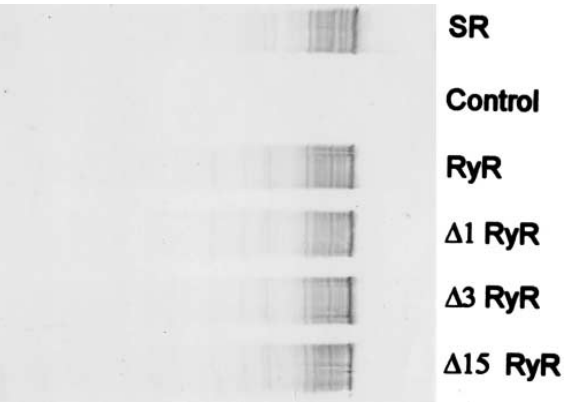

Fig. 2. Expression of full-length and truncated RyRs in HEK293 cells. Transfected cells were incubated with Laemmli SDS sample buffer for 5 min at 95–100°C and separated by 3–12% SDS-PAGE. Ryanodine receptor proteins were detected on immunoblots using monoclonal antibody RyRD110.

3. Results and discussion

3.1. Expression of RyR cDNAs in HEK293 cells

The expression plasmid pCMV5RyR encoding the entire skeletal muscle RyR protein sequence was constructed and used to transfect HEK293 cells. Similarly, three truncated forms were constructed and expressed in HEK293 cells. In agreement with previous studies [14,19,20], full-length cDNA could be also expressed in CHO and COS cells, although with lower levels than in HEK293 cells. Transient expression of

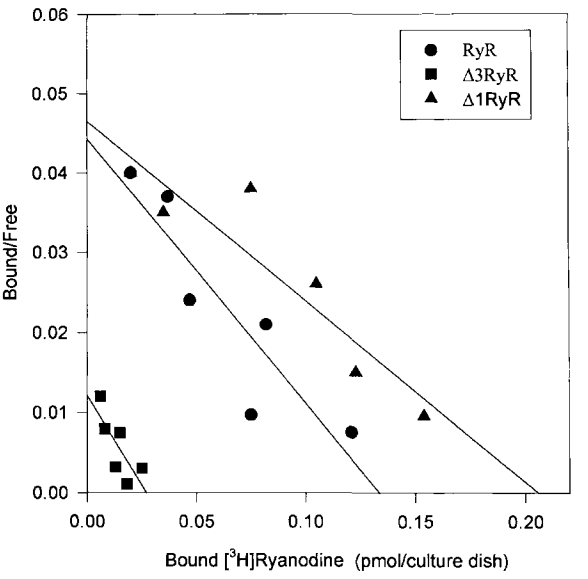

Fig. 3. [^{3}H]Ryanodine binding to full-length and truncated RyRs. Membranes of 1/5 cell culture dish were incubated with 0.5–16 nM [^{3}H]ryanodine for 4 h at room temperature as described in Methods (see Section 2). Scatchard analysis of [^{3}H]ryanodine binding yielded the following averaged (±S.D.) B_{max} (pmol/dish) and K_d (nM) values, respectively (n=3): Full-length RyR: 0.11±0.06, 2.9±0.3; Δ1RyR: 0.13±0.10, 2.3±1.5; Δ3RyR: 0.03±0.01, 2.7±1.0; Δ15 RyR: No specific [^{3}H]ryanodine binding was detected for cells transfected with Δ15 RyR cDNA.

RyR was detected by immunoblotting using mAb RyRD110 raised against rabbit skeletal muscle RyR (Fig. 2). In cells transfected with full-length and truncated cDNAs, there was a major high molecular weight protein band corresponding to rabbit skeletal muscle 560-kDa RyR polypeptide, whereas only background staining was observed in the control (vector only) lane. In addition, several lower molecular weight bands were present. These likely represent degradation products of the expressed RyRs, since they were absent in the control lane. Densitometry of blots indicated that full-length and truncated RyRs were expressed at similar levels in HEK293 cells.

3.2. [^{3}H]Ryanodine binding to membranes

Possible differences in the function of the expressed deletion mutants were investigated by determining the [^{3}H]ryanodine-binding properties of cell membrane fractions and by carrying out single channel measurements. Scatchard analysis of [^{3}H]ryanodine-binding data indicated the presence of a single high-affinity [^{3}H]ryanodine-binding site with similar affinities for the full-length, Δ1 and Δ3 constructs (Fig. 3). Membranes of cells transfected with full-length and Δ1 RyR cDNAs had a similar B_{max} value of specific [^{3}H]ryanodine binding. Lower levels of [^{3}H]ryanodine binding were observed in membranes obtained from cells transfected with Δ3 RyR cDNA, whereas no specific [^{3}H]ryanodine binding could be detected in cells transfected with Δ15 RyR cDNA (not shown). The observation of different [^{3}H]ryanodine-binding levels was further pursued by determining the sedimentation behavior of CHAPS-solubilized, [^{3}H]ryanodine-labeled RyRs [16]. A single peak of bound [^{3}H]-radioactivity with a sedimentation value of 30 S was apparent when cells were transfected with full-length and

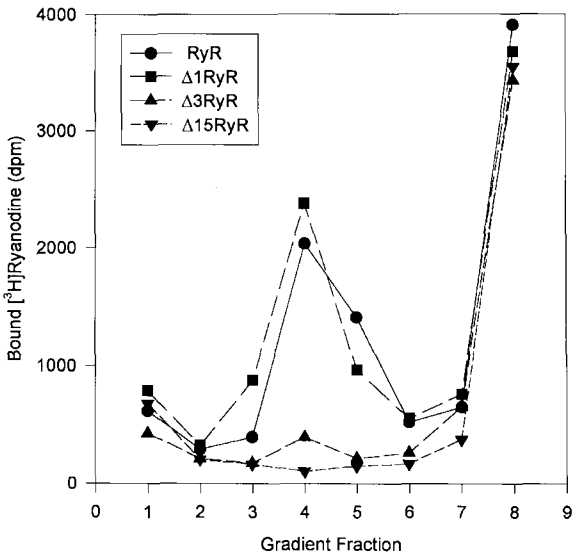

Fig. 4. [^{3}H]Ryanodine-binding sedimentation profile of CHAPS-solubilized, transfected cells centrifuged through a linear sucrose density gradient. Cells from two culture dishes were incubated for 1 h at room temperature in 1.5 ml of a solubilizing solution containing 5 mg/ml PC, 1.45% CHAPS and 20 nM [^{3}H]ryanodine and then centrifuged through a 7–20% linear sucrose gradient as described in Methods. Gradient fractions (labeled from bottom to top) were analyzed for [^{3}H]-radioactivity. A similar [^{3}H]-radioactivity profile was obtained when CHAPS-solubilized heavy SR vesicles were centrifuged through a parallel gradient, indicating presence of tetrameric 30 S RyR complexes [16] in gradient fraction 4.

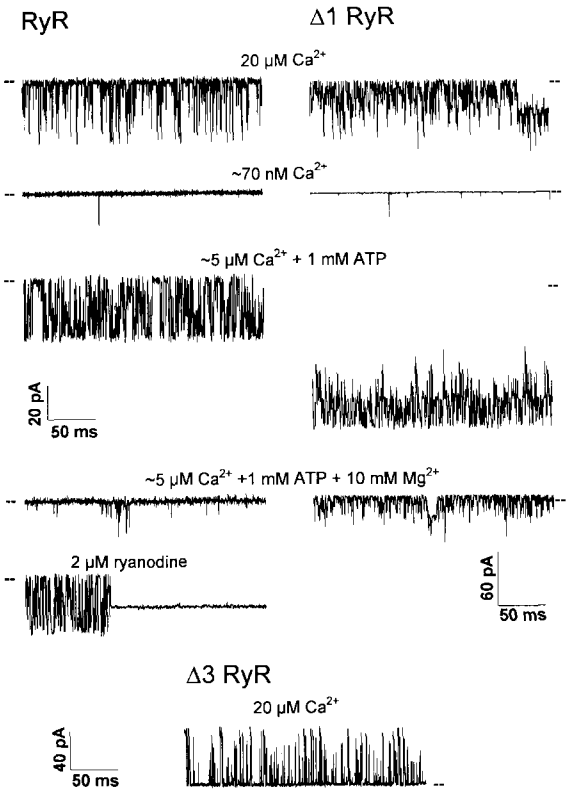

Fig. 5. Single channel activities of full-length and truncated RyRs. Expressed full-length RyR (left panel), Δ1 RyR (right panel) and Δ3 RyR (bottom trace) were purified, reconstituted in lipid bilayer vesicles, and incorporated into planar lipid bilayers. Single channel currents, shown as downward deflections (RyR and Δ1 RyR) from closed level (- -), were recorded in symmetrical 0.35 M KCl, 10 mM KHEPES, pH 7.3 media containing 20 μM free Ca^{2+} (top traces) and following the successive addition of EGTA (second traces), Ca^{2+} and ATP (third traces) and Mg^{2+} (fourth traces) to the cis (SR cytosolic) side of the bilayer chamber to yield the indicated concentrations. The left fifth trace is from a separate experiment obtained after the addition of 2 μM ryanodine to the cis chamber. Holding potential was −40 mV. Right panel traces were obtained from a bilayer in which 4 Δ1 RyRs had been incorporated. P_o values of left and right panels were as follows: 0.09 and 0.12 (first traces), ~0 and 0.001 (second traces), 0.53 and 0.85 (third traces), and 0.005 and 0.04 (fourth traces). The bottom trace shows a recording of Δ3 RyR in 0.35 M cytosolic/0.02 M luminal KCl, 10 mM KHEPES, pH 7.3 medium containing 20 μM free Ca^{2+}. The holding potential was +40 mV. Current deflections are upward.

Δ1 and Δ3 RyR cDNAs (Fig. 4). In agreement with data of Fig. 3, cells transfected with Δ3 RyR cDNA showed a decreased level of bound [^{3}H]ryanodine. Cells transfected with Δ15 RyR cDNA did not exhibit a peak of bound [^{3}H]-radioactivity. The presence of RyRs in gradient fractions was also tested using a monoclonal antibody to the skeletal muscle RyR. Immunoblots showed the presence of a 560-kDa RyR protein band in gradient fraction 4 (see Fig. 4) for all transfected cells and variable amounts of the 560-kDa band as well as lower molecular weight immunoreactive material in the top gradient fractions (Fractions 6–8) (not shown). In gradient fractions 7 and 8, the highest levels of the 560-kDa band were seen in cells transfected with Δ15 RyR cDNAs. Predominant presence of lower molecular protein bands indicated an increased sensitivity of the Δ3 RyR to proteolysis. These results suggest that deletion of C-terminal amino acids impairs the assembly of a tetrameric RyR channel complex. Another

possibility is the formation of an incorrectly folded receptor complex that is more readily dissociated into its monomeric subunits by detergents [21].

3.3. Single channel measurements

Channel activity of expressed and purified RyRs was recorded in symmetrical 0.35 M KCl medium with different additions in the cis (SR cytosolic) bilayer chamber. In 350 mM symmetrical KCl solution, full-length and Δ1 RyR channels had a mean conductance of 792 ± 10 pS (± S.D., $n = 6$) and 764 ± 40 pS ($n = 5$), respectively, which was essentially identical to that of native RyR (not shown). In 20 μM cis (SR cytosolic) Ca^{2+}, channel activities were comparable to those of native RyR (Fig. 5 left and right panels, top traces). The mean channel open probabilities (P_os) were 0.16 ± 0.18 (± S.D., $n = 5$) and 0.30 ± 0.15 ($n = 3$), respectively, for full-length and Δ1 RyRs. Reducing cis Ca^{2+} to ~70 nM by adding EGTA to the cis chamber decreased channel activities to near zero (Fig. 5, second traces). Po increased to 0.71 ± 0.07 ($n = 4$) and 0.85 ($n = 2$) (Fig. 5, third traces) when cis Ca^{2+} was raised to ~5 μM and millimolar ATP was added to the cis chamber. Further addition of 10 mM Mg^{2+} reduced channel activities to 0.009 ± 0.008 ($n = 3$) and 0.02 ($n = 2$) (Fig. 5, fourth traces). In separate experiments, addition of 10 μM ruthenium red completely abolished channel activities (data not shown). Addition of 2 μM ryanodine to the cis side locked the expressed full-length channel in a sub-conductance state (Fig. 5 left panel, fifth trace). Thus, the expressed skeletal muscle full-length and Δ1 RyRs exhibited a conductance and pharmacology indistinguishable from that of native receptors.

Δ3 RyR showed a single channel activity with a conductance (~740 pS) resembling that of full-length channels in two out of a total of five experiments (Fig. 5C). In both cases, channels disappeared too quickly (≤3 s) to allow determination of their pharmacology. These results suggest that removal of the 3 C-terminal amino acids from the skeletal muscle RyR polypeptide does not affect channel conductance but has serious deleterious effects on channel stability in lipid bilayers. No Δ15 RyR single channel activity resembling that of native or expressed full-length RyR was observed in five experiments.

In conclusion, three truncated RyR cDNAs were used to show that the most C-terminal 15 amino acids play an important role in the assembly of skeletal muscle RyR capable of [^{3}H]ryanodine binding and displaying a stable channel activity in lipid bilayers.

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