The human cardiac muscle ryanodine receptor–calcium release channel: identification, primary structure and topological analysis

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Rapid Ca^{2+} efflux from intracellular stores during cardiac muscle excitation–contraction coupling is mediated by the ryanodine-sensitive calcium-release channel, a large homotetrameric complex present in the sarcoplasmic reticulum. We report here the identification, primary structure and topological analysis of the ryanodine receptor–calcium release channel from human cardiac muscle (hRyR-2). Consistent with sedimentation and immunoblotting studies on the hRyR-2 protein, sequence analysis of ten overlapping cDNA clones reveals an open reading frame of 14901 nucleotides encoding a protein of 4967 amino acid residues with a predicted molecular mass of 564569 Da for hRyR-2. In-frame insertions corresponding to eight and ten amino acid residues were found in two of the ten cDNAs isolated, suggesting

that novel, alternatively spliced transcripts of the hRyR-2 gene might exist. Six hydrophobic stretches, which are present within the hRyR-2 C-terminal 500 amino acids and are conserved in all RyR sequences, may be involved in forming the transmembrane domain that constitutes the Ca^{2+} -conducting pathway, in agreement with competitive ELISA studies with a RyR-2-specific antibody. Sequence alignment of hRyR-2 with other RyR isoforms indicates a high level of overall identity within the RyR family, with the exception of two important regions that exhibit substantial variability. Phylogenetic analysis suggests that the RyR-2 isoform diverged from a single ancestral gene before the RyR-1 and RyR-3 isoforms to form a distinct branch of the RyR family tree.

INTRODUCTION

The rapid release of Ca^{2+} ions from intracellular stores is a fundamental part of excitation–contraction coupling in cardiac and skeletal muscle and is mediated by a ryanodine-sensitive calcium-release channel (RyR) located in the junctional portion of the sarcoplasmic reticulum (SR) [1–3]. The mammalian cardiac [4–6] and skeletal [7–9] muscle RyRs have been isolated and shown to exist as large oligomeric complexes with apparent sedimentation coefficients of 30 S [5,6,9]. Ryanodine-binding, isoelectric-focusing and cross-linking studies have indicated a cooperatively coupled, negatively charged, homotetrameric arrangement for the skeletal 30 S complex [10]. Electron microscopy has shown that the subunits display 4-fold symmetry with a large cytoplasmic assembly and a smaller transmembranous region [9,11,12], giving an overall mushroom-like structure. The isolated RyRs are morphologically identical with the 'foot' structures that bridge the junctional gap between SR and plasma membrane [13], suggesting that the RyR complex might also play a structural role in maintaining the junctional framework [14].

The primary structure of three distinct isoforms of mammalian RyR expressed in the skeletal muscle of rabbit, human and pig (RyR-1) [15–17], rabbit cardiac muscle (RyR-2) [18,19] and rabbit brain (RyR-3) [20] have been deduced by cDNA cloning and sequencing, as has that of bullfrog skeletal muscle α and β

RyRs [21] and the *Drosophila* RyR [22]. A transcript of approx. 16 kilobases encoding a protein of approx. 560 kDa that comprises a hydrophobic C-terminal one-tenth and a hydrophilic N-terminal nine-tenths has been a consensus observation for all the cloned RyRs. A 2.4 kb transcript derived from the 3'-terminal region of the RyR-1 gene has also been found in rabbit brain [23]. Expression studies of the mammalian full-length RyR cDNAs [18,24–26] have been shown to produce functional Ca^{2+} release channels.

Muscle excitation–contraction coupling comprises the activation of SR Ca^{2+} release channels after plasma membrane depolarization [27]. In cardiac muscle, the elementary events underlying excitation–contraction coupling are referred to as 'calcium sparks' and are attributable to the opening of ryanodine-sensitive Ca^{2+} -release channels [28–31]. Although there is a general structural and functional similarity between skeletal and cardiac muscle RyRs, the native and reconstituted cardiac RyR channels show greater sensitivity to Ca^{2+} , but lower sensitivity to Mg^{2+} and ATP, than the skeletal RyR [1–3]. These observations, together with morphological studies indicating that the cardiac RyR is not in contact with the plasma membrane [32] and functional studies showing that entry of external Ca^{2+} is required for cardiac muscle contraction [1–3], suggest that Ca^{2+} -induced Ca^{2+} release might be the physiological mechanism that triggers Ca^{2+} efflux in cardiac SR.

Here we report the identification, primary structure and

Abbreviations used: Ins(1,4,5) P_3 R, Ins(1,4,5) P_3 receptor; MR1, MR2, modulator regions; ORF, open reading frame; RyR, ryanodine receptor; hRyR, human ryanodine receptor; rRyR, rabbit ryanodine receptor; SR, sarcoplasmic reticulum.

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topological analysis of the human cardiac muscle RyR (hRyR-2). Sedimentation and immunochemical analysis indicate that both the human and rabbit cardiac RyR are similar proteins. Cloning and sequence analysis indicates that the hRyR-2 is a protein of 4967 amino acids and that novel splice variants might exist. Six hydrophobic regions in the highly conserved C-terminal region are observed, and a predicted cytoplasmic loop connecting two of these regions is shown by competitive ELISA to be on the cytosolic surface of the native protein. Phylogenetic analysis indicates the possible evolutionary relationship between the various members of the RyR channel family, and the divergence of RyR-2 from the ancestral RyR before RyR-1 and RyR-3.

MATERIALS AND METHODS

Isolation of human and rabbit cardiac muscle SR microsomes

Crude SR microsomes were prepared from human and rabbit cardiac muscle and rabbit skeletal muscle by differential centrifugation [9]. Human heart biopsy material was stored at -80°C (National Heart and Lung Institute, London, U.K.). Freshly dissected and minced rabbit tissues, or partly thawed human tissue, were homogenized with an Ultra-Turrax T25 homogenizer in ten volumes of homogenization buffer (10 mM Hepes, pH 7.2, containing 0.25 M sucrose, 0.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM PMSF, 1 mM benzamidine, 10 μM leupeptin, 1 μM pepstatin A and 100 nM aprotinin). Homogenates were centrifuged for 25 min at 4000 *g* (Beckman JA20) and the supernatant re-centrifuged for a further 60 min at 100000 *g* (Beckman 50.2Ti). Microsomal pellets were resuspended in four volumes of homogenization buffer by using ten strokes of a Dounce homogenizer, then rapidly frozen and stored at -80°C until required. All procedures were performed at 4°C .

RyR-2 antibody production

A peptide corresponding to the conserved RyR-2 residues 4676–4699 and 4674–4697 of the rabbit RyR-2 (rRyR-2) and hRyR-2 respectively was synthesized on an Applied Biosystems 430A Peptide Synthesizer and coupled via an additional C-terminal cysteine to keyhole limpet haemocyanin by using the bifunctional reagent sulpho-SMCC (Pierce), in accordance with the manufacturer's instructions. Rabbits were injected subcutaneously with 250 μg of the peptide–keyhole-limpet haemocyanin conjugate in Freund's complete adjuvant, followed by three booster injections at 28-day intervals in Freund's incomplete adjuvant. Antisera were tested for specificity by ELISA and immunoblotting with either coupled or uncoupled peptide and rabbit skeletal and cardiac muscle SR microsomes. Rabbit antiserum pAb129 was found to recognize specifically the cardiac muscle RyR and not the skeletal RyR, and was used at an optimum dilution of 1:500 for immunoblot analysis.

SDS/PAGE and immunoblot analysis

SDS/PAGE was performed with 5% (w/v) separating and 3% (w/v) stacking gels [33] cast in a Bio-Rad minigel system. Human and rabbit SR microsomes (50 μg) were denatured for 5 min at 95°C in loading buffer [0.1 M Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 50 $\mu\text{g}/\text{ml}$ Bromophenol Blue]. Electrophoresis was performed at a constant current (20 mA per gel) and gels were either stained with Coomassie Brilliant Blue R250 or electrophoretically transferred on to Immobilon-P [poly(vinylidene difluoride)] membrane (Millipore) at 15°C for 1 h at 400 mA, then for 15 h at 1500 mA. After blocking for 1 h with 4% (w/v) non-fat dried

milk proteins in PBS, the blot was incubated for 2 h at 23°C with pAb129. The immunoblot was developed with peroxidase-coupled secondary antibody and 3,3'-diaminobenzidine/ H_2O_2 .

Competitive ELISA with intact or permeabilized cardiac and skeletal muscle SR microsomes

The competitive ELISA was performed by the method described by Grunwald and Meissner [34]. Antigen-coated plates were prepared by incubating polystyrene microtitre plates with cardiac SR microsomes (10 μg per well) for 4 h at 23°C in 50 mM NaHCO_3 , pH 9.0, fixed with 10% (v/v) methanol, then blocked by incubation with 5% non-fat dried milk proteins in PBS at 4°C overnight. Detergent-permeabilized cardiac and skeletal SR microsomes were prepared by incubation in 125 mM Tris/HCl, pH 7.5, containing 750 mM NaCl, 1 mM CaCl_2 , 0.25% CHAPS, 0.2 mM PMSF, 1 mM benzamidine, 10 μM leupeptin, 1 μM pepstatin A and 100 nM aprotinin for 1 h at 23°C . Control SR microsomes were treated in parallel without CHAPS. The treated samples were first diluted 5-fold with deionized water then serially diluted to the required concentration with 25 mM Tris/HCl, pH 7.5, containing 150 mM NaCl with or without 0.05% CHAPS. To these diluted SR samples was added the cardiac-specific antibody pAb129 (1:50 final) in 4% milk proteins, 25 mM Tris/HCl, pH 7.5, containing 150 mM NaCl. After overnight incubation at 4°C , the free antibody was detected by adding 100 μl of the solution to the antigen-coated plates for 2 h at 23°C . Wells were washed with PBS/Tween-20 and the antibody bound to the plates was determined with peroxidase-coupled secondary antibody and *o*-phenylenediamine. The fraction of free antibody was calculated from the ratio of D_{490} for antibody incubated with and without SR microsomes.

Cloning of human cardiac muscle RyR cDNA

Overlapping cDNA clones comprising the complete coding sequence of the human cardiac muscle RyR were isolated from a single λZAPII cDNA library (Stratagene) derived from a 22-year-old normal male. Two human cardiac muscle RyR cDNAs (Card 2, corresponding to nt 3959–5464, and Card 13, corresponding to nt 149–1927) were obtained by screening 10^6 phage plaques with two rabbit RyR-2 fragments amplified by PCR. Labelling of Card 2 and Card 13 and subsequent cDNA probes with [α - ^{32}P]dCTP employed a random primer oligonucleotide labelling kit (Pharmacia). Screening of 10^6 plaques on nitrocellulose filters with [α - ^{32}P]dCTP-labelled DNA probes was performed in accordance with the library manufacturer's instructions (Stratagene). Clones spanning the region –121 to 7300 bp that comprised HC1 (3258–4898 bp), HC2 (4097–5550 bp), HC3 (4690–7300 bp), HC4 (–121 to 2617 bp) and HC5 (1139–4037 bp) were identified with the Card 2 and Card 13 probes. A hRyR-2 PCR product encompassing 9372–9814 bp was amplified from the human library DNA by using rRyR-2 primers, and subsequent screening with this probe yielded clones HC6 (8030–10446 bp) and HC7 (9441–11940 bp). Positives obtained with the 3' *EcoR*I fragment of HC3 (5443–7300 bp) were rescreened with clone HC6 (8030–10446 bp) to identify a single spanning clone HC8 (6131–8292 bp). The most 3' clone, HC9 (12595–15610 bp), was isolated with a rabbit kidney RyR-2 cDNA clone, RK5 (13894–15801 bp) [35]. The final and largest clone isolated, HC10 (8831–13605 bp), was obtained after screening first with clone HC7 (9441–11940 bp) and then subsequent rescreening with clone HC9 (12595–15610 bp) to ensure a clone overlapping both HC7 and HC9. pBluescript SK(–) phagemids were prepared from each of the eluted positive phage

isolates by using the excision protocol *in vivo* in accordance with the manufacturer's instructions (Stratagene). All other DNA manipulations were performed with standard protocols [36].

DNA sequencing

The library screening procedures yielded a total of over 100 cDNAs. To identify the minimum number of clones needed to span the entire coding region (HC1–HC10), each was end-sequenced by the dideoxy termination method [37]. A series of unidirectional nested deletions was made for each of the clones HC1–HC10 by using the Erase-a-Base System (Promega). The nucleotide sequence of both DNA strands of deletion mutants was determined with the Taq DyeDeoxy® Terminator Cycle Sequencing Kit on an automated DNA sequencer (Applied Biosystems 373A). For cDNA clones that could not be deleted because of inappropriate restriction sites (HC1 and HC4), a series of synthetic oligonucleotide primers were made to enable sequence analysis.

Analysis of human RyR-2 cDNA and protein sequences

Nucleotide sequence data from individual clones were assembled into a full-length contig and the open reading frame (ORF) was translated with the AssemblyLIGN and MacVector sequence analysis programs (IBI, Kodak). Analysis of the full-length protein sequence was performed with LaserGene (DNASTar). Alignment of RyR sequences was by the Clustal method [38] using the PAM250 weight table and a gap penalty of 3 for the initial pairwise alignment. A phylogenetic tree was generated from the final alignment in the form of a cladogram in which the length of each pair of branches represented the distance between sequence pairs. The percentage identity between sequence pairs was calculated directly from the initial pairwise matrix used in the first step of the alignment. The identity plot was produced with the UWGCG sequence analysis program, Plotsimilarity, from the aligned hRyR-1, hRyR-2 and rRyR-3 sequences with a 10-amino-acid window. The hydropathy plot was calculated from the full-length hRyR-2 by using the Kyte and Doolittle [39] algorithm and a 21-amino-acid window.

RESULTS

Immunoblot analysis of human and rabbit cardiac SR microsomes

Figure 1 shows a Coomassie-stained gel (left panel) and a parallel immunoblot of human and rabbit cardiac SR microsomes probed with the RyR-2 specific antiserum pAb129 (right panel). Both human and rabbit cardiac microsomes possess high-molecular-mass proteins and a similar protein staining pattern, consistent with a common tissue origin. Immunoblot analysis with pAb129 reveals immunoreactivity with a single high-molecular-mass band corresponding to the lowest mobility protein (R_F 0.16). This immunoreactive protein in human and rabbit cardiac muscle microsomes is consistent with the molecular mass of approx. 565 kDa predicted for the rabbit cardiac muscle RyR [18,19]. No immunoreactivity with RyR-1 in skeletal muscle microsomes was observed with pAb129 (results not shown), confirming its isoform specificity for RyR-2 [35]. The differential staining intensity of immunoblots suggests a greater relative RyR-2 enrichment, of approx. 2-fold, in the rabbit. Because the preparation procedures were identical, this might be a genuine difference in relative abundance of the cardiac RyR between rabbits and humans, or it might be due to degradation caused by the requirement for freezing and storage of the human sample before homogenization. Sedimentation analysis of the CHAPS-solubilized

Figure 1 SDS/PAGE and immunoblot analysis of human and rabbit cardiac muscle SR

Left panel: Human (lane 1) and rabbit (lane 2) cardiac muscle SR (50 µg of protein per lane) was subjected to SDS/PAGE (5% gel) and stained with Coomassie Brilliant Blue. Right panel: two identical lanes were transferred on to Immobilon membrane and probed with a cardiac RyR-specific antibody, pAb129. Arrows indicate the positions of molecular mass standards (kDa).

rabbit and human cardiac muscle RyR indicated an apparent sedimentation coefficient of approx. 30 S (results not shown). Specific [3 H]ryanodine binding in the approx. 30 S peak fraction was 163 and 303 fmol/mg of protein for human and rabbit respectively (results not shown), a 2-fold difference, in agreement with the relative intensity of the RyR protein observed on immunoblot analysis (Figure 1).

Isolation of the human RyR-2 cDNA

Sequential rounds of library screening resulted in the isolation of ten overlapping cDNA clones, HC1–HC10 (Figure 2), that encompassed the entire ORF of hRyR-2. The ten clones were sequenced entirely on both strands to yield a contiguous nucleotide sequence of 15731 bp containing a 14901 bp ORF (open box

Figure 2 Map of human cardiac RyR cDNA clones

The cDNA clones HC1–HC10 isolated from a human cardiac muscle cDNA library are shown relative to the full-length hRyR-2 cDNA sequence. The nucleotide sequence of both strands of each cDNA clone was determined. A partial restriction map is illustrated above the open box on the full-length cDNA representing the ORF, with solid bars for the 5' and 3' untranslated regions. Arrows denote the location of sequence insertions. Clone HC3 has a unique 128 bp sequence at its 3' end, containing several stop codons, that is absent from clone HC8, and has no similarity with any database sequence. Clone HC6 contains a 67 bp sequence insertion between residues 8129 and 8130 that is absent from clone HC8 and follows the GT/AT rule [57] for intron–exon boundaries, suggesting that it is an unspliced intron. Clone HC9 contains a 17 bp insert between residues 13914 and 13915 that is not present in several smaller clones isolated in this region (results not shown), nor in the rRyR-2 sequence [18,19]. This insert does not follow the GT/AT rule and leads to a frame shift that terminates the ORF.

Figure 3 For caption see page 483.

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Figure 3 Alignment of the hRyR-2 protein sequence with other members of the RyR family of calcium-release channels

The deduced amino acid sequence (single-letter code) of the human cardiac muscle RyR (Hum 2) was aligned with those of the bullfrog skeletal muscle α and β RyRs (Fro A and Fro B) [21], human skeletal muscle RyR (Hum 1) [16], pig skeletal muscle RyR (Pig 1) [17], rabbit skeletal muscle RyR (Rab 1) [15,16], rabbit cardiac RyR (Rab 2) [18,19], rabbit brain RyR (Rab 3) [20] and *Drosophila* RyR (Dros) [22]. Optimal alignment was made by using the Clustal method [38] with gaps (–) inserted to maintain maximum identity.

in Figure 2). The encoded 4967 amino acid residues predicts a protein with molecular mass of 564 569 Da for hRyR-2, consistent with pAb129 staining of a single high-molecular-mass

band in human and rabbit cardiac muscle microsomes (Figure 1). The initiation methionine codon was within the sequence GAACCATGG, consistent with the eukaryotic consensus in-

Figure 4 Phylogenetic tree of the RyR family of calcium-release channels

A phylogenetic tree was generated from the optimal alignment of available full-length RyR sequences shown in Figure 3 to determine the ancestral relationships between sequences. Branch distances from the ancestral node correspond to sequence divergences.

initiation sequence CA(G/A)CCATGG [40] and the corresponding rabbit cardiac RyR sequence GAGCCATGG [18,19]. The 121 bp of 5'-untranslated sequence upstream of the initiation codon is 81% GC and without a TATA box, like the corresponding region of rRyR-2 [18,19]. However, unlike rRyR-2, the 709 bp of 3'-untranslated sequence, which begins after the TAA termination codon (Figure 2), is 59% AT and does not contain the polyadenylation signal sequence AATAAA or extend to the polyadenylation site, suggesting a larger 3'-untranslated region for the hRyR-2 transcript. Five of the cDNA clones (HC2, 3, 6, 7 and 9) were found to contain additional nucleotide sequence insertions, two of which (HC2 and 7) did not disrupt the ORF (see legend to Figure 2). The 24 bp insert in HC7 (GTCACAGGATCCCAACGCAGCAAG), absent from HC10, was found between residues 11145 and 11146 and encodes the eight amino acids VTGSQRSK. An identical inserted amino acid sequence has been reported for rRyR-2 between residues 11146 and 11147 [18]. The 30 bp insert in HC2 (CTTTGCCATTGACTCGCTGTGTGGCTTTGG), absent from HC1, was located between residues 4437 and 4438 and encodes the ten amino acids FAIDSLCGFG. This is a novel sequence that has not been previously observed in the rRyR-2 sequence [18,19].

Analysis of the human RyR-2 sequence and comparison with other RyRs

The two pairs of tandem repeat sequence motifs of 114–119 amino acids [16,18–21] were found in hRyR-2 between amino acid residues 853–966, 967–1080, 2692–2810 and 2811–2925 (Figure 3). The average identity across these four homologous repeats is approx. 27%. Figure 3 shows a Clustal alignment [38] of the amino acid sequence of the eight known full-length RyRs with that of the hRyR-2. Although large gaps have to be introduced to span stretches of amino acids found only in the invertebrate *Drosophila* RyR sequence, there is a high level of identity (more than 60%) observed along the whole length of the alignment, in particular in the C-terminal region.

Two notable regions of very low identity between RyRs were present between residues 1310 and 1423 and residues 4208 and 4489 of hRyR-2, comprising 114 and 282 amino acids residues respectively, a consistent feature for all RyRs [18,19,41]. The first variable region (1310–1423) is characterized by substantial deletions in both the rRyR-3 and frog RyR-β sequences, coinciding with additional sequence insertions from *Drosophila* for which there is no corresponding sequence in all other RyRs. The second variable region (4208–4489) has few of the insertions/deletions seen in the first variable region and is characterized by its high content of charged residues, in particular glutamate, as exemplified by the skeletal muscle RyR-1 sequence of PEPEPEPEPE, previously suggested as a putative Ca²⁺-binding site [42]. The alignment also shows that the RyR-2 epitope for pAb129 lies in the only low-identity stretch of residues present within the C-terminal region of high identity of the RyRs.

The overall identity between RyRs, based on the initial pairwise comparison of RyR sequences (Figure 3), indicated that for each subtype of RyR there are high percentage identity scores, with 87% identity between rRyR-3 and frog rRyR-β, and 88% identity between rabbit, pig, human RyR-1 and frog RyR-α. Notably, the sequence identity of 98.6% for rabbit and human RyR-2 is the highest value obtained from the pairwise comparison.

The results of the alignment and percentage identity analysis were used to generate the phylogenetic tree shown in Figure 4. The tree is presented in the form of a cladogram and portrays the

Figure 5 Sequence similarity plot of the RyR isoforms and hydropathy plot of the hRyR-2

(A) A plot of sequence identity was generated from an optimal alignment of the human skeletal muscle (Hum 1), human cardiac muscle (Hum 2), and rabbit brain (Rab 3) RyR (methods as described in the legend to Figure 3) by using a window of ten amino acids. The solid horizontal line represents the average identity between all three isoforms of 65%. The position of the epitope for the RyR-2-specific antiserum pAb129 is shown with an arrow. (B) A hydropathy plot of the human cardiac muscle RyR made by using the Kyte and Doolittle algorithm with a window of 21 amino acids. Six conserved hydrophobic regions of the protein, which may constitute the membrane-spanning segments, are indicated by vertical arrows.

Figure 6 Competitive ELISA with intact and permeabilized cardiac and skeletal SR vesicles

Intact and permeabilized cardiac (A) or skeletal (B) SR vesicles were serially diluted, then incubated with the cardiac RyR-specific antiserum pAb129 as described in the Materials and methods section. Free antibody was detected by binding to microtitre plates coated with denatured cardiac SR. The amount of free antibody is plotted as a fraction of the total antibody detected in the absence of SR vesicles. Results are means $\pm$ S.E.M. for 5 experiments.

evolutionary divergence from a putative ancestral RyR and subsequent ancestors (nodes), with branch length indicating the degree of sequence divergence from the predicted ancestor. The arthropod sequence (*Drosophila*) shows an early divergence from the remaining vertebrate RyR sequences [21], which diverge at a much later stage into three closely related families of RyRs. The analysis shows that the RyR-2 subtype was the first to arise from the common ancestor lineage, which subsequently led to the subtypes RyR-1 and RyR-3 (Figure 4).

Analysis of RyR-2 transmembrane topology and competitive ELISA with pAb129

Figure 5 shows an identity plot (Figure 5A) based on the aligned RyR sequences from hRyR-1, hRyR-2 and rRyR-3, and an analysis of hydropathic index for hRyR-2 (Figure 5B). Apart from the two large variable regions between residues 1310 and 1423 and residues 4208 and 4489 of the hRyR-2 mentioned above, the average identity of 65% shown as a horizontal line across the plot illustrates the substantial similarity that exists between the three mammalian RyR isoforms. By comparing the conserved regions of RyR-1, RyR-2 and RyR-3 illustrated in Figure 5(A) with the hRyR-2 hydropathy plot shown in Figure 5B, six putative transmembranous sequences that are well conserved in all three isoforms are suggested in the C-terminal region (hRyR-2 residues 4499–4519, 4572–4593, 4719–4744, 4767–4789, 4809–4829 and 4844–4867, shown by arrows). Previous estimates of the number of transmembrane-spanning segments based on analyses of the RyR-1 sequence range from four to twelve [15,16,43].

The epitope for the RyR-2-specific antibody pAb129 corresponds to a predicted cytosolic loop (4594–4718) present between the second and third putative transmembrane sequences. To test this prediction, competitive ELISA studies were performed on intact and permeabilized cardiac and skeletal SR microsomes [34]. Figure 6(A) illustrates that in the presence of either intact or permeabilized cardiac SR, pAb129 depletion occurred simultaneously, indicating that the antibody epitope is cytoplasmically exposed, in agreement with the transmembrane predictions above (see Figure 7). However, this result does not preclude the possibility of an alternative transmembrane topology with an even number of helices other than six. Figure 6(B) illustrates a control experiment with skeletal muscle SR where no antibody

Figure 7 Working model of RyR transmembrane topology

A working model of the transmembrane topology of the RyRs with six putative transmembrane segments. The epitope for the RyR-2-specific antibody pAb129 is shown in the cytoplasmic loop present between transmembrane segments 2 and 3. The relative lengths of regions between putative transmembrane segments are shown by solid lines.

depletion occurs, thus demonstrating the isoform specificity of pAb129.

DISCUSSION

In the present study we describe the identification of the human cardiac RyR–calcium release channel as an approx. 30 S oligomeric complex comprising high-molecular-mass (approx. 560 kDa) subunits (Figure 1, and results not shown), the cloning and sequencing of the corresponding full-length cDNA (Figures 2 and 3) and a topological and phylogenetic analysis (Figures 4 to 6). This is the first report of the biochemical characterization of the human cardiac RyR with a demonstration of novel transcripts and the use of an epitope-specific antibody to address RyR-2 transmembrane topology.

To avoid potential allelic differences that can occur between various individuals, a cDNA library derived from a single human heart was used to isolate ten overlapping cDNA clones, HC1–HC10. The full-length cDNA sequence of hRyR-2 contains an ORF of 14901 bp encoding a 564569 Da protein of 4967 amino

acid residues. The similar sequence length and predicted molecular mass for both hRyR-2 and rRyR-2 [18,19] corroborate our observation that an RyR-2-specific antibody, pAb129, recognizes the same mobility high-molecular-mass band in human and rabbit cardiac SR microsomes (Figure 1).

The identical sedimentation properties of the CHAPS-solubilized hRyR-2 and rRyR-2 as an approx. 30 S oligomer (results not shown) suggest that, as shown for RyR-1 [9,10], the RyR-2 complex also exists as a homotetrameric complex. Although there have been several reports that the $\text{Ins}(1,4,5)\text{P}_3$ receptor [$\text{Ins}(1,4,5)\text{P}_3\text{R}$] in certain cells comprises a heterotetrameric assembly, with subunits derived from distinct $\text{Ins}(1,4,5)\text{P}_3\text{R}$ -1, -2 and -3 isoforms [44–46], no evidence for a hetero-oligomeric RyR complex has yet been reported. In tissues such as avian and amphibian skeletal muscle, where more than one RyR isoform has been found, immunoprecipitation studies with specific antibodies have shown the homo-oligomeric nature of the RyR complexes [47,48].

The alignment of RyR sequences in Figure 4 reveals that the most closely related of all are hRyR-2 and rRyR-2, with an identity of 98.6%, suggesting an evolutionary requirement for greater structural conservation of the RyR-2 subtype. A potential ATP-binding site with the characteristic nucleotide-binding motif GWGNFG is situated in the middle of a predicted β -strand and α -helix between residues 2618 and 2653. This lies within the proposed modulator region (MR1) that spans residues 2618–3015 [19], which is conserved in hRyR-2. Three further sites are also present in the hRyR-2 between residues 1324–1329, 2336–2341 and 4351–4356 [18], although none of these predicted sites has been confirmed. As in the rabbit sequence, the hRyR-2 MR1 sequence has three potential calmodulin-binding sites, between residues 2774–2806, 2876–2897 and 2997–3015, and a calmodulin-dependent protein kinase phosphorylation site at residue 2808, which, when phosphorylated in the dog cardiac RyR, has been shown to increase the probability of channel opening [49].

The hydropathy plot of hRyR-2 (Figure 5B) suggests that the whole of this putative MR1 is surface exposed and significantly hydrophilic, consistent with the observation that this region is protease-sensitive [50]. In RyR-1, only weak calmodulin binding to this region has been found [51], in contrast with stronger binding of calmodulin, Ca^{2+} and Ruthenium Red to sites within a second modulator region (MR2) suggested between residues 3614 and 4457 [15,51]. However, unlike MR1, which is conserved between RyRs (Figure 5A), MR2 overlaps one of the variable regions that in hRyR-2 is from residues 4208–4489 and is rich in charged residues. The corresponding variable region of RyR-1 contains a proline–glutamate repeat sequence found uniquely in the skeletal RyRs between residues 4489 and 4499 that is thought to be involved in Ca^{2+} -induced Ca^{2+} release [52]. These observations, together with the proximity of the MR2/variable region (4208–4489) to the proposed channel-forming carboxy-domain, suggest that the segment between residues 4208 and 4489 might be responsible for the differences observed between cardiac and skeletal RyRs. These differences include the increased sensitivity of the cardiac RyRs to Ca^{2+} , and reduced sensitivity to Mg^{2+} and Ruthenium Red [1–3]. Thus the unique modulator region for each class of RyR probably confers the distinct functional and biochemical properties of the various RyR isoforms.

Conserved hydrophobic segments adjacent to the putative modulator regions have been used in previous predictions of the RyR transmembrane topology to suggest between four and twelve membrane-spanning sequences [15,16,19,43]. Inclusion of the hRyR-2 sequence in the alignment of putative transmembrane segments supports the presence of at least six membrane-spanning sequences for the RyRs (Figure 5). These are all highly conserved

regions in the C-terminal region of the RyRs. This transmembrane topology is consistent with the competitive ELISA results with pAb129 (Figure 6), illustrating that this epitope is present on the cytoplasmic side of the membrane, a model of which is shown in Figure 7. This putative transmembrane channel-forming C-terminal domain is also consistent with the observed location of the ryanodine-binding site at the C-terminal region of RyR-1 [53,54]. However, further studies are required for a full definition of the transmembrane topology of the RyRs.

The RyR-1 isoform has three conserved glutamic acid-rich stretches between residues 1873–1923, 2025–2090 and 3675–3750 (Figure 3), which could be low-affinity calcium-binding sites. These sites are also present, although to a smaller extent, in the other classes of RyR. In hRyR-2 the first of these regions is between residues 1853 and 1891 and contains 12 of the 39 glutamate residues found in RyR-1; the second, between residues 2012 and 2055, has only five of the fifteen glutamate residues. Similar differences are observed with RyR-3 in these two regions (Figure 3). Interestingly, the third glutamic acid-rich sequence, between residues 3649 and 3720, lies within the proposed MR2 [15]. Unlike the other two, this third glutamic acid-rich region is much more highly conserved between the RyRs, suggesting that it might be an important regulatory site common to all RyRs. We have found an insertion within this region encoding eight amino acids (VTGSQRSK) (HC7; Figure 2) between residues 3715 and 3716, identical with the sequence insertion found in rRyR-2 [18] between residues 3716 and 3717. Alternative transcripts with or without this insertion might provide a means for altering the binding affinity of this putative site for calcium.

A second insertion that we have observed, not previously seen in any other RyR, is the ten amino acids (FAIDSLCGFG) between residues 1479 and 1480 (HC2; Figure 2). This segment might also contribute to functional diversity, as it is adjacent to the variable region illustrated in our alignment between residues 1310 and 1423 of hRyR-2 (Figure 3), suggesting that this is another potential modulatory region within the RyR isoforms. The presence of alternate RNA transcripts of the hRyR-2 implies a greater diversity in intracellular Ca^{2+} signalling via RyR-2 than has previously been shown. Similar RNA splice variants have been reported for the $\text{Ins}(1,4,5)\text{P}_3\text{R}$ within the $\text{Ins}(1,4,5)\text{P}_3$ -binding site and also adjacent to putative sites for phosphorylation and ATP binding, where it is thought that they might alter affinities for ligands [55]. Significantly, no spliced variant has so far been identified in the conserved channel portion of either the RyR family or the $\text{Ins}(1,4,5)\text{P}_3\text{R}$ family of proteins, presumably reflecting the structural importance of this region.

The phylogenetic tree constructed from the aligned sequences (Figure 4) suggests that all RyRs are descended from a common ancestral gene and that RyR-2 diverged first from the ancestral vertebrate RyR to form a distinct branch of the RyR family tree. The tree indicates that the divergence into three main classes of vertebrate RyR was very close and occurred after the divergence from the invertebrate lineage. This is consistent with the observation that the murine and human genes for each class of RyR are located on different evolutionarily linked chromosomes [56].

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