

Primary structure and functional expression from cDNA of the cardiac ryanodine receptor/calcium release channel

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The sequence of 4968 (or 4976 with an insertion) amino acids composing the ryanodine receptor from rabbit cardiac sarcoplasmic reticulum has been deduced by cloning and sequencing the cDNA. This protein is homologous in amino acid sequence and shares characteristic structural features with the skeletal muscle ryanodine receptor. *Xenopus* oocytes injected with mRNA derived from the cardiac ryanodine receptor cDNA exhibit Ca^{2+} -dependent Cl^- current in response to caffeine, which indicates the formation of functional calcium release channels. RNA blot hybridization analysis with a probe specific for the cardiac ryanodine receptor mRNA shows that the stomach and brain contain a hybridizable RNA species with a size similar to that of the cardiac mRNA. This result, in conjunction with cloning and analysis of partial cDNA sequences, suggests that the brain contains a cardiac type of ryanodine receptor mRNA.

Ryanodine receptor; Calcium release channel; cDNA cloning; cDNA expression; RNA blot hybridization analysis; Rabbit heart

1. INTRODUCTION

The ryanodine receptor in the sarcoplasmic reticulum (SR) functions as a calcium release channel and is most likely involved in the Ca^{2+} release that triggers muscle contraction [1]. This receptor protein is thought to form a homo-tetrameric complex with the characteristic 'foot' structure [2] which spans the gap between the SR and transverse tubule (T-tubule) membranes. Cloning and sequence analysis of cDNA have revealed that the rabbit skeletal muscle ryanodine receptor is composed of 5037 amino acid residues with the carboxy-terminal transmembrane region which probably forms the calcium release channel and the remaining portion which apparently constitutes the foot structure [3]. It has also been shown that the skeletal muscle ryanodine receptor, expressed in Chinese hamster ovary cells, functions as an intracellular calcium release channel [4].

The mechanism of excitation–contraction (E–C) coupling in cardiac muscle differs from that in skeletal muscle in that entry of extracellular Ca^{2+} is required for contraction of cardiac muscle, but not of skeletal muscle [1]. Expression of the cardiac and the skeletal

muscle dihydropyridine receptor cDNA in dysgenic mouse skeletal muscle has been shown to restore E–C coupling which resembles that of cardiac and skeletal muscle, respectively [5–7]. However, possible involvement of the ryanodine receptor in determining the type of E–C coupling remains to be investigated. Previous studies have shown that the cardiac and the skeletal muscle ryanodine receptor exhibit some differences in properties including molecular size, sensitivity to ligands and antigenicity [8–14].

The present investigation deals with cloning and sequencing of cDNA encoding the ryanodine receptor from rabbit heart and with expression in *Xenopus* oocytes of functional calcium release channels from the cloned cDNA. Evidence has been obtained to suggest that a cardiac type of ryanodine receptor mRNA is present in the brain and probably also in the stomach.

2. MATERIALS AND METHODS

2.1. Cloning and sequencing of cDNAs

Total RNA was extracted [15] from adult rabbit heart and poly(A)⁺ RNA was isolated [16]. An oligo(dT)-primed, size-selected (≥ 1 kilobase pairs (kb)) cDNA library [17], constructed in λ gt10 using rabbit heart poly(A)⁺ RNA, was screened with the *Sma*I(13290)/*Sma*I(15181) fragment from clone pRR616 [3] to yield λ HRR10 and λ HRR12; restriction endonuclease sites are identified by numbers (in parentheses) indicating the 5'-terminal nucleotide generated by cleavage. The cDNA inserts of λ HRR10 and λ HRR12 were subcloned into the *Eco*RI site of pBluescript KS(+) (Stratagene) to yield pHRR10 and pHRR12, respectively. A randomly primed, size-selected (≥ 1 kb) cDNA library, constructed in pBluescript KS(+) as in [3], was screened four times with different probes: the

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Abbreviations: SR, sarcoplasmic reticulum; T-tubule, transverse tubule; E–C coupling, excitation–contraction coupling; IP_3 , inositol 1,4,5-trisphosphate

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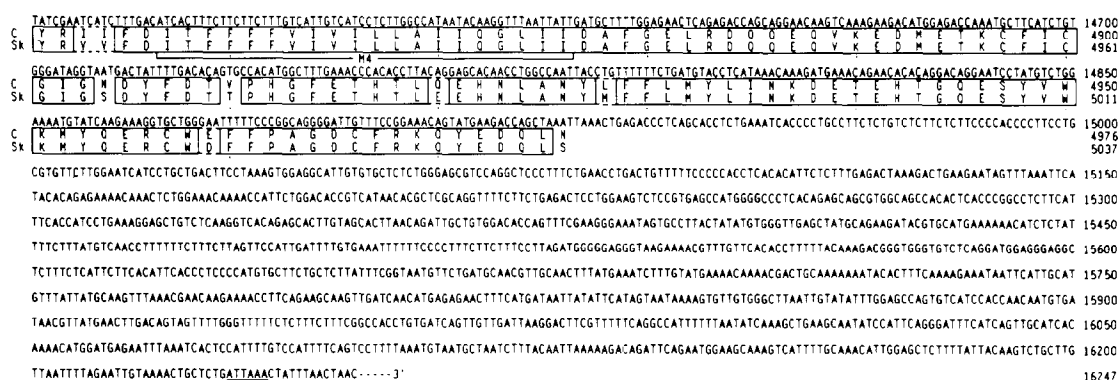


Fig. 1. Nucleotide sequence of cloned cDNA encoding the rabbit cardiac ryanodine receptor together with the preceding genomic DNA sequence (top) and alignment of the deduced amino acid sequence (in one-letter code) of the rabbit cardiac ryanodine receptor (C, middle) with that of the rabbit skeletal muscle ryanodine receptor (Sk, bottom). The sequence data for the rabbit skeletal muscle ryanodine receptor have been taken from [3]. The nucleotide sequences determined with the cloned cDNA and genomic DNA are shown in capital and small letters, respectively. Nucleotide residues are numbered in the 5' to 3' direction from the first residue of the ATG initiation triplet and the preceding residues are indicated by negative numbers. Amino acid residues are numbered from the initiating methionine. Numbers of the nucleotide and amino acid residues at the right-hand end of the individual lines are given. Sets of identical amino acid residues are enclosed with solid lines, and sets of conservative residues [26] with broken lines. Deletions and insertions in the amino acid sequence of the rabbit skeletal muscle ryanodine receptor, as compared with that of the rabbit cardiac ryanodine receptor, are indicated by gaps (—) and triangles (with the number of inserted residues in parentheses), respectively. The putative mRNA start site is marked with an asterisk. Sequences of the GC box and the polyadenylation signal are underlined. Nucleotide residue 16247 is followed by a poly(dA) tract. The putative transmembrane segments M1–M4 are indicated; the termini of each segment are tentatively assigned by comparison with the rabbit skeletal muscle ryanodine receptor. The nucleotide differences observed among the individual clones are as follows: G (pHRR105, pHRR158 and three other clones) or A (pHRR160) at residue 5341; insertion (pHRR10 and another clone) or deletion (pHRR60 and three other clones) of residues 1146–1169 (overlined). The resulting amino acid substitutions are as follows: E or K at residue 1781; insertion/deletion of residues 3716–3723. The first-named (or inserted) residues are given in the sequences presented.

0.50-kb *EcoRI*(vector)/*HincII*(12025) fragment from pHRR12 yielded five positive clones including pHRR60; the 0.72-kb *EcoRI*(vector)/*HincII*(7584) fragment from pHRR60 yielded pHRR105; the 0.38-kb *EcoRI*(vector)/*EcoRI*(5444) fragment from pHRR105 yielded four positive clones including pHRR158 and pHRR160; and the 0.24-kb *EcoRI*(vector)/*BglII*(1921) fragment from pHRR160 yielded pHRR251. A synthetic primer complementary to nucleotide residues 1235–1251, prepared using an automatic DNA synthesizer (Applied Biosystems), was elongated by the procedures described previously [3] and the resulting clones were selected with the 0.29-kb *EcoRI*(vector)/*BglII*(1220) fragment from pHRR251 to yield pHRR404, which carried the 5'-terminal cDNA sequence. Another oligo(dT)-primed, size-selected (≥ 1 kb) cDNA library, constructed in pBluescript KS(+) as in [3], was screened with the 2.1-kb *PvuII*(14114)/*EcoRI*(vector) fragment from pHRR12 to yield pHRR501. Nested deletions were made [18] and DNA sequencing [19] was carried out on both strands, except for portions of the 3'-noncoding sequence following nucleotide residue 14999. The cDNA clones used for nucleotide sequence analysis were as follows: pHRR404 (carrying nucleotides –313 to 1251), pHRR251 (934–2512), pHRR160 (1690–5508; residues 1949–2149 were not sequenced), pHRR158 (2714–5614; residues 2993–4841 were not sequenced), pHRR105 (5071–9003), pHRR60 (6869–12497 with a deletion of 1146–1169; residues 7205–8784 and 11704–12233 not sequenced), pHRR10 (10975–14998; residues 11258–14675 not sequenced), pHRR12 (11527–16207) and pHRR501 (13321–16247; residues 13545–15993 not sequenced).

A rabbit genomic DNA clone (λ HRRG3), which contained a 5'-flanking sequence and the putative first exon (extending to nucleotide residue 48) of the cardiac ryanodine receptor gene, was isolated by screening a Charon 4A library [20] with the *PstI*(–292)/*PstI*(–153) fragment from pHRR404 as probe. The ~15-kb *EcoRV* fragment and the ~5-kb *BamHI* fragment from λ HRRG3 were subcloned into pBluescript SK(–) for sequence analysis.

An oligo(dT)-primed, size-selected (≥ 1 kb) cDNA library was con-

structed in λ gt10 using poly(A)⁺ RNA from adult rabbit brain as in [17]. The library was screened with the 2.1-kb *PvuII*(14114)/*EcoRI*(vector) fragment from pHRR12 to yield 9 positive clones including λ BRR58 and λ BRR59. The cDNA inserts of the 9 clones were subcloned into pBluescript KS(+) and analysed with restriction endonucleases (digestion with *HinfI*, with *DraI* plus *SacI* and with *EcoRI* plus *HincII*). Both ends of the cDNAs carried by the plasmids pBRR58 and pBRR59 were sequenced.

2.2. Synthesis of specific mRNA

Recombinant plasmids carrying the cDNA for the cardiac ryanodine receptor with (pSPCRR200) or without the 24-nucleotide insertion (pSPCRR51) were constructed as follows. The *SacII*(–49)/*AatI*(1107) fragment from pHRR404 and the 4.3-kb *AatI*(1107)/*SacII*(vector) fragment from pHRR251 were ligated to yield pCRR10. pCRR10 was digested with *SacII* and ligated with the synthetic *SacII*-*SalI* adaptor 5'-GTGCGACGC-3' (prepared using the automatic DNA synthesizer) to yield pCRR13. The *Clal*(2352)/*BamHI*(4867) fragment from pHRR160, the *BamHI*(4867)/*EcoRI*(5444) fragment from pHRR158 and the *EcoRI*/*Clal* fragment from pBluescript SK(–) were ligated to yield pCRR21. The 2.4-kb *SalI*(adaptor)/*Clal*(2352) fragment from pCRR13, the *Clal*(2352)/*EcoRI*(5444) fragment from pCRR21 and the *EcoRI*/*SalI* fragment from pBluescript KS(+) were ligated to yield pCRR31. The *XmaI*(5418)/*PstI*(8956) fragment from pHRR105, the *PstI*(8956)/*AccI*(11649) fragment from pHRR60, the 4.6-kb *AccI*(11649)/*EcoRI*(vector) fragment from pHRR12 and the *EcoRI*/*XmaI* fragment from pBluescript KS(+) were ligated to yield pCRR40. The 5.5-kb *SalI*(adaptor)/*XmaI*(5418) fragment from pCRR31, the 12.6-kb *XmaI*(5418)/*SacI*(vector) fragment from pCRR40 and the 1.1-kb *SacI*/*SalI* fragment from pBluescript SK(–) were ligated to yield pCRR51. The *Clal*(10483)/*PvuII*(10999) fragment from pHRR60, the *PvuII*(10999)/*SacII*(11227) fragment from pHRR10 and the *SacII*/*Clal* fragment from pBluescript SK(–) were ligated to yield pCRR100. The *XmaI*(5418)/*Clal*(10483) fragment from pCRR40, the *Clal*(10483)/*SacII*(11227) fragment from

pCRR100 and the 13.5-kb vector-containing *Sac*II(11227)/*Xma*I(5418) fragment from pCRR51 were ligated to yield pCRR200. Finally the 16.3-kb *Sac*II fragment containing the entire protein-coding sequence from pCRR51 (or pCRR200) was cloned into the *Sac*II site of pSP64AX [21] to yield pSPCRR51 (or pSPCRR200). mRNA specific for the cardiac ryanodine receptor was synthesized in vitro [22], using *Xho*I-cleaved pSPCRR51 (or pSPCRR200) as template. Transcription was primed with the cap dinucleotide m⁷G(5')ppp(5')G (1 mM) [23].

2.3. Electrophysiological measurements

The mRNA specific for the cardiac ryanodine receptor was injected into *Xenopus laevis* oocytes (mRNA concentration 1.0 µg/µl; average volume injected per oocyte, ~50 nl). The injected oocytes were incubated at 19°C for 4–6 days as in [24]. All electrophysiological measurements were performed in nominally Ca²⁺-free Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl₂, 10 mM Hepes, pH adjusted to 7.2 with NaOH) unless otherwise specified. Whole-cell currents were recorded as in [24].

3. RESULTS

3.1. DNA cloning

An oligo(dT)-primed cDNA library prepared from rabbit heart poly(A)⁺ RNA was screened by hybridization with a probe derived from the rabbit skeletal muscle ryanodine receptor cDNA. A fragment from an initial clone thus isolated was used to probe a randomly primed cDNA library for adjacent cDNA sequences and such procedures were repeated successively (see section 2.1).

To locate the mRNA start site, we cloned and sequenced rabbit genomic DNA corresponding to the 5'-terminal region of the cDNA. Primer extension experiments with the synthetic primers complementary to nucleotide residues -245 to -206 and to residues -265 to -236 (for residue numbers, see Fig. 1) yielded major products of ~130 and ~100 nucleotides, respectively. On the basis of these results and of the fact that eukaryotic mRNAs generally start with an A residue [25], the start site of the cardiac ryanodine receptor mRNA was tentatively assigned to the A residue -334 (Fig. 1). There are three overlapping copies of the GC box (residues -398 to -393, -394 to -389 and -390 to -385) upstream of the mRNA start site, but neither the CAAT box nor the TATA box is found. The skeletal muscle ryanodine receptor gene contains two GC box sequences at similar positions and a CAAT box sequence located between them [3]. The genomic DNA sequence corresponding to residues -313 to 48 of the cDNA is uninterrupted and residue 48 (followed by GTAAGC) is encompassed by a potential splice donor site [25]. This indicates that the coding sequence for the amino-terminal 16 amino acid residues of the cardiac ryanodine receptor is followed by an intron, as is the case for the skeletal muscle counterpart [3].

3.2. Protein structure

Fig. 1 shows the amino acid sequence of the rabbit cardiac ryanodine receptor, deduced from the cDNA

sequence using the open reading frame of the rabbit skeletal muscle ryanodine receptor cDNA. The nucleotide sequence surrounding the translational initiation codon agrees reasonably well with the consensus sequence [27]. Nucleotide residues 11146–11169 (encoding amino acid residues 3716–3723) are deleted in some clones. The insertion/deletion may be accounted for by alternative RNA splicing. The rabbit cardiac ryanodine receptor is composed of 4968 (or 4976 with the insertion) amino acid residues, its calculated *M_r* (including the initiating methionine) being 565057 (or 565901 with the insertion). The 3'-noncoding region of the cDNA is 1319 nucleotides long (excluding the poly(dA) tract); the polyadenylation signal ATTTAA [28] is found 14 nucleotides upstream from the poly(dA) tract.

The amino acid sequence of the rabbit cardiac ryanodine receptor shows 66% identity with that of the rabbit [3] (or human [29]) skeletal muscle ryanodine receptor; for evaluating sequence similarity, insertions/deletions have been counted as one substitution regardless of their length. The hydropathicity profile of the cardiac ryanodine receptor (Fig. 2A) is similar to that of the skeletal muscle counterpart in that there are four highly hydrophobic segments (referred to as M1, M2, M3 and M4) in the carboxy-terminal tenth of the molecule, that the remaining region is largely hydrophilic and that there is no hydrophobic amino-terminal sequence indicative of the signal sequence. Fig. 2B shows the profile of local amino acid sequence identity between the rabbit cardiac and skeletal muscle ryanodine receptors. The carboxy-terminal region that encompasses segments M3 and M4 is highly conserved, whereas other regions, for example, the region immediately preceding segment M1 and the region around position 1350 are rather divergent. The cardiac ryanodine receptor contains four repeated sequences occurring in two tandem pairs (amino acid residues 853–966, 967–1082, 2692–2810 and 2811–2925), as described for the skeletal muscle counterpart [29].

3.3. RNA blot hybridization analysis and cloning of partial cDNA sequences from brain

RNA preparations from different rabbit tissues were subjected to blot hybridization analysis with a probe derived from the 3'-noncoding region of the cardiac ryanodine receptor cDNA (Fig. 3). The heart contained a hybridizable RNA species of ~17 kilobases (lane 1). The size of this RNA agrees with our assignment of the mRNA start site and the polyadenylation site. A small amount of a hybridizable RNA species with a similar size was found in the stomach (lane 4) and in the brain (lane 2). No hybridizable RNA species was detected in the skeletal muscle (lane 3) and kidney (lane 5).

To identify the hybridizable RNA present in the brain, we screened a cDNA library derived from rabbit brain poly(A)⁺ RNA using a cardiac ryanodine recep-

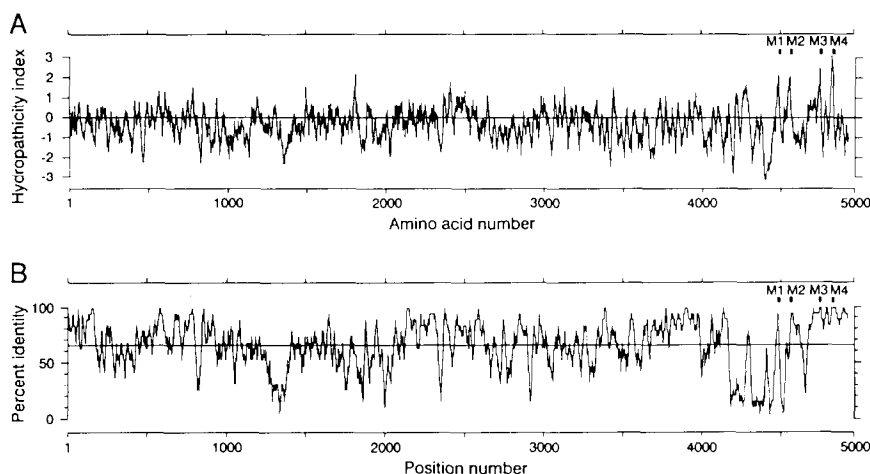


Fig. 2. (A) Hydropathicity profile of the rabbit cardiac ryanodine receptor. The averaged hydropathicity index [30] of a nonadecapeptide composed of amino acid residues $i-9$ to $i+9$ is plotted against i , where i represents the amino acid number. (B) Profile of local amino acid sequence identity between the rabbit cardiac and skeletal muscle ryanodine receptors. The percent sequence identity of a segment corresponding to positions $j-9$ to $j+9$ is plotted against j , where j represents the position number; deletions and insertions (see Fig. 1) have been counted as one position regardless of their length. The horizontal line in B indicates the overall percent sequence identity between the two ryanodine receptors (66%). The positions of the putative transmembrane segments M1–M4 are shown by filled boxes.

tor cDNA probe and obtained 9 positive clones. Restriction endonuclease analysis showed that all these clones yielded fragments common to those derived from the 3'-terminal region of the cardiac ryanodine

receptor cDNA (see section 2.1). Sequence analysis of two of these clones showed that they carried at least the sequences of nucleotide residues 14389–14687 and 15944–16247 (pBRR58) and of residues 14759–14981 and 15971–16247 (pBRR59) of the cardiac ryanodine receptor cDNA. These results indicate that the cardiac ryanodine receptor gene is transcribed in the brain. However, the brain mRNA may not be identical with the cardiac ryanodine receptor mRNA because transcription may be initiated at different promoters and/or because the transcription product may be subject to alternative splicing. The same may hold for the putative stomach ryanodine receptor mRNA.

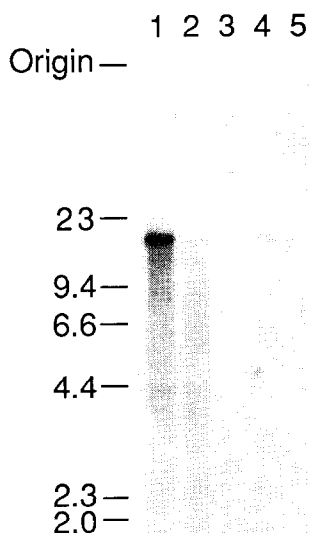


Fig. 3. Autoradiogram of blot hybridization analysis of RNA from adult rabbit tissues with a cDNA probe specific for the cardiac ryanodine receptor mRNA. Total RNA from heart (10 μ g, lane 1) and poly(A)⁺ RNA from brain (5 μ g, lane 2), skeletal muscle (10 μ g, lane 3), stomach (30 μ g, lane 4) and kidney (30 μ g, lane 5) were analysed as in [31]; the hybridization probe used was the 0.75-kb *HincII*(15463)/*EcoRI*(vector) fragment from pHRR12, labelled by the random primer method [32]. Autoradiography was performed at -70°C for 7 days with an intensifying screen. The size markers used were the *HindIII* cleavage products of phage λ DNA (sizes in kilobases).

3.4. Functional expression of cDNA

Recombinant plasmids carrying the entire protein-coding sequence of the cardiac ryanodine receptor cDNA (pSPCRR51 encoding 4968 amino acids and pSPCRR200 encoding 4976 amino acids with the insertion) were constructed and transcribed in vitro. Each of the resulting mRNAs was injected into *Xenopus* oocytes. The oocytes were tested for calcium release channel activity by measuring Ca^{2+} -dependent Cl^{-} current [33,34] that would occur in response to caffeine.

Fig. 4A(a) exemplifies a caffeine-induced response of an injected oocyte in nominally Ca^{2+} -free Ringer's solution. An inward current occurred after a substantial delay (9 ± 3 s (mean $\pm$ SD), $n = 16$, n indicating the number of oocytes; the dead-space time in the perfusion system has been subtracted). At -70 mV membrane potential, the peak inward current elicited by 50 mM caffeine (applied for 20 s) was 1.5 ± 1.3 μA ($n = 14$, excluding 5 unresponsive oocytes). The caffeine response was almost completely abolished by intracellular injection of the calcium-chelating agent

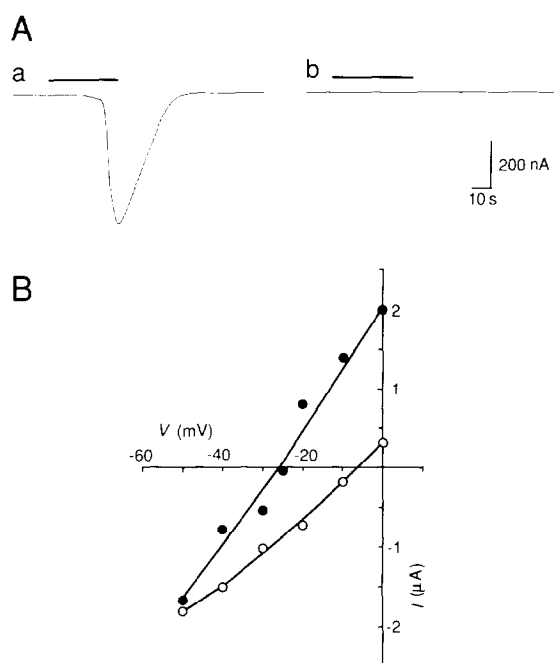


Fig. 4. (A) Caffeine response recorded from a *Xenopus* oocyte injected with the mRNA derived from the cloned rabbit cardiac ryanodine receptor cDNA. Whole-cell currents evoked by bath application of 50 mM caffeine were recorded under voltage clamp at -70 mV membrane potential in nominally Ca^{2+} -free Ringer's solution before (a) and after (b) intracellular injection of EGTA. Inward current is downward. The duration of caffeine application is indicated by bars without taking into account the dead-space time in the perfusion system (~ 7 s). EGTA was injected ionophoretically into the oocyte as in [35], except that the duration of injection was 10 min. (B) Peak current-voltage (I - V) relations (inward current shown as negative current) obtained from an injected oocyte in nominally Ca^{2+} -free Ringer's solution (●) and in the solution in which half of the Cl^- was replaced by methanesulphonate (○). Currents evoked by bath application of 50 mM caffeine for 10 s were recorded under voltage clamp at different membrane potentials. The data shown were from oocytes injected with the mRNA derived from clone pSPCRR51. Essentially the same results were obtained with oocytes injected with the mRNA derived from clone pSPCRR200.

EGTA (Fig. 4A(b)). No detectable response (< 10 nA) was observed in 31 non-injected oocytes; some non-injected oocytes showed a marginal outward current (21 ± 7 nA, $n = 10$) or a marginal inward current (21 ± 7 nA, $n = 5$) or both (an outward current of 20 nA followed by an inward current of 30 nA, $n = 1$).

Fig. 4B shows the membrane potential dependence of the caffeine response. The reversal potential of the caffeine-induced current, measured in nominally Ca^{2+} -free Ringer's solution (filled circles), was -23 ± 3 mV ($n = 6$). This is close to the equilibrium potential of chloride ions observed in *Xenopus* oocytes [33]. The reversal potential shifted to -6 ± 3 mV ($n = 6$) when the external Cl^- concentration was halved by substitution with methanesulphonate (open circles), but it did not significantly change when the external Na^+ was replaced by K^+ (-26 ± 3 mV, $n = 5$) or when the external K^+ was replaced by Na^+ (-24 ± 3 mV, $n = 5$).

These results indicate that the caffeine-induced current is carried by chloride ions and that this Cl^- current results from Ca^{2+} being released from an intracellular store in response to caffeine. Thus we conclude that functional calcium release channels are formed in *Xenopus* oocytes by expression of the cardiac ryanodine receptor cDNA.

4. DISCUSSION

The structural similarity demonstrated in the present investigation suggests that the cardiac ryanodine receptor, like its skeletal muscle counterpart [3], consists of two main parts, that is, the carboxy-terminal channel region which contains four putative transmembrane segments (M1-M4) and the large cytoplasmic region which corresponds to the foot structure; the presence of several additional potential transmembrane segments in the skeletal muscle ryanodine receptor has been proposed [29]. The carboxy-terminal region, including segments M3 and M4, is highly conserved between the cardiac and skeletal muscle ryanodine receptors. This region also shows remarkable amino acid sequence similarity to the carboxy-terminal region of the inositol 1,4,5-trisphosphate (IP_3) receptor [36]. Both the ryanodine receptor and the IP_3 receptor mediate release of Ca^{2+} from intracellular stores, as demonstrated by reconstitution of the purified protein into phospholipid bilayer membranes or vesicles [10-13,37] and by functional expression of the cloned cDNA (see section 3.4 and [4,38]). Thus the carboxy-terminal region of both the receptors may be important in forming intracellular membrane channels.

The SR calcium release channel is activated or inhibited by various modulators, including Ca^{2+} , adenine nucleotides, caffeine, calmodulin and polycationic compounds [1]. The regions encompassing amino acid residues 1336-1347 and 2010-2021 of the cardiac ryanodine receptor bear some resemblance to the EF-hand [39], but are not well conserved in the skeletal muscle ryanodine receptor. The cardiac ryanodine receptor has four copies of the nucleotide-binding consensus sequence GXGXXG [40] (residues 1324-1329, 2337-2342, 2626-2631 and 4352-4357), one of which (residues 2337-2342) is conserved in the skeletal muscle counterpart. Two potential calmodulin-binding sites [41] (residues 3581-3604 and 4257-4285) are found in the cardiac ryanodine receptor and one of them (residues 3581-3604) is particularly well conserved in the skeletal muscle counterpart. The observation that the region preceding and close to segment M1 is relatively divergent in amino acid sequence between the cardiac and skeletal muscle ryanodine receptors does not seem to favour our previous suggestion that this region contains putative modulator-binding sites [3]. It has been reported that the cardiac ryanodine receptor is phosphorylated by cyclic AMP-dependent protein

kinase [42]. The cardiac ryanodine receptor contains seven potential cyclic AMP-dependent phosphorylation sites [43] (serine residues 2056, 2808, 4701 and 4702 and threonine residues 2044, 2057 and 4283), all of which are located on the putative cytoplasmic side according to our transmembrane model; residue 4283 is conserved in both the rabbit and human skeletal muscle ryanodine receptors.

Several investigators have observed caffeine-induced Ca^{2+} release in neurons [44–46]. Electron-microscopic studies have revealed that junctions between the surface membrane and the subsurface cisternae of endoplasmic reticulum in neurons have structural features similar to those of the junctions between the T-tubule membrane and the terminal cisternae of SR in striated muscle [47,48]. Cloning and determination of partial cDNA sequences and RNA blot hybridization analysis have provided evidence that the brain contains a cardiac type of ryanodine receptor mRNA (see section 3.3). These findings together suggest that the cardiac type (and possibly other types) of ryanodine receptor may be present in the junctional membrane of the subsurface cisternae of neurons, taking part in Ca^{2+} signalling.

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REFERENCES

- [1] Fleischer, S. and Inui, M. (1989) *Annu. Rev. Biophys. Biochem.* 18, 333–364.
- [2] Franzini-Armstrong, C. (1970) *J. Cell Biol.* 47, 488–499.
- [3] Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T. and Numa, S. (1989) *Nature* 339, 439–445.
- [4] Penner, R., Neher, E., Takeshima, H., Nishimura, S. and Numa, S. (1989) *FEBS Lett.* 259, 217–221.
- [5] Tanabe, T., Beam, K.G., Powell, J.A. and Numa, S. (1988) *Nature* 336, 134–139.
- [6] Tanabe, T., Mikami, A., Numa, S. and Beam, K.G. (1990) *Nature* 344, 451–453.
- [7] Tanabe, T., Beam, K.G., Adams, B.A., Niidome, T. and Numa, S. (1990) *Nature* (in press).
- [8] Inui, M., Saito, A. and Fleischer, S. (1987) *J. Biol. Chem.* 262, 1740–1747.
- [9] Inui, M., Saito, A. and Fleischer, S. (1987) *J. Biol. Chem.* 262, 15637–15642.
- [10] Imagawa, T., Smith, J.S., Coronado, R. and Campbell, K.P. (1987) *J. Biol. Chem.* 262, 16636–16643.
- [11] Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q.-Y. and Meissner, G. (1988) *Nature* 331, 315–319.
- [12] Hymel, L., Schindler, H., Inui, M. and Fleischer, S. (1988) *Biochem. Biophys. Res. Commun.* 152, 308–314.
- [13] Anderson, K., Lai, F.A., Liu, Q.-Y., Rousseau, E., Erickson, H.P. and Meissner, G. (1989) *J. Biol. Chem.* 264, 1329–1335.
- [14] Imagawa, T., Takasago, T. and Shigekawa, M. (1989) *J. Biochem. (Tokyo)* 106, 342–348.
- [15] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) *Biochemistry* 18, 5294–5299.
- [16] Aviv, H. and Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* 69, 1408–1412.
- [17] Mikami, A., Imoto, K., Tanabe, T., Niidome, T., Mori, Y., Takeshima, H., Narumiya, S. and Numa, S. (1989) *Nature* 340, 230–233.
- [18] Henikoff, S. (1987) *Methods Enzymol.* 155, 156–165.
- [19] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
- [20] Maniatis, T., Hardison, R.C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G.K. and Efstratiadis, A. (1978) *Cell* 15, 687–701.
- [21] Yokoyama, S., Imoto, K., Kawamura, T., Higashida, H., Iwabe, N., Miyata, T. and Numa, S. (1989) *FEBS Lett.* 259, 37–42.
- [22] Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) *Nucleic Acids Res.* 12, 7035–7056.
- [23] Konarska, M.M., Padgett, R.A. and Sharp, P.A. (1984) *Cell* 38, 731–736.
- [24] Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., Mishina, M., Haga, T., Haga, K., Ichiyama, A., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. and Numa, S. (1986) *Nature* 323, 411–416.
- [25] Breathnach, R. and Chambon, P. (1981) *Annu. Rev. Biochem.* 50, 349–383.
- [26] Dayhoff, M.O., Schwartz, R.M. and Orcutt, B.C. (1978) in: *Atlas of Protein Sequence and Structure*, vol. 5 (Dayhoff, M.O. ed.) suppl. 3, pp. 345–353, National Biomedical Research Foundation, Silver Springs, MD.
- [27] Kozak, M. (1984) *Nucleic Acids Res.* 12, 857–872.
- [28] Goeddel, D.V., Leung, D.W., Dull, T.J., Gross, M., Lawn, R.M., McCandliss, R., Seeburg, P.H., Ullrich, A., Yelverton, E. and Gray, P.W. (1981) *Nature* 290, 20–26.
- [29] Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N.M., Lai, F.A., Meissner, G. and MacLennan, D.H. (1990) *J. Biol. Chem.* 265, 2244–2256.
- [30] Kyte, J. and Doolittle, R.F. (1982) *J. Mol. Biol.* 157, 105–132.
- [31] Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C. and Sakmann, B. (1986) *Nature* 321, 406–411.
- [32] Feinberg, A.P. and Vogelstein, B. (1983) *Anal. Biochem.* 132, 6–13.
- [33] Barish, M.E. (1983) *J. Physiol.* 342, 309–325.
- [34] Miledi, R. and Parker, I. (1984) *J. Physiol.* 357, 173–183.
- [35] Fukuda, K., Kubo, T., Akiba, I., Maeda, A., Mishina, M. and Numa, S. (1987) *Nature* 327, 623–625.
- [36] Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989) *Nature* 342, 32–38.
- [37] Ferris, C.D., Haganir, R.L., Supattapone, S. and Snyder, S.H. (1989) *Nature* 342, 87–89.
- [38] Miyawaki, A., Furuichi, T., Maeda, N. and Mikoshiba, K. (1990) *Neuron* 5, 11–18.
- [39] Kretsinger, R.H. (1976) *Annu. Rev. Biochem.* 45, 239–266.
- [40] Wierenga, R.K. and Hol, W.G.J. (1983) *Nature* 302, 842–844.
- [41] Blumenthal, D.K., Takio, K., Edelman, A.M., Charbonneau, H., Titani, K., Walsh, K.A. and Krebs, E.G. (1985) *Proc. Natl. Acad. Sci. USA* 82, 3187–3191.
- [42] Takasago, T., Imagawa, T. and Shigekawa, M. (1989) *J. Biochem. (Tokyo)* 106, 872–877.
- [43] Krebs, E.G. and Beavo, J.A. (1979) *Annu. Rev. Biochem.* 48, 923–959.
- [44] Kuba, K. (1980) *J. Physiol.* 298, 251–269.
- [45] Lipscombe, D., Madison, D.V., Poenie, M., Reuter, H., Tsien, R.W. and Tsien, R.Y. (1988) *Neuron* 1, 355–365.
- [46] Thayer, S.A., Perney, T.M. and Miller, R.J. (1988) *J. Neurosci.* 8, 4089–4097.
- [47] Rosenbluth, J. (1962) *J. Cell Biol.* 13, 405–421.
- [48] Henkart, M., Landis, D.M.D. and Reese, T.S. (1976) *J. Cell Biol.* 70, 338–347.