# Molecular cloning and characterization of a human brain ryanodine receptor

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Received 14 July 1997; revised version received 29 September 1997

**Abstract We have cloned and sequenced the cDNA of the human brain ryanodine receptor (RyR3), which is composed of 4866 amino acids and shares characteristic structural features with the rabbit RyR3. Northern blot analysis shows that the human RyR3 mRNA is abundantly expressed in hippocampus, caudate nucleus and amygdala as well as in skeletal muscle. The human RyR3 mRNA is also detected in several cell lines derived from human brain tumors. Functional expression of RyR3 and a chimeric RyR suggests that RyR3 forms a calcium-release channel with a very low $Ca^{2+}$ sensitivity.**



*Key words:* Ryanodine receptor; Calcium release channel; cDNA cloning; RNA blot hybridization; Chimeric RyR; Functional expression

## 1. Introduction

Cytoplasmic $Ca^{2+}$ plays important roles in cell excitability, neurotransmitter release, muscle contraction and other biological processes [1]. The concentration of cytoplasmic $Ca^{2+}$ can be increased either by $Ca^{2+}$ entry across the plasma membrane or by $Ca^{2+}$ release from intracellular stores. While extracellular $Ca^{2+}$ enters through voltage-gated $Ca^{2+}$ channels (VGCC) and other types of $Ca^{2+}$-permeable channels [2], the two major classes of intracellular $Ca^{2+}$ release channels, the inositol 1,4,5-trisphosphate receptors ($IP_3Rs$) [3] and the ryanodine receptors (RyRs), are responsible for $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores [4–6]. $Ca^{2+}$ release through $IP_3Rs$ has been observed in many cells, as a ubiquitous mechanism to regulate intracellular $Ca^{2+}$ concentration. On the other hand, three types of RyRs, RyR1, RyR2, and RyR3, are expressed in specific tissues; RyR1 is expressed mainly in skeletal muscle [7], RyR2 in cardiac muscle and brain [8] and RyR3 in brain and smooth muscle [9,10]. In brain, RyR3 is expressed highly in limited areas which include hippocampus, caudate nucleus, corpus callosum, and thalamus [10,11]. Furthermore, RyR3 is also expressed in non-excitable cells [12] such as human T-lymphocytes, and is suggested to contribute to cell proliferation [13].

Successful recombinant expression of RyR1 and RyR2 in myotubes from RyR1-deficient (dyspedic) mice, homozygous for a disrupted skeletal muscle isoform of RyR gene [14], has led us to elucidate the indispensable roles of RyR1 and RyR2 in the molecular mechanisms of excitation-contraction coupling in skeletal muscle and in cardiac muscle, respectively [15,16]. VGCC directly activates RyR1 in skeletal muscle [15,17], and possibly in neuron [18], and $Ca^{2+}$ entering through the VGCC is a trigger to open RyR2 in cardiac muscle ($Ca^{2+}$-induced $Ca^{2+}$ release). Despite the interesting distribution of RyR3 expression in brain and in smooth muscle, however, functions of RyR3 have remained enigmatic. $Ca^{2+}$ is assumed to be a major physiological ligand for RyR3, but several lines of evidence suggest that $Ca^{2+}$-induced $Ca^{2+}$ release of RyR3 is different from that of other RyRs [9,13,19]. An endogenous RyR3 is suggested to be responsible for a much lower $Ca^{2+}$ sensitivity of residual $Ca^{2+}$ release activity observed in skeletal muscle cells from the RyR1-deficient mice [19]. RyR3 is insensitive, at least on some occasions, to caffeine [9,13], which is the most widely employed compound for RyR activation. The fact that RyR3 is expressed in non-excitable cells practically lacking VGCC may even imply that RyR3 is regulated by quite different mechanisms from those for other types of RyR. Mutant mice lacking RyR3 show no gross abnormality except for increased locomotor activity [20]. In spite of these circumstantial pieces of evidence, molecular-physiological properties of RyR3 have remained unclear, partly because none of the trials of expressing RyR3 from its cDNA in a recombinant expression system has been successful so far. Here we have cloned and sequenced the human RyR3 cDNA and characterized properties of the human RyR3. We have also examined the functional expression of the wild-type human RyR3 and a chimeric RyR3-RyR2, using myotubes from the RyR1-deficient mice [15].

## 2. Materials and methods

### 2.1. cDNA cloning

Oligo(dT)- and random-primed cDNA libraries, constructed in phage λgt10 using poly(A)$^+$ RNA isolated from human brain (caudate nucleus) were obtained from Clontech (USA). The cDNA libraries were screened ($\sim 3.0 \times 10^5$ plaques) with the *Pst*I (9790)/*Eco*RI (11834) fragment from the rabbit RyR3 cDNA clone pBRR74 [10] to

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*Abbreviations:* VGCC, voltage-gated calcium channel; RyR, ryanodine receptor; MH, malignant hyperthermia; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB001025.


*PII* S0014-5793(97)01275-1

yield λhBRR79; the restriction endonuclease sites are identified by numbers (in parentheses) indicating the 5′-terminal nucleotide generated by cleavage: nucleotide residues are numbered in the 5′ to 3′ direction, beginning with the first residue of the ATG triplet encoding the putative initiating methionine. The cDNA insert of λhBRR79 was subcloned into the *Eco*RI site of pBluescript SK$^-$ (Stratagene) to yield phBRR79. The library was screened nine times with different probes: the 2.4-kbp *Eco*RI (vector)/*Dra*I (2395) fragment from pBRR331 [10] yielded three positive clones including λhBRR22, λhBRR61 and λhBRR112; the 1.2-kbp *Pma*CI (4750)/*Apa*I (5912) fragment from pBRR133 [10] yielded λhBRR51, 52, 53; the 1.3-kbp *Eco*RI (vector)/*Hin*cII (14656) fragment from pBRR110 [10] yielded two positive clones including λhBRR91 and λhBRR93; the 1.3-kbp *Kpn*I (6249)/*Hin*dIII (7523) fragment from pBRR121 [10] and the 1.1-kbp *Xba*I (8405)/*Pst*I (9494) fragment from pBRR92 [10] yielded λhBRR140, λhBRR141 and λhBRR411; the 1.0-kbp *Eco*RI (vector)/*Eco*RI (13335) fragment from λhBRR93 and the 0.8-kbp *Spe*I (10569)/*Eco*RI (11408) fragment from λhBRR79 yielded λhBRR161; the 0.8-kbp *Eco*RI (vector)/*Eco*RI (11815) fragment from λhBRR161 yielded λhBRR407. Furthermore, two additional clones were derived from RT-PCR as follows: 1 μg human brain poly (A)$^+$ RNA (Clontech) was subjected to Moloney's murine leukemia virus RNase H$^-$ reverse transcriptase (Gibco BRL) with random primer. The synthesized first strand cDNA was amplified with a DNA Thermal Cycler (Perkin-Elmer Corp.), according to the manufacturer's specifications (TaKaRa LA PCR kit). Following the hot start (1 min at 94°C) the samples were subjected to 30 cycles of 20 s at 98°C and, 5 min at 68°C. Primer pairs for phBRR501 were synthetic 25-nucleotide oligomers at bases 2949–2973 (upstream primer, AGTGGATAAACTTGCAGAAAATGCA) and 3495–3519 (downstream primer, TGGGGAGCTGCTGATCACCAATAAA) of the cloned phBRR61 and phBRR51, respectively, and those for phBRR502 were synthetic 20-nucleotide oligomers at bases 11369–11388 (upstream primer, TTGATGAATCTGGACAGCAC) and 12353–12372 (downstream primer, ACGTGTTAGAAATTGCGGGT) of the cloned phBRR79 and phBRR91, respectively.

The cDNA clones used for nucleotide sequence analysis were as follows: phBRR22 (carrying nucleotides −86 to 1263), phBRR61 (991–3103), phBRR501 (2949–3519), phBRR51 (3435–5253), phBRR53 (4444–7346), phBRR411 (7330–9900), phBRR407 (10112–11814), phBRR79 (8358–11408), phBRR502 (11369–12372) and phBRR91 (11468–15486). All cDNA inserts except phBRR501 and phBRR502 were subcloned into the *Eco*RI site of pBluescript SK$^-$. The 0.6-kbp *Hin*dIII (2956)/*Bcl*I (3506) fragment of phBRR501 was subcloned into the *Bam*HI/*Hin*dIII site, and the 0.5-kbp *Apa*I (11451)/*Acc*I (11930) fragment of phBRR502, into the *Acc*I/*Apa*I site of pBluescript SK$^-$.

Both strands of the resulting cDNA and the reverse transcriptase PCR products were sequenced by the dideoxy chain termination method [21].

### 2.2. Physiological analysis

The entire protein coding sequence of the human RyR3 was inserted into the *Eco*RI/*Not*I site of pCI-neo (Promega) to yield phNRR9. The cDNA insert was constructed from the following fragments: *Eco*RI (vector)/*Mro*I (1232) derived from λhBRR22, *Mro*I (1232)/*Hin*dIII (2956) from λhBRR61, *Hin*dIII (2956)/*Bcl*I (3506) from phBRR501, *Bcl*I (3506)/*Pma*CI (4750) from λhBRR51, *Pma*CI (4750)/*Pst*I (7339) from λhBRR53, *Pst*I (7339)/*Cla*I (9559) from λhBRR411, *Cla*I (9559)/*Spe*I (10569) from λhBRR79, *Spe*I (10569)/*Apa*I (11451) from λhBRR407, *Apa*I (11451)/*Eco*RI (11815) from phBRR502, *Eco*RI (11815)/*Eco*RI (14861) from λhBRR91. The expression plasmid carrying the chimeric ryanodine receptor cDNA between the human RyR3 and the rabbit RyR2 was constructed as follows. The *Sal*I (vector)/*Pma*CI (5038) fragment from the rabbit RyR2 cDNA [8] and the *Pma*CI (4750)/*Not*I (vector) fragment from the human RyR3 cDNA were ligated into the *Sal*I/*Not*I site of pCI-neo. Culture of myotubes from RyR1-deficient (dyspedic) mice and cDNA injection were described previously [15]. Fluorescence changes (in arbitrary units) were measured after loading Fluo-3 AM into myotubes [22]. Caffeine was applied by local ejection from a wide-tipped pipette (10–50 μm diameter). Bath solution was normal rodent Ringer which has the following composition (mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4 with NaOH. Temperature was 20–22°C.

### 2.3. Northern blot analysis

For Northern blot analysis of human brain and other tissues, sheets of multiple tissue Northern (MTN) Blots (Clontech) were purchased. Each lane of a sheet of MTN Blots contained approximately 2 μg of poly(A)$^+$ RNA from the following human brain regions; amygdala, caudate nucleus, corpus callosum, hippocampus, whole brain, substantia nigra, subthalamic nucleus and thalamus. Each lane of another type of MTN Blots contained approximately 2 μg of poly(A)$^+$ RNA from the following human tissues; heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. The result of tissue distribution was confirmed by an additional sheet of MTN Blots of a different lot. Each lane of the blot for cell lines contained approximately 20 μg of total RNA from the following human cell lines, prepared as previously [10], SK-N-MC (derived from neuroblastoma in origin), IMR-32 (neuroblastoma), HEL-299 (lung fibroblast), H4 (neuroglioma), SK-N-SH (neuroblastoma), HEK-293 (embryonal kidney cell) and U373 (astrocytoma). Because probes derived from partial cDNAs of the human RyR3 gave only faint signals, the probe used for Northern blot analysis was 14.9-kbp *Nal*I (vector)/*Not*I (vector) fragment from phNRR9, unless otherwise specified. The probe was generated with the Klenow fragment of DNA polymerase and [$^{32}$P]dCTP [23] by random oligonucleotide primer. The blots were hybridized in 50% formamide at 42°C and washed three times with 0.3×SSC, 0.1% SDS at 50°C.

### 2.4. Luminescence assay

Luminescence assay was carried out as previously [24]. In brief, the cells ($1\times10^5$ cells/well) were transferred to the calcium assay solution with the following condition (mM): 140 NaCl, 5 KCl, 1.5 $MgCl_2$, 2.5 $CaCl_2$, 5 glucose and 10 HEPES, pH7.4 with NaOH, containing 2.5 μM coelenterazine, the intermediary substrate of aequorin, and incubated at 37°C for 6 h. The system for luminescence assay was the spectrofluorometer CAF-110 (Jasco) [13], connected to luminescence unit PL-03 (Jasco). The mobilization of intracellular $Ca^{2+}$ was initiated by the injection of caffeine (final concentration 10 mM) or ryanodine (final concentration 100 μM). The total amount of aequorin activity was measured after permeabilizing the cells with digitonin at a final concentration of 200 μg/ml. Ethanol concentration was 0.5% or less in the cuvette, which did not affect $Ca^{2+}$ release under these experimental conditions.

## 3. Results and discussion

### 3.1. cDNA sequence and amino acid sequence analysis

Hybridization screening and sequencing have resulted in identification of a number of overlapping cDNA clones. The complete nucleotide sequence of the human RyR3 cDNA was determined by sequence analysis of nine overlapping clones. The primary structure of this protein was deduced by using the open reading frame corresponding to the amino acid sequence of the rabbit RyR3 [10]. The deduced amino acid sequence is 4866 amino acid long with the calculated molecular mass being 551 046. The amino acid sequence comparison reveals 96%, 69%, 67% identities, between human RyR3/rabbit RyR3, human RyR3/rabbit RyR2, human RyR3/rabbit RyR1, respectively. Fig. 1A shows the homology score between the human RyR3 and the rabbit RyR3 plotted along the entire amino acid sequences, demonstrating a high homology between the two. Interestingly, however, there are two regions of about 20 amino acid residues each, where the homology score is significantly lower than the rest. The local sequence divergence may indicate possibility of alternative RNA splicing at those regions. The hydropathicity profile of the human RyR3 is similar to those of other RyRs in that there is no hydrophobic amino-terminal sequence indicative of the signal sequence, that the remaining region is largely hydrophilic and that there are four highly hydrophobic segments (referred to as M1, M2, M3 and M4) in the carboxy-terminal tenth of the molecule (data not shown). The carboxy-terminal

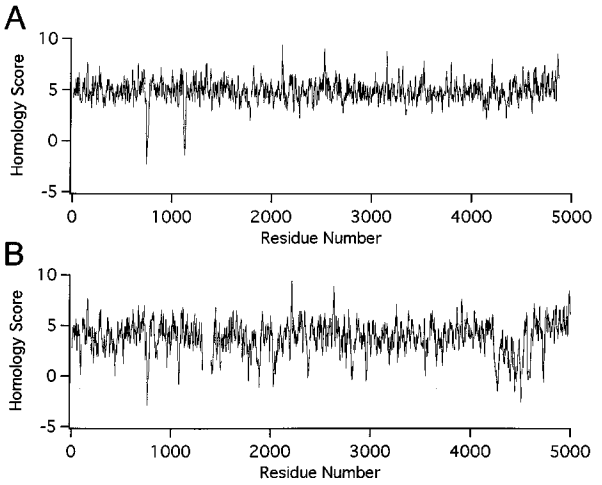


Fig. 1. Amino acid sequence homology between RyRs. The amino acid sequence deduced from the human RyR3 was aligned with those of the rabbit RyR2 and RyR3, and homology scores between the human RyR3 and the rabbit RyR3 (A) and between the human RyR3 and the rabbit RyR2 (B) were plotted along the entire sequences. Homology score was calculated as a mean of weight values in the Dayhoff PAM250 matrix [41] with the window size of 10. A gap was treated as one substitution regardless of its length, and its weight was assumed to be the minimum value of the matrix.

region that encompasses the M3 and M4 segments is particularly well conserved in all RyRs. On the other hand, there are rather divergent regions. For example, as in Fig. 1B where the homology score between the human RyR3 and the rabbit RyR2 is plotted, a stretch of about 100 amino acid residues in the region around amino acid residue 1300 is missing in RyR3; RyR2 has an EF-hand consensus sequence [25] (encompassing amino acid residues 1336–1347) [8] and a nucleotide-binding consensus sequence GXGXXG [26] (amino acid residues 1324–1329) [8] in this region. Furthermore, the region immediately preceding the M1 segment is divergent. The human RyR3 contains four repeated sequences occurring in two tandem pairs (amino acid residues 841–954, 955–1070, 2600–2711 and 2712–2791), as described for the rabbit RyR2 and RyR3 and the human RyR1 and RyR2 [8,10,27,28].

Arginine residue 613 or 614, replacement of which with cysteine in the RyR1 has been found to be associated with malignant hyperthermia (MH) of swine [29] and human [30], is conserved in the three types of RyRs.

### 3.2. Functional analysis of RyR3

Both RyR1 and RyR2 mediate release of $Ca^{2+}$ from intracellular stores, as demonstrated by functional expression of the cloned cDNAs [8,31]. Until now, there has been no direct demonstration that RyR3 functions as a calcium release channel. Therefore, we examined functional expression of the human RyR3, using myotubes from mice lacking skeletal muscle isoform of RyR [15]. First, we tried to express the wild-type human RyR3 in myotubes from the RyR1-deficient (dyspedic) mice by injection of the cDNA into nuclei. But an endogenous caffeine response of dyspedic myotubes obscured a difference between caffeine responses of non-injected myotubes and of myotubes injected with the RyR3 cDNA (10 mM caffeine, data not shown). This result was not unexpected because the human RyR3 in human T-lymphocytes shows no response to caffeine [13]. Since caffeine is believed to exert its activating effect on RyR by enhancing $Ca^{2+}$ sensitivity, we planned to confer an effective $Ca^{2+}$ sensitivity on RyR3 in order to circumvent the technical difficulties in demonstrating $Ca^{2+}$-release activity of RyR3. Considering the possibility that the deleted region around amino acid residue 1300 may contain a region determining $Ca^{2+}$ sensitivity, we constructed a chimeric RyR molecule so that the missing region is supplemented with the sequence of RyR2, which has a high $Ca^{2+}$ sensitivity. The chimeric molecule of the RyR consists of one third of the rabbit RyR2 amino acid sequence in the amino-terminus and two thirds of the human RyR3 sequence in the carboxy-terminus (Fig. 2A). Fig. 2B shows a caffeine response of the chimeric RyR expressed in dyspedic myotubes. The chimeric RyR responded to 1 mM caffeine (Fig. 2B, $n=5$ out of 20). In contrast, the non-injected dyspedic myotubes never responded to 1 mM caffeine (Fig. 2B, $n=0$ out of 20), nor did the dyspedic myotubes injected with the RyR3 cDNA to 1mM caffeine (data not shown, $n=0$ out of 20). Therefore, we conclude that the chimeric RyR can produce intracellular $Ca^{2+}$ release channel which can respond to caffeine.

Based on the structure prediction of RyR from the amino acid sequence, the channel-forming region of the RyRs resides in the carboxy-terminal tenth of molecule of the RyRs [7,8,10,27]. The simplest interpretation of our results is that the carboxy-terminal two thirds of the human RyR3 has the $Ca^{2+}$ release channel activity, and that the amino-terminal

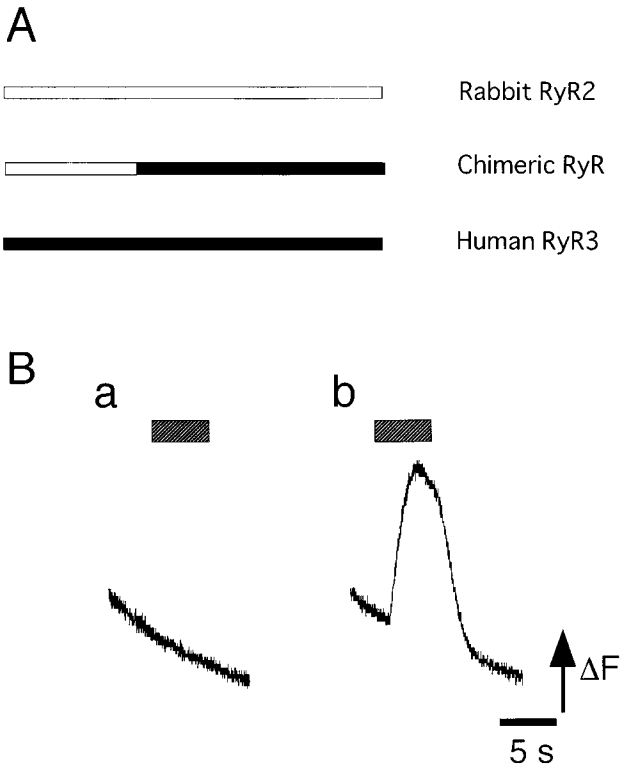


Fig. 2. Response to caffeine of a chimeric human RyR3. A: Schematic representation of the structure of the chimeric RyR composed of the rabbit RyR2 (open boxes) and the human RyR3 (filled boxes). B: Intracellular $Ca^{2+}$ transients in response to caffeine in dyspedic myotubes expressing chimeric the human RyR3. a: Non-injected dyspedic myotubes never respond to 1 mM caffeine ($n=20$). b: Dyspedic myotubes in which the chimeric human RyR3 cDNA was injected responded to 1 mM caffeine ($n=5$ out of 20). Baseline shifts may be due to dye bleaching.

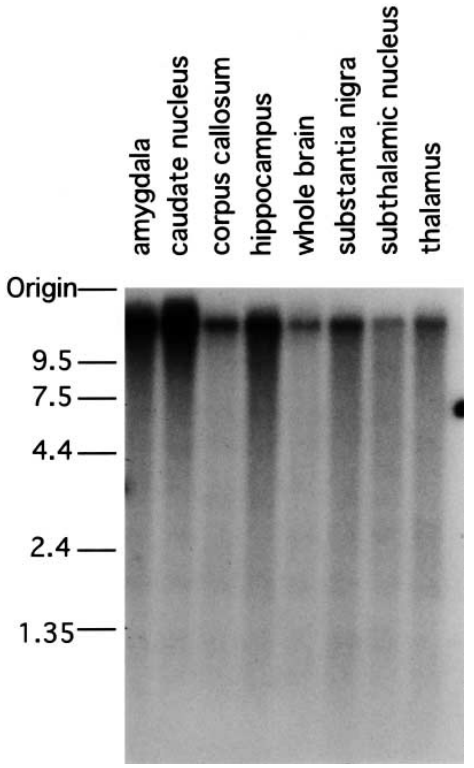


Fig. 3. Distribution of human RyR3 in brain. Northern blot analysis of different regions of human brain with cDNA probes for human RyR3 mRNAs. 2 μg of poly(A)$^+$ RNA was applied in each lane. Autoradiography was performed at −70°C for 7 days with an intensifying screen.

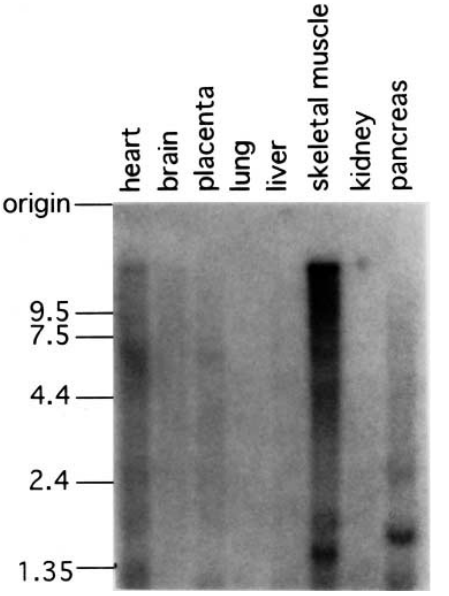


Fig. 4. Human RyR3 expression in tissues. Northern blot analysis of different tissues of human with cDNA probes for the human RyR3 mRNAs. 2 μg of poly(A)$^+$ RNA was applied in each lane. Autoradiography was performed at −70°C for 7 days with an intensifying screen.

third of RyR sequence contains the region determining caffeine and/or $Ca^{2+}$ sensitivity.

### 3.3. Tissue-specific distribution of RyR3 mRNA

The tissue-specific transcription of the human RyR3 gene was studied by Northern blot analysis of mRNA isolated from a variety of human tissues. Although only a weak signal is observed when mRNA prepared from whole brain is used, a ~16-kbp RNA species hybridizable with a human RyR3 cDNA probe is found abundantly in restricted areas of the brain (caudate nucleus, amygdala and hippocampus), and at a lower level in corpus callosum, substantia nigra and thalamus (Fig. 3). This pattern of the restricted distribution of the mRNA expression is similar to that of the rabbit brain RyR3 [10,11].

This limited distribution of RyR3 in the brain raises a number of possibilities. It has been reported that RyR is directly coupled to the L-type $Ca^{2+}$ channel also in the brain [18]. While the P-type and other types of $Ca^{2+}$ channels are expressed in whole area of brain, the R-type $Ca^{2+}$ channel is expressed highly limited regions of the brain, caudate nucleus and hippocampus [32]. A similar distribution of the R-type $Ca^{2+}$ channel and RyR3 leads to a speculation that RyR3 can directly interact with the R-type $Ca^{2+}$ channel in these areas. Furthermore, since the regions of RyR3 expression roughly correspond to the areas where 'delayed neuronal death' occurs after hypoxia in the human brain [10], this type of ryanodine receptor may be important in the pathological state. The increased locomotor activity of mice lacking RyR3 may reflect the distribution of RyR3 [20].

Outside of the brain, an RNA species hybridizable with a human RyR3 cDNA probe is detected in skeletal muscle (Fig.

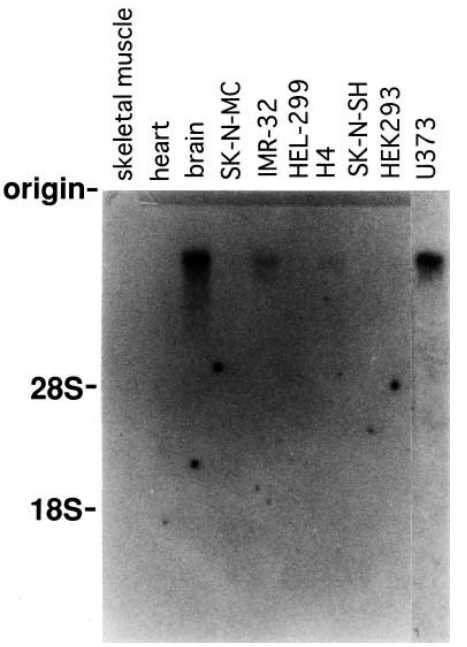


Fig. 5. Distribution of human RyR3 in human cell lines. Northern blot analysis of human RyR3 mRNA expression in rabbit skeletal muscle, rabbit heart, rabbit whole brain and human cell lines; neuroblastoma (SK-N-MC, IMR-32), lung fibroblast (HEL-299), neuroglioma (H4), neuroblastoma (SK-N-SH), embryonal kidney cell (HEK293), astrocytoma (U373) with cDNA probes for human RyR3 mRNAs. 20 μg of total RNA was applied in each lane. Autoradiography was performed at −70°C for 4 days with an intensifying screen.

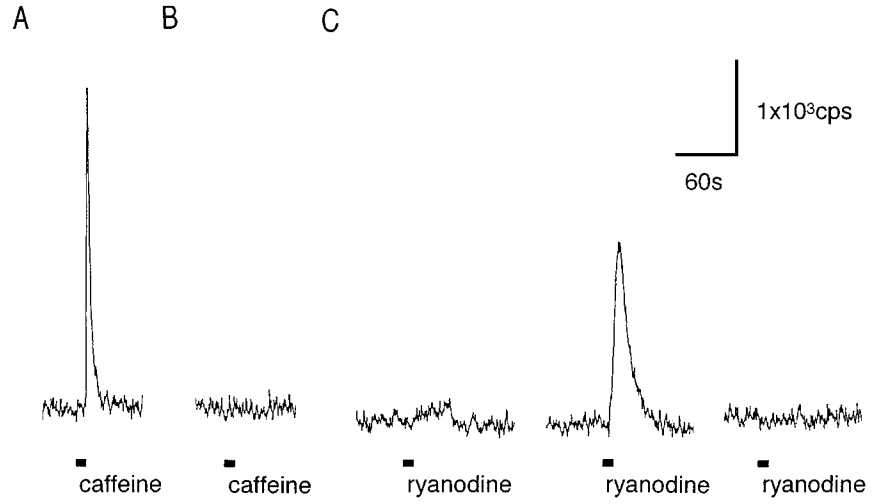


Fig. 6. Intracellular $Ca^{2+}$ of U373 cells in response to application of caffeine and ryanodine. A: CHO cells transfected with the rabbit RyR2 cDNA; R3-7 [24] shows a clear increase of intracellular $Ca^{2+}$ in response to caffeine ($n=11$ out of 11). B: Caffeine (10 mM) caused no effect in U373 cells ($n=7$). C: Examples of responses of U373 cells to ryanodine (100 μM). Ryanodine evoked $Ca^{2+}$ transients variable in amplitude ($n=2$ out of 4; left and middle traces). Some cells remained refractory to application of ryanodine ($n=2$ out of 4; right trace). The variability of the responses cannot be ascribed to ryanodine not reaching its site of action as was described previously [31]. Addition of caffeine and ryanodine is indicated by bars. cps, counts per second.

4). The size of the RNA species in these tissues is ~16 kbp, the same as in the brain. The faint signals suggest the existence of RyR3 mRNA in heart. The distribution of mRNA outside of the brain is different from that of rabbit, since expression of RyR3 can be hardly detected in rabbit skeletal muscle [10]. Because RyR1 mRNA is plentifully present in skeletal muscle, we cannot exclude a small contribution of cross-hybridization of the RyR3 probe to RyR1 mRNA, but the findings that a probe derived from the 3′-non-coding region of the human RyR3 cDNA detects signals in skeletal muscle (data not shown) and that the heart mRNA gives a much lower hybridization signal despite of a similar or even slightly higher homology of RyR3 to RyR2 than to RyR1, together with isolation of RyR3 cDNA from skeletal muscle cDNA library [33], indicate that RyR3 is really expressed in the human skeletal muscle. In fact, RyR3 was reported to be detected in skeletal muscle in other species, such as mouse [34], chicken [35] and bullfrog [36].

In non-mammalian skeletal muscle, two types of RyR isoforms, α-isoform (homologous to RyR1) and β-isoform (homologous to RyR3), have been identified [36]. In chick skeletal muscle, expression of the α- and β-isoforms is differently regulated in the stages of development. Therefore these two types of isoforms are thought to have different roles in skeletal muscle [35,37]. The different contents of RyR3 gene products are also observed in mammalian skeletal muscle, being more abundant in the diaphragm and soleus, and at a lesser level in the extensor digitorum longus [33]. In this study, we showed that RyR3 expression is much higher in human skeletal muscle than in rabbit skeletal muscle [10]. This observation suggests that the expression level of RyR3 in skeletal muscle varies among mammalian species, although the possibility that the differential tissue distribution of RyR3 accounts for this observation cannot be excluded. It is well known that an acute increase of intracellular $Ca^{2+}$ in human skeletal muscle causes MH [30]. Although MH is reported to be associated with the mutation of RyR1 [38], only 5% of MH have the mutation of Arg to Cys substitution at position 614 in RyR1 gene [30,39]. The abundant expression of RyR3 in human skeletal muscle raises a possibility of involvement of RyR3 in a variant form of MH. RyR3 may be also related to other disorders of intracellular $Ca^{2+}$ regulation, such as central core disease [39] and neuroleptic malignant syndrome [40].

We have evaluated human cell lines originating from brain tumors, since the tissue-specific distribution of the RyRs in the human brain may represent cell-specific $Ca^{2+}$ regulation related to proliferation. The RyR3 mRNA is found to be expressed in several human cell lines (Fig. 5). Abundant expression is observed in U373, a cell line originating from a malignant astrocytoma, weak expression is detected in IMR-32, derived from a malignant neuroblastoma, and faint expression in H4 from a malignant neuroglioma. Although RyR2 expression is detected in IMR-32, expression of other types of RyRs is not detected in U373 or H4 (data not shown). Expression of RyR3 is not detected in SK-N-MC or SK-N-SH, although these cell lines are also malignant neuronal cells in origin.

There was an increase of intracellular $Ca^{2+}$ in U373 and H4, in response to ryanodine but not to caffeine, although in the same experimental condition, caffeine response was always observed in CHO cells expressing the rabbit RyR2 [24] (Fig. 6). The positive ryanodine response and the negative caffeine response of U373 and H4 are the same as observed for T-lymphocytes, which are features distinguishing RyR3 [13]. The $Ca^{2+}$ sensitivity of $Ca^{2+}$-induced $Ca^{2+}$ release of the endogenous RyR3 observed in muscle cells from the RyR1-deficient mice is reported to be significantly lower than that of RyR1 [19]. Together with the results of expression of the RyR3 and the chimeric RyR, we conclude that RyR3 has a very low caffeine and/or $Ca^{2+}$ sensitivity. Since it is questionable whether a $Ca^{2+}$ concentration high enough to activate RyR3 can be attained in physiological conditions, it is intriguing to speculate that RyR3 may have a different activation mechanism. In pathological situations, however, when such a

high $Ca^{2+}$ concentration happens to occur, RyR3 may take part in maintaining and spreading pathological states.

*Acknowledgements:* We thank Ms. Miwa Kataoka for technical assistance. This investigation was supported in part by research grants from the Ministry of Education, Science and Culture of Japan and Shimizu Immunological Research Grant for 1995 and NIH R01AR43301 and MDA (P.D.A.).

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