The Ryanodine Receptor/Calcium Channel Genes Are Widely and Differentially Expressed in Murine Brain and Peripheral Tissues

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Abstract. Ryanodine receptors (RyRs) are intracellular calcium release channels that participate in controlling cytosolic calcium levels. At variance with the probably ubiquitous inositol 1,4,5-trisphosphate-operated calcium channels (1,4,5-trisphosphate receptors), RyRs have been mainly regarded as the calcium release channels controlling skeletal and cardiac muscle contraction. Increasing evidence has recently suggested that RyRs may be more widely expressed, but this has never been extensively examined. Therefore, we cloned three cDNAs corresponding to murine RyR homologues to carry a comprehensive analysis of their expression in murine tissues. Here, we report that the three genes are expressed in almost all tissues analyzed, where tissue-specific patterns of expression were observed. In the uterus and vas deferens, expression of RyR3 was localized to the smooth muscle component of these organs. In the testis, expression of RyR1 and RyR3 was detected in germ cells. RyR mRNAs were also detected in in vitro-cultured cell lines. RyR1, RyR2, and RyR3 mRNA were detected in the cerebrum and in the cerebellum. In situ analysis revealed a cell type-specific pattern of expression in the different regions of the central nervous system. The differential expression of the three ryanodine receptor genes in the central nervous system was also confirmed using specific antibodies against the respective proteins. This widespread pattern of expression suggests that RyRs may participate in the regulation of intracellular calcium homeostasis in a range of cells wider than previously recognized.

Increases in the cytosolic calcium concentration [Ca^{2+}] are observed after extracellular stimulation of many cells, where they contribute to the integrated intracellular signaling pathways that control cellular functions (Berridge, 1993). [Ca^{2+}] can be increased either by the opening of calcium channels on the plasma membrane, to generate an influx of Ca^{2+} from extracellular fluids, or by the activation of Ca^{2+} release channels located on intracellular membranous compartments of the ER that are specialized for storing Ca^{2+} (Pozzan et al., 1994). Two different families of calcium release channels located on these intracellular stores are known: one is sensitive to inositol 1,4,5-trisphosphate (InsP3) and is referred to as the InsP3 receptor (Ferris and Snyder, 1992; Mikoshiba, 1993); the other is identified by its ability to bind the plant alkaloid ryanodine (Ry) hence its name, Ry receptor (RyR) (McPherson and Campbell, 1993b; Sorrentino and Volpe, 1993).

Ca^{2+} release through InsP3 receptors has been described in almost all cell types, and it is viewed as an ubiquitous mechanism to regulate intracellular calcium release. InsP3 receptors are activated to release calcium by InsP3 binding. This way, production of InsP3 by phospholipase C links calcium release from InsP3 receptors to the stimulation of the receptors for various growth factors and hormones on the cell surface (Berridge, 1993). Three genes coding for RyRs have so far been identified. These include the RYR1 gene encoding the skeletal muscle isoform (Takeshima et al., 1989; Zorzato et al., 1990), as well as the RYR2 gene expressing the isoform present in the heart (Nakai et al., 1990; Otsu et al., 1990). More recently, a third RyR (RyR3) has been cloned (Giannini et al., 1992; Hakamata et al., 1992). The RyRs/calcium channels, although distinct from the InsP3 receptors, share with them several structural features. However, despite these structural analogies, InsP3 is not an effective agonist of calcium release through RyRs. Cyclic adenosine diphosphoribose, a metabolite of nicotinamide adenine dinucleotide, has been recently shown to be able to stimulate Ca^{2+} release through RyRs, independently of InsP3 generation (Galione, 1993; Lee et al., 1993). Interestingly, cyclic adenosine diphosphoribose has been detected in many and possibly all cells (Howard et al., 1993). Less is known about the expression pattern of RyRs. Along with skeletal and cardiac muscle, the brain is the only other

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1. Abbreviations used in this paper: Ca^{2+}, cytosolic calcium concentration; CNS, central nervous system; InsP3, inositol 1,4,5-trisphosphate; Ry, ryanodine; RyRs, ryanodine receptors; tRNA, transfer RNA.

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tissue where the presence of RyR has been well documented (McPherson and Campbell, 1993a). A thorough biochemical characterization has shown that the cardiac RyR2 is the major isoform present in several regions of the central nervous system (McPherson and Campbell, 1993a; Sharp et al., 1993), while RyR1 was shown to be exclusively expressed in cerebellum Purkinje cells (Kuwajima et al., 1993). RyR3 mRNA has been found by Northern blot analysis and antibodies against RyR (Lesh et al., 1993), as well as in the brain and stomach by Northern blot analysis (Nakai et al., 1990; Otsubo et al., 1990). During the last years, the existence of RyRs in several noncontractile cells such as neurons, chromaffin cells, and secretory cells has been documented (Kasai et al., 1993; Kasai and Petersen, 1994; Takasawa et al., 1993; Thorn et al., 1993). RyRs have also been studied in sea urchin eggs (Galione et al., 1993; Lee et al., 1993; McPherson et al., 1992). Most of these data are, however, mainly based on pharmacological evidence, and with few exceptions, identification of RyRs in different tissues and cell types is largely incomplete. Furthermore, from the existing data, it is not possible to determine which of the three known RyRs is present in a given tissue or cell, nor whether more than one isoform is present. In addition, other intracellular RyR/calcium channels that are not well characterized have been described, differing in their biochemical and/or pharmacological properties from cardiac and skeletal muscle RyRs, making the issue of RyR identification in nonmuscle cells even more intricate (Schmid et al., 1990).

During our initial studies on the expression pattern of the type 3 RyR, we realized that it was likely that RyR1 and RyR2 could also be expressed in tissues that do not contain an evident muscle or neuronal component, as well as in several cell lines. This prompted us to isolate cDNA clones for the murine RyRs and to investigate the expression in murine tissues of these genes by RNase protection assay, because of its high sensitivity and specificity, and by in situ hybridization to identify the cells expressing these receptors within a given tissue. Expression of the three RyR proteins in the central nervous system (CNS) was also investigated by Western blot, with antisera raised against recombinant protein sequences specific for the three different RyR isoforms.

Materials and Methods

Cloning of Murine cDNAs for RyR1, RyR2, and RyR3

Standard protocols were used to isolate murine cDNA for RyR1, RyR2, and RyR3 by screening a mouse brain cDNA library with a human RyR3 complementary DNA under low stringency conditions (Mattié et al., 1994; Sorrentino et al., 1993). Three plasmids, pMB9, pMB3, and pMBIII7, were isolated, and the three inserts were identified by nucleotide sequence analysis as partial cDNA corresponding to RyR1, RyR2, and RyR3, respectively. The sequence data used in this study are available from EMBL/GenBank/DDBJ under accession numbers X83932-X83934.

RNase Protection Analysis

Total RNA from adult mouse tissues was purified by the guanidine isothiocyanate/CsCl method as previously described (Morrone et al., 1989). mRNA levels have been determined by RNase protection because of the high sensitivity and specificity of this technique. The EcorI-Styl fragment isolated from pMB9 was subcloned into pBSK plasmid (Stratagene, La Jolla, CA). This construct was linearized at an internal Avai site, located 350 bp upstream the Styl site, to generate an antisense RyR1 probe. An internal 1,661-bp EcoRI fragment from pMB3, corresponding to a partial mouse RyR2 cDNA, was subcloned into pBSKS. The construct was linearized at the internal Styl site located 307 bp upstream to the 3' end of the subclone. The RyR3 MTa11 mouse cDNA subclone was linearized with BglII to produce a RyR3-specific, 410-bp cRNA probe. RyR1, RyR2, and RyR3 antisense cRNA probes were obtained by transcription of the above-mentioned constructs in the presence of [α-32P]UTP (800 Ci/mmol; Amersham Corp., Arlington Heights, IL) with a polymerase transcription kit (T3/T7; Stratagene), according to the manufacturers instructions. After purification on an 8% sequencing gel, 2.5 × 10^6 cpm of the three cRNA probes were hybridized to total RNA from indicated tissues in 30 μl of a buffer containing 40 mM Pipes pH 6.4, 1 mM EDTA, 0.4 M NaCl, and 80% formamide overnight at 55°C. Afterwards, the hybrids were treated with RNase A (40 μg/ml; Sigma Immunochemicals, St. Louis, MO) and RNase T1 (2 μg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 h at 37°C. Samples were further treated with proteinase K (50 μg; Boehringer Mannheim) in the presence of 0.4% SDS at 37°C for 30 min, phenol-chloroform extracted, ethanol precipitated, and analyzed on an 8% sequencing gel (Marziali et al., 1993). 10 μg of total heart RNA and 5 μg of total skeletal muscle (thigh or diaphragm) RNA were analyzed with RyR2 and RyR1 cRNA probes respectively. 50 μg of transfer RNA was hybridized to each probe as a negative control. Both RNase protection and in situ hybridization experiments have been performed with several different probes for each gene, including sequences corresponding to regions of divergence among the three RyRs.

In Situ Hybridization

Adult mouse tissues were excised and directly frozen in OCT compound on dry ice. Serial 10-μm cryosections were stored at −80°C up to 2 mo. Before hybridization, sections were thawed in cold acetone, fixed for 15 min in 4% paraformaldehyde, and acetylated. After a denaturing step at 70°C in 2× SSC 50% formamide, sections were dehydrated in graded ethanols. Probes used were derived from many portions of the cDNA probes isolated. Single-strand sense and antisense cRNA probes were obtained by transcription with T3 or T7 RNA polymerase in the presence of [α-35S] CTP, >1000 Cpm/μl, Amersham). Probes were digested to 50–100 nucleotides by mild alkaline hydrolysis. Hybridization was carried out as described (Dollé and Duboule, 1989). After washing and RNase treatment, sections were dehydrated in graded ethanols containing 300 mM Na4 acetate, and were dipped in NB T2 emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 in water. After 2–3 wk, slides were developed with D19 developer (Kodak) washed in distilled water, and fix in AH-4 fixer (Kodak). The slides were counterstained with Toluidine blue, and coverslips were mounted using Eukitt (O. Kindler GmbH, Freiburg, Germany).

Fusion Proteins and Polyclonal Antisera

The nucleotide sequence corresponding to the region of low homology situated between the transmembrane domains 4 and 5 (divergent region 1 or DI) of the RyR1 gene was amplified using the oligonucleotides mSk52T (5'TGGATCCGCCAGCCGCGCCTCGG) and mSk32T (5'CAGAATTCCGAGGCTGTCGCCC). The same region of the RyR2 gene was amplified using the oligonucleotides mCa52T (5'TGGATCCGCCAGCCGCGCCTCGG) and mCa32T (5'CAGAATTCCGAGGCTGTCGCCC). The oligo ryr3DI-5' (5'G'IV_d3ATCCCTCd3ATGCTGAGCGGAGGGAGAGAGG) and mCa3'2T (5'CCGAATTCATTATTTTCTTCAGAA). The oligo ryr3DI-5' (5'TGGATCCCTTGCTGAGGGCGCCTCGG) and ryr3DI-3' (5'CCGAATTCCGAGGCTGTCGCCC) were used to amplify the DI region of the RyR3 gene. The fragments were digested with EcoRI and BamHI and subcloned in the pGEX2T plasmid (Pharmacia, Freiburg, FRG) digested with the same enzymes. The resulting plasmids were transformed in the JM101 strain of Escherichia coli. Recombinant proteins were induced with isopropyl-β-D-thiogalactopyranoside as described (Smith and Johnson, 1988). Cells were washed in PBS containing 1% Triton X-100 and 100 mM EDTA (PBSTE) and sonicated. The bacterial lysate was cleared by centrifugation. Glutathione agarose beads (Pharma-acia) were then added and incubated for 10 min at 4°C. After several washes with PBS, the recombinant proteins were eluted with 50 mM Tris, pH 8, containing 10 mM reduced glutathione. Polyclonal rabbit antisera were developed against these GST fusion proteins according to standard protocols (Ausubel et al., 1993).
Preparation of Microsomal Membranes Proteins and Western Blot

Bovine brains from a local slaughter house were dissected to separate specific areas. Microsomal membranes were prepared by homogenization with a Dounce homogenizer of 1 g of tissue in 5 ml of a solution containing 320 mM sucrose, 0.1 mM PMSF, and 5 mM Hepes-NaOH pH 7.4 (buffer A). The homogenate was centrifuged at 10,000 g for 5 min at 4°C. Microsomal membranes were obtained as a pellet by centrifugation at 100,000 g for 60 min at 4°C. Microsomes were resuspended in buffer A and stored at −80°C. Protein concentration of the microsomal fraction was quantified using a protein assay kit (Bio Rad Laboratories, Hercules, CA). For Western blot analysis, 100 μg of microsomal proteins were separated on 5% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) following standard procedures. Membranes were blocked for 3 h in blocking buffer containing 150 mM NaCl, and 50 mM Tris-HCl, pH 7.4, with 0.2% Tween 20 and 5% dry milk) and then incubated overnight with a 1 to 3,000 dilution of the immune sera in the same buffer. Specific antigen detection was performed using the Amplified Alkaline Phosphatase Immunoblot reagents from Bio Rad. For competition experiments, 6 μl of each undiluted antiserum were preincubated with 100 μg of recombinant protein in 500 μl of blocking buffer. After 4 h, the absorbed antisera were diluted and blotted as described above.

Cell Cultures

Cells were passaged in DME medium containing 10% fetal calf serum. Cells, unless otherwise indicated, were typically grown for 2 d before being treated with TGFβ3 at 3 ng/ml. Cells, either treated with TGFβ3 (+) or untreated (−) were then harvested after 24 h and processed for RNA preparation. RD cells, a human rhabdomyosarcoma cell line, were kindly provided by Dr. M. Bouche (University of Rome, Rome, Italy) (Aguanno et al., 1990). HC1 cells, a murine mammary epithelial cell line, were provided by N. Hynes (Friedrich Miescher Institute, Basel, Switzerland) (Hynes et al., 1990). HACAT cells, a human keratinoocyte cell line, were kindly provided by N. Fusenig (D.K.F.Z., Heidelberg, Germany). Hep 3B cells derived from a human hepatoma, and A459 cells derived from a human lung carcinoma, have been previously described (Morrone et al., 1989). Friend erythroleukemia cells are murine proerythrocytes that can be induced to differentiate to the proerythroblast stage (Coccia et al., 1992). Differentiation of C2C12 cells was achieved by culturing cells in low serum as described (Airey et al., 1991).

Results

RyR Genes Are Widely Expressed in Mouse Tissues

Initial studies on RyR1 and RyR2 mRNA distribution have detected expression of the former in skeletal muscle (Take-shima et al., 1989; Zorzato et al., 1990), and of the latter in the heart, brain, and possibly stomach (Nakai et al., 1990; Ota et al., 1990). On the contrary, RyR3 was found to be widely expressed in mink tissues and in some rabbit tissues (Gianmini et al., 1992; Hakamata et al., 1992). To study the expression of RyRs in mouse tissues, partial cDNAs for RyR1, RyR2, and RyR3 were isolated from a mouse brain cDNA library as described in Materials and Methods. Specific cRNA probes derived from these plasmids were subsequently used for RNase protection experiments on total RNA prepared from murine tissues (Fig. 1).

As expected, the highest levels of RyR1 expression were detected in skeletal muscle (note that for RNase protection of RyR1, only 5 μg of total RNA from diaphragm and thigh were analyzed, while 50 μg of total RNA from other tissues were used). Actually, by comparing the expression levels of RyR1 in different muscles, we observed higher levels of RyR1 mRNA in diaphragm than in thigh muscles. RyR3 mRNA was also expressed in skeletal muscle, although at lower levels than RyR1, as previously described in mink (Gianmini et al., 1992). Again, similarly to what observed for RyR1 mRNA, the levels of RyR3 mRNA in the diaphragm were higher than those observed in thigh muscles. No signal was detected when RyR2 cDNA was used to probe RNA extracted from skeletal muscle.

RyR2 was expressed at the highest level in the heart, as previously reported (for RNase protection of RyR2, only 10 μg of RNA from the heart was analyzed, while 50 μg of RNA from other tissues were used). RyR3 mRNA was also detected in the heart (Fig. 1), but at variance with RyR2, which is expressed in myocytes, RyR3 is preferentially expressed in the Purkinje cells of the cardiac conduction system (Gorza et al., 1995). In agreement with the results obtained in mink tissues the RyR3 gene appeared to be widely transcribed in mouse tissues, being detectable in the cerebrum, cerebellum, lung, esophagus, spleen, gut, kidney, stomach, submaxillary gland, thymus, testis, adrenal gland, and ovary. 50 μg of total RNA from each tissue was used in RyR3 RNase protection experiments.

To our surprise, although not completely unexpectedly, both RyR1 and RyR2 transcripts were detected in a large array of tissues. Both RyR1 and RyR2 mRNAs were found, although expressed at different levels, in mouse cerebrum and cerebellum. Significantly higher levels of RyR1 mRNA were also observed in the esophagus and testis compared to other organs; however, lower amounts were found in the spleen, gut, kidney, stomach, submaxillary gland, thymus, adrenal gland, and ovary. RyR2 mRNA was detected in esophagus, gut, kidney, stomach, thymus, testis, adrenal gland, and ovary, and at higher levels in the lung. No hybridization of any of the three probes was visible against tRNA, which was used as a negative control. In contrast with results from [3H]Ry binding, we did not observe large amount of RyRs mRNAs in the liver (Shoshan-Barmatz et al., 1991). Whether this suggests the presence of other RyRs different from the three genes we have studied, or the presence of a Ry-binding protein in the liver that is different from RyRs, is not clear at the moment.

![Figure 1](https://example.com/figure1.png)
In Situ Hybridization Reveals a Differential Expression Pattern of the Three RyR Genes in the Mouse Brain

RyR2 has been shown to be the major RyR protein present in brain (McPherson and Campbell, 1993a). However, RNase protection experiments indicated that also RyR1 and RyR3 mRNAs were expressed in both mouse cerebrum and cerebellum (Fig. 1). To systematically study the pattern of distribution of the three RyR isoforms in the mouse brain, RyR1, RyR2, and RyR3 cRNA probes, prepared as described in the methods section, were hybridized to sagittal and coronal brain cryosections. RyR1 mRNA expression was detected as a strong positive signal in Purkinje cells (Figs. 2 A, 3 C1, and 4 D1), which appeared to be the only RyR1-positive cell population in the cerebellum, in agreement with a previous report (Kuwajiima et al., 1992). However, RyR1 probe also yielded a specific signal in the dentate gyrus of the hippocampus and in the less strongly stained CA3 and CA1 cells of the Ammon's horn (Figs. 2 A, 3 Bl, and 4 Bl and Cl). In more anterior regions, RyR1-specific expression was observed on caudate/putamen nuclei (Figs. 2 A and 4 Al), on the olfactory tubercle (Figs. 2 A and 3), and on the olfactory bulb (Fig. 2 A, where it is specifically expressed in the mitral cell layer (Fig. 3 Al). Expression of RyR1 was also observed in the cortex (Figs. 2 A and 4 Al). As expected, a broader pattern of expression was revealed with the RyR2 cRNA probe. In the cerebellum, RyR2-specific grains were mainly localized to the granular cell layer (Figs. 2 C, 3 C2, and 4 D2). In the cerebrum, intense staining was also revealed in the hippocampus (Figs. 2 C, 3 B2, and 4 B2 and C2), both in the dentate gyrus and in the Ammon's horn (CA3–CA1). However, RyR2 expression was also detected at the level of the medial habitual nuclei, in the amygdala (Fig. 4 B2), and in more anterior regions, in the cortex (Figs 2 and 3) and in the granular cell layer of the olfactory bulb (Figs. 2 C and 4, A2 and B2). RyR3 mRNA expression was evident in several structures in the mouse brain. The RyR3 probe also appeared to preferentially stain the granular cell layer in the cerebellum, although the signal was much weaker than that produced by the RyR2 probe (Figs. 2 E, 3 C3, and 4 D3). RyR3 specific signal was also detected in the hippocampus, but with a pattern different from those of RyR1 and RyR2. RyR3 probe, in fact, labeled the dentate gyrus less strongly than the Ammon's horn (Figs. 2 E, 3 D3, and 4 B3 and C3). In this latter structure, RyR3 was found to be more enriched in the CA1 region compared to CA3 (Figs. 2, 3 B3, and 4, B3 and C3), with a distribution similar to InsP3 receptors (Sharp et al., 1993). A sparse signal was also detected with the RyR3 probe in the thalamic and hypothalamic regions, as well as in the caudate/putamen nuclei (Figs. 2 E and 4 B3). In the olfactory bulb, RyR3 mRNA was detected in the mitral cell layer and in the granular cell layer, overlapping both RyR1 and RyR2 expression (Figs. 2 E and 3 A3). Strong labeling was also revealed in the olfactory tubercle (Fig. 2 E). Finally, the RyR3-specific signal was detected in the lateral septum (Fig. 4 A3) and, in more posterior sections, in a region were the dorsalis raphae nuclei are located (Fig. 4 D3). RyR1-, RyR2-, and RyR3-labeled sense probes gave no signal on parallel sagittal (Fig. 2, B, D, and F) or coronal (not shown) sections.
Figure 3. Dark-field micrographs of sagittal brain cryosections showing details of RyR1 (A1, B1, and C1), RyR2 (A2, B2, and C2), and RyR3 (A3, B3, C3) patterns of expression in the olfactory bulb (A-A3), in the hippocampus (B1-B3), and in the cerebellum (C1-C3). Toluidine staining of parallel sections is shown in A4, B4, and C4. CA1-CA3, fields of Ammon’s horn of the hippocampus; DG, dentate gyrus; ML, molecular layer; GL, granular layer; P, Purkinje cells; EPL, external plexiform layer; MCL, mitral cell layer; IGL, internal granular layer. Bar in A4, 240 μm; B4, 180 μm; C4, 180 μm.

Differential Expression of the Three RyR Proteins in Specific Regions of the Central Nervous System

The distribution of the three RyRs in the central nervous system was further investigated using specific antisera developed against the three different RyR isoforms. These antisera identify three distinct proteins of ~500 kD in molecular mass which display a clear tissue specificity of expression. The anti RyR1 antiserum reacted specifically with a high molecular weight protein that is present in the sarcoplasmic reticulum of skeletal muscle, while the anti-RyR2 and the anti-RyR3 antisera recognized similar but distinct bands in cardiac muscle and the hippocampus, respectively (Fig. 5 A, lanes labeled with a minus). The specificity of these three antisera was further demonstrated by competition experiments using the recombinant proteins initially used to immunize the rabbits. As shown in Fig. 5 A, competition of the antiserum against the RyR1 with the recombinant RyR1 protein prevented the detection of the band expressed in skeletal muscle and a comparable situation was observed when antisera against RyR2 and RyR3 were competed by the respective proteins (Fig. 5 A, lanes labeled with a plus). Heterologous proteins did not compete the antisera (not shown). Interestingly, the three proteins recognized by these antibodies present a slight difference in their apparent molecular weight. It has been reported that the RyR2 proteins have a faster mobility with respect to RyR1. Here, we show that the
RyR3 protein runs slightly ahead of the RyR2 (Fig. 5 A). These relative differences in the apparent molecular weight of the three RyRs proteins are in agreement with the expected length of these proteins as deduced by the nucleotide sequences of the respective cDNAs (Coronado et al., 1994). These antibodies were then used to analyze the presence of RyRs in microsomal fractions prepared from specific areas of the CNS regions (Fig. 5 b). In agreement with in situ hybridization data, RyR1 protein was predominantly localized in microsomal fractions prepared from the cerebellum, but also from the hippocampus and striatum. No clear signal was detectable with the antiserum against RyR1 in other regions of the CNS, at least under our experimental conditions. RyR2 isoform was present in microsomal proteins prepared from all regions examined. RyR3 was detected, at the highest levels, in fractions prepared from hippocampus, striatum, and cerebral cortex; the least of expression was observed in the brain stem. Interestingly, with respect to other regions of CNS, RyR3 protein appears to be even more abundant in the cerebral cortex than expected, based on in situ hybridization results. Despite RyR3, mRNA is detected in total RNA prepared from skeletal and cardiac muscles; expression of RyR3 protein was not detected in preparations of microsomes from these tissues, even when higher amounts of protein were loaded on gels (not shown). This is very likely to result from the low levels of RyR3 protein present in these two tissues, especially skeletal muscle, compared with its expression in brain. Preliminary results from large scale purification experiments do, however, demonstrate the presence of the RyR3 protein, although at low levels, in these tissues (Conti, A., unpublished observations).

In Situ Hybridization on Mouse Peripheral Tissues
RNase protection experiments have shown that a message specific for RyR1, RyR2, and RyR3 can be detected in several mouse peripheral tissues, although at much lower levels than in brain or muscle. (Fig. 1). On these bases, some of these tissues were chosen for in situ hybridization experiments. Probes specific for the three RyRs were in situ hybridized to serial transversal sections of the organs contained in the mediastinal cavity (including heart, lungs, thymus, and
Figure 5. Expression of the three RyR proteins in the CNS. (A) Antibodies against RyR1, RyR2, and RyR3 recognize a single band in microsomes prepared from skeletal muscle, heart, and hippocampus, respectively. The minus (−) refers to antibodies not preincubated with recombinant protein; the plus (+) refers to experiments where the antibodies were preincubated with the recombinant protein used as antigen in the respective immunization protocol. (B) Localization of the isoforms of RyR in different areas of the CNS using antibodies specific for the different isoforms. Skeletal muscle microsomes were prepared from the posterior legs of a mouse. Cardiac microsomes were prepared from the entire heart, cleaned from large vessels. 5 μg of microsomal proteins from skeletal muscle and from heart were loaded on gel. 100 μg of microsomal proteins were loaded for all other samples.

In these sections, RyR1 mRNA was detected only in the esophagus (Fig. 6 A). In different sections (not shown), the labeling corresponded to both the inner and outer layers of striated muscle fibers; the signal could be followed to the entrance in the stomach, and it abruptly disappeared as soon as the esophageal striated muscle was substituted by the smooth muscles of the stomach. As expected, the RyR2 probe gave a strong signal over the whole heart (not shown). In addition, specific labeling was observed in pulmonary vessels (Fig. 6 B). This is consistent with reports describing "myocardial extrusions or sleeves" over pulmonary veins in rodents, which express cardiac muscle specific protein isoforms in line with the embryological origin of these veins from cardiac tissue (Lyons et al., 1990).

Both RyR1 and RyR3 mRNAs are transcribed in the spleen (Fig. 1). RyR3 expression was thus investigated by in situ hybridization. In cryosections of the spleen, RyR3 cRNA antisense probe clearly showed a particular pattern of positivity (Fig. 6 F). Grains were reproducibly observed in defined areas that were not superimposable to either red or white pulp, but rather appeared, at a higher magnification, to be distributed in the periphery of spleen sinusoids (not shown). No signal was detected using the sense control probe (Fig. 6 E). An RyR3-specific signal was clearly observed only in the peripheral region of the uterine muscle wall (Fig. 6 H). A similar pattern was observed in the vas deferens, where RyR3 transcripts were detected over the entire muscular tu-

Figure 6. Low power dark-field micrographs of mouse thoracic organs (A and B) spleen (E and F), uterus (G and H), and vas deferens (C and D) cryosections. Sections were in situ hybridized to RyR1 (A), RyR2 (B), and RyR3 (D, F, and H) cRNA antisense probes, as well as to control RyR3 sense cRNA probes (C, E, and G). E, esophagus; PV, pulmonary vessels. Bars in A and B, 1 mm; C and D, 0.1 mm; F, 0.45 mm; H, 0.3 mm.

nic (Fig 6 D). Again, no signal was detected using the sense control probe (Fig. 6, C and G). No specific signals (compared with pictures obtained with sense control probes) were observed when parallel sections were hybridized to RyR1 and RyR2 cRNA probes (not shown).

Several other tissues, such as the lung, stomach, thymus, submaxillary, and adrenal glands, were found to express at least one of the three RyR genes by RNase protection (Fig. 1). Unfortunately, despite repeated attempts, when those tissues were analyzed by in situ hybridization, no clear pattern was produced by antisense versus sense probes, probably because of the very high background observed with the sense control probes (not shown).

RyR1 and RyR3 mRNA Are Expressed in Germ Cells in Mouse Testis

RNase protection experiments revealed the presence of relatively high levels of RyR1 and RyR3 mRNA in total RNA
prepared from mouse testis (Fig. 1). In situ hybridization experiments were performed on transversal cryosections of adult mouse testis with the three cRNA probes mentioned in the above section. A clear positive signal was visible at the level of seminiferous tubules on sections hybridized with RyR1 (Fig. 7, A and B) and RyR3 (Fig. 7, D and E). In both cases, only a part of the tubules present in the section were stained by the probes. Considering that germinal cells at different stages of differentiation are present in contiguous seminiferous tubules in adult mice testis, this hybridization pattern suggests that RyR1 and RyR3 are expressed in the germinal cell compartment. This was confirmed when sections were observed at higher magnification. Silver grains were particularly dense over germ cells and, more specifically, in areas rich in spermatocytes and spermatids (Fig. 7, C and F). Almost no signal was present on the large spermatogonia and Sertoli cells. These results were also confirmed by RNase protection analysis performed on RNA extracted from elutriated spermatocytes, spermatids, and Sertoli cells, which demonstrated RyR1 and RyR3 expression in purified populations of spermatocytes and spermatids, but not in Sertoli cells (not shown).

RyR Genes Are Transcribed in Human and Murine Cell Lines

Expression of the three RyRs was studied by RNase protection in human and murine cell lines of different lineage. Given our initial finding that RyR3 mRNA is inducible in mink Mv1Lu cells by TGFβ (Giannini et al., 1992), RyR mRNAs expression was analyzed in cells treated with the growth factor and compared to untreated cells (Fig. 8). RyR1 mRNA was detected in the human HACAT and A549 cell lines and RyR2 mRNA was detected in Hep3B and HeLa cells, and their expressions were not modulated by TGFβ (Fig. 8 A). On the contrary, in HeLa cells, TGFβ treatment for 48 h induced expression of RyR1 but concomitantly, inhibited expression of RyR3 mRNA, compared to the untreated control. Murine NIH3T3 fibroblasts expressed relatively low levels of RyR1 mRNA, both in actively growing or stationary conditions (not shown). In these cells, treatment with TGF for 48 h resulted in a twofold increase of RyR1 expression (Fig. 8 B). A similar situation was observed for RyR1 in NIH3T3 cells transformed by the src oncogene; these cells, however, in contrast with the non-transformed parent cell line, expressed RyR2 mRNA in addition to RyR1 (Fig. 8 B). Constitutive expression of RyR1 mRNA was observed in the C5 epithelial cell line. In the mammary epithelial cell line HCl1, RyR1 mRNA levels were influenced by TGFβ treatment. In these cells, a constant increase in the RyR1 mRNA levels was detected upon continued treatment with TGFβ for 48 h. In Friend erythroleukemia cells, basal levels of expression of RyR1 and RyR3 were detected. Expression of both genes was not modulated by TGFβ treatment (Fig. 8) or by induction of differentiation (not shown).

RyR3 mRNA Expression in Myoblasts Is Linked to Expression of the Differentiated Phenotype

We have found that, along with expressing high levels of RyR1, skeletal muscle also expresses low levels RyR3 mRNA (Giannini et al., 1992; Fig. 1 in this paper). To verify whether RyR3 expression in muscle cells in constitutive or dependent on differentiation, we analyzed the expression of RyR3 in the C2C12 cells. C2C12 cells are myoblasts that have the potential to differentiate to muscle cells under appropriate culture conditions (Airey et al., 1991). Expression of RyR1 has been shown to be induced in these cells after induction of differentiation (Airey et al., 1991). As shown in Fig. 8 C, low levels of RyR3 mRNA are expressed in undifferentiated C2C12 cells, and they increase severalfold after induction of differentiation. TGFβ is an inhibitor of differentiation in these cells. Treatment of C2C12 with TGFβ reduced the expression of both RyR1 and RyR3, thus reinforcing the hypothesis that upregulation of RyR3 mRNA, in differentiated in C2C12, is linked to expression of the differentiated phenotype in these cells. Expression of the
Figure 8. RNase protection analysis of RyR1, RyR2, and RyR3 expression in in vitro-cultured cell lines. The plus (+) indicates cultures treated with TGFβ for 48 h except for HCl1 cells that were treated with TGF/3 from 12 to 48 h, as indicated in the figure, C2C12 cells that were cultured in differentiating conditions for GT = 4 d either in the presence or the absence of TGFβ, as also indicated in the figure.

RyR3, along with RyR1, was observed also in the myogenic cell line BC3HI (not shown) and in the human rhabdomyosarcoma cell line RD (Fig. 8 A).

Discussion

After skeletal and cardiac muscles, the CNS is the tissue where the expression of RyRs has been more extensively investigated (McPherson and Campbell, 1993a). Studies on intracellular calcium release channels in the CNS have shown the presence, within neurons, of both InsP3 receptors and RyRs (Sharp et al., 1993; Walton et al., 1991). This is consistent with the existence of two distinct intracellular calcium release processes in brain areas (Verma et al., 1990, 1992). These studies, however, did not distinguish between the different RyR isoforms. Here, we report the specific pattern of expression of the three RyRs in the cerebrum and the cerebellum. The relative abundance of the three isoforms agrees with previous published results that have indicated that the RyR2 is the major isoform present in brain (McPherson and Campbell, 1993a). Our data indicate that RyR3 is more represented than RyR1, although the latter is relatively well expressed in Purkinje neurons of the cerebellum. By means of in situ hybridization analysis a preferential expression of the three RyRs has been observed in different areas of the CNS, such as the cerebellum, hippocampus, striatum, and olfactory bulb. A distinct cell type-specific expression was also evident within these regions. Localization differences especially in neuronal cell populations, may reflect a differential usage of these channels to probably achieve a higher level for functional diversification. Such a pattern recalls that of the InsP3 receptors (Mikoshiba, 1993; Sharp et al., 1993). The different isoforms of InsP3 receptors have been shown previously to be also differentially expressed in the CNS (De Smedt et al., 1994; Furuichi et al., 1990; Nakagawa et al., 1991; Ross et al., 1992; Sharp et al., 1993). Although in some cases the expression of members of these two types of intracellular calcium release channels may partially overlap, it is interesting to note that the overall distribution of InsP3 receptors in the brain differs from that of RyRs (Sharp et al., 1993; Verma et al., 1990, 1992). This intriguing pattern of different types of intracellular calcium release channels and relative isoforms is likely to reflect the complexity of the mechanisms that are involved in the generation of oscillatory changes in intracellular calcium, as suggested by models that propose an interplay between two calcium release systems for generation of such patterns (Berridge, 1993). The predominance of one mechanism of calcium release on the other, as well as the intracellular localization of these channels, are certainly important in the regulation of the resulting neuronal function.

During the last few years, both pharmacological and molecular studies have shown that cells with intracellular calcium release pools that are regulated by channels with properties similar to those of RyRs of muscles are present, not only in the brain, but also in the peripheral tissues (Berridge, 1993; Fasolato et al., 1991; Kasai and Petersen, 1994). Evidence suggesting the presence in these tissues of calcium-operated channels differing from InsP3 receptors have been obtained studying the molecular mechanisms that regulate the concentration and diffusion of calcium in the cytosol (Pozzan et al., 1994). With the development of digital calcium imaging techniques, it has become clear that changes in intracellular calcium concentration take place as spatio-temporal events, such as calcium waves and oscillations, regulating a variety of functions (i.e., fertilization of eggs, cellular mobility, and release of hormones and neurotransmitters) (Augustine and Neher, 1992; Brundage et al., 1991; Kasai and Augustine, 1990; Miyazaki, 1988; Smith and Augustine, 1988). Such waves and oscillations seem to be generated by the concerted action of more types of intracellular calcium release channels, probably localized in distinct regions of the cell (Kasai et al., 1993; Thorn et al., 1993). However, whether these cells expressed the same RyRs present in muscle cells has not yet been clarified nor it is clear if they express more than one of the three known RyR isoforms.

This appears to be true also for smooth muscle cells which, although provided with InsP3-operated calcium
models of muscular differentiation such as the C2C12 and the purified smooth muscle cells is currently being performed to the case for neurons and germ cells. Analysis of RyRs in be also expressed in smooth muscle cells, as it appears to be associated with smooth muscle cells, RyR1 and/or RyR2 mRNA has been reported in aorta smooth muscle cells (Moschella and Marks, 1993), and expression of RyR3 mRNA in smooth muscle cells has also been suggested (Hakamata et al., 1992). Accordingly, we have found by in situ hybridization that in vas deferens and uterus, RyR3 is expressed in correspondence of areas that are rich in smooth muscle cells. However, it should be noted that in tissues such as the stomach gut, aorta, etc., where RyR3 expression has been associated with smooth muscle cells, RyR1 and/or RyR2 mRNA have also been detected (see Fig. 1 in this manuscript and Giannini et al., 1992; Moschella and Marks, 1993). Therefore, it is possible that more than one RyR may be also expressed in smooth muscle cells, as it appears to be the case for neurons and germ cells. Analysis of RyRs in purified smooth muscle cells is currently being performed to answer this question.

In skeletal muscle, both RyR1 and RyR3 are expressed. Expression of the RyR3 has also been studied in in vitro models of muscular differentiation such as the C2C12 and the BC3H1 myogenic cell lines. In these cells, we have found that increasing levels of RyR3 mRNA are associated with expression of the differentiated phenotype. Detection of RyR3 along with RyR1 in mammalian skeletal muscle is in agreement with detection of caffeine responsiveness in muscle preparation from mice where the RyR1 gene had been functionally altered by homologous recombination techniques (Takeshima et al., 1994). This result apparently contrasts with the reported absence of caffeine response in cells expressing RyR3 (Giannini et al., 1992). More recent data, however, indicate that the lack of appreciable responses of caffeine could be caused by the low levels of RyR3 expressed in those cells, rather than revealing the distinctive property of the RyR3 isoform (Rossi, D., and V. Sorrentino, unpublished results).

Since RyRs are known to be expressed in neurons and smooth muscle cells, it could be claimed that detection of RyRs in many tissues may be caused by the fraction of neuronal and smooth muscle cells present in these tissues. Several evidences argue against this possibility: (a) In situ hybridization analysis of RyRs in several tissues, including the stomach, gut, aorta, spleen, etc., did not always label areas enriched in smooth muscle cells. (b) The relative levels of RyRs expression vary from tissue to tissue in a way that does not correlate with the presence of neuronal and smooth muscle cells. In the liver, to our surprise, we did not detect significant levels of RyRs mRNAs. (c) In the testis, both RyR1 and RyR3 mRNAs were distinctly expressed in germ cells. It is, therefore, more likely that RyRs expression in peripheral tissues is not limited to neurons and muscle cells. This is also supported by detection of RyRs mRNAs in several cell lines of different lineage, such as fibroblasts, epithelial, and hematopoietic cells, as reported in Fig. 8.

What determines the choice of a given cell to express one RyR instead of another, or a specific combination of more than one isoform, is not clear. In at least a few cases, the em-

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bryological origin of the cells may dictate the preference for one RyR isoform instead of others, as observed in pulmonary vessels (Lyons et al., 1990). It is, however, more likely that the regulatory properties of the different isoforms may influence this decision (Ehrlich et al., 1994; Percival et al., 1994). In this context, it is noteworthy to recall that expression of RyR mRNAs, at least in vitro, seems to be influenced in part by TGFβ. In HeLa cells, for example, TGFβ induces the expression of the RyR1 mRNA and abolishes expression of RyR3 mRNA. These findings, together with other previously reported data (Giannini et al., 1992), suggest that the expression of the different isoforms may be coordinately regulated, and that TGFβ and other growth factors in general may have a modulatory role in determining quantitative and qualitative changes in RyRs expression in at least certain cells. Such changes may have influence on the generation of intracellular calcium signals (Berridge, 1993; Kasai and Petersen, 1994).

The low levels at which RyRs are expressed in many cells have been an obstacle to their localization outside skeletal and cardiac muscles and the brain. In this paper, we demonstrate that many cells express different isoforms of RyRs in addition to the InsP3 receptor isoforms (De Smedt et al., 1994; Ferris and Snyder, 1992; Mikoshiba, 1993). From such a complex pattern, it emerges that the calcium release channels, i.e., an important component of the machinery controlling intracellular calcium levels, may vary from cell to cell. The reason for such a finely tuned diversification will probably become clearer when we know more about the regulatory properties of the different RyR isoforms and their functional interaction with InsP3 receptors, and when more information on intracellular localization of all these channels becomes available. The data presented here about the expression of RyR genes, as well as our preliminary results at the protein level, suggest that the actual amounts of RyRs present in many cells are rather low. However, given the larger conductance of RyRs with respect to that of the InsP3 receptors, it may be expected that specific localization of even a few RyR channels to specific cellular microdomains may be effective in regulating the concentration of calcium in these intracellular districts. Clearly, much work remains to be done; however, the results reported here call for further investigation about RyRs to verify the extent of the contribution of these channels to the regulation of intracellular calcium homeostasis.

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