J. Membrane Biol. 189, 181–190 (2002)
DOI: 10.1007/s00232-002-1012-x

The Journal of
Membrane
Biology

Expression and Cellular Localization of a Modified Type 1 Ryanodine Receptor and L-Type Channel Proteins in Non-Muscle Cells

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Received: 11 March 2002/Revised: 17 June 2002

Abstract. Functional and molecular biological evidence exists for the expression of ryanodine receptors in non-muscle cells. In the present study, RT-PCR and 5′-rapid amplification of cDNA 5′-end (5′-RACE analysis) provided evidence for the presence of a type 1 ryanodine receptor/Ca^{2+} channel (RyR_1) in diverse cell types. In parotid gland-derived 3-9 (epithelial) cells, the 3′-end 1589 nucleotide sequence for a rat RyR shared 99% homology with rat brain RyR_1. Expression of this RyR mRNA sequence in exocrine acinar cells, endocrine cells, and liver in addition to skeletal muscle and cardiac muscle, suggests wide tissue distribution of the RyR_1. Positive identification of a 5′-end sequence was made for RyR_1 mRNA in rat skeletal muscle and brain, but not in parotid cells, pancreatic islets, insulinoma cells, or liver. These data suggest that a modified RyR_1 is present in exocrine and endocrine cells, and liver. Western blot analysis showed L-type Ca^{2+} channel-related proteins in parotid acinar cells, which were of comparable size to those identified in skeletal and cardiac muscle, and in brain. Immunocytochemistry carried out on intact parotid acini demonstrated that the dihydropyridine receptor was preferentially co-localized with the IP_3 receptor in the apical membranes. From these data we conclude that certain non-muscle cells express a modified RyR_1 and L-type Ca^{2+} channel proteins. These receptor/channels may play a role in Ca^{2+} signaling involving store-operated Ca^{2+} influx via receptor-mediated channels.

Key words: Ryanodine receptor — IP_3 receptors — L-type channels — Dihydropyridine receptors — Parotid cells — Immunofluorescence

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Introduction

Ca^{2+} mobilization through receptor-integral Ca^{2+} channels such as the inositol 1,4,5-trisphosphate receptor (IP_3R) and ryanodine receptor (RyR) is important in regulating Ca^{2+} levels and cell activation in diverse cell types. The RyR/Ca^{2+} channel complex, a homotetramer with a molecular weight of approximately 560 kDa, was originally identified by binding to the plant alkaloid ryanodine. The RyRs have been divided into three subtypes. RyR_1 and RyR_2 are enriched in skeletal muscle and cardiac muscle, respectively, and are well-characterized in these tissues (Sutko & Airey, 1996). RyR_3 has been cloned and identified in such tissues as brain and smooth muscle (Giannini et al., 1992). The basic RyR subtypes have several common structural features including the large N-terminal domain comprising the foot region, and the transmembrane domain near the C-terminus that is presumed to form the cation channel. The modulatory region between the N-terminal and the transmembrane domain contains the binding domains of Ca^{2+}, adenine nucleotides, and calmodulin (CaM), as well as phosphorylation sites that may modulate receptor function. The different RyR isoforms are products of distinct genes and share a significant degree (60–70%) of amino acid sequence identity, particularly in the carboxyl-terminal region (Takeshima, 1993; Sutko & Airey, 1996). Despite the fact that the carboxyl-terminal region (3′-end) represents only 20% of the full-length RyR_1, this domain can by itself operate as a functional Ca^{2+} release channel (Bhat et al., 1997a,b). These findings are compatible with the concept that the carboxyl-terminal end of the RyR constitutes the pore of the Ca^{2+} release channel.

Comprehension of RyR function depends on acknowledging that there are important differences in the way RyR channels are regulated. In cardiac muscle, surface membrane dihydropyridine-sensitive

(L-type) channels (DHPR) mediate the influx of a small amount of Ca^{2+} during depolarization that activates RyR_2 to release additional Ca^{2+} (i.e., CICR) (Franzini-Armstrong & Protasi, 1997; Franzini-Armstrong, Protasi & Ramesh, 1999). Excitation-contraction coupling in mammalian skeletal muscle occurs by depolarization of the T-tubule membrane, which leads to Ca^{2+} release from the sarcoplasmic reticulum (Franzini-Armstrong & Protasi, 1997). In this process the DHPR in close association with the foot region of the RyR_1 functions as a voltage sensor, which in response to depolarization activates the RyR_1 by a mechanism that does not require Ca^{2+} influx but involves direct protein-protein interactions (Franzini-Armstrong et al., 1999).

During the last several years, the existence of RyRs in non-excitable mammalian cells, including lung, renal, and exocrine acinar cells, has been established (Giannini et al., 1995; Bennett et al., 1996; Tunwell & Lai, 1996; DiJulio et al., 1997; Leite et al., 1999; Zhang et al., 1997, 1999). While high expression of RyRs typically occurs in muscle cells (Shoshan-Barmatz & Ashley, 1998), the level of expression of RyR in most non-excitable cells is very low (Bennett et al., 1996), frequently making their detection difficult. In an earlier study, a RyR/channel with distinct properties was identified in rat parotid acinar cells (Zhang et al., 1997, 1999). The sequence of this RyR showed high sequence identity to ~240 nucleotides of the 3′-end of the RyR_1 gene previously identified in rabbit brain (Takeshima et al., 1993; Zhang et al., 1997).

In the present study, a transcript identified by RT-PCR in exocrine and endocrine cells, as well as liver, was found to contain elements homologous to the 3′-region of the RyR_1 gene (Takeshima et al., 1993). A determination of the RyR partial sequence in rat parotid cell cultures by RT-PCR and rapid amplification of cDNA 5′-end (5′-RACE analysis) confirmed the presence of a RyR similar to that in rat brain and extended the sequence to 1589 bases. However, preliminary evidence indicates that the nucleotide sequence of the RyR in parotid and certain other tissues is not identical to skeletal muscle RyR_1 at the 5′-end. We also report in this study that parotid cells possess at least two L-type Ca^{2+} channel-related cell proteins in common with muscle cells that appear to preferentially co-localize with inositol 1,4,5-trisphosphate (IP_3) receptors. The unique localization of the DHPR and the divergent properties of the RyR presages a novel mechanism involved in the physiological regulation of Ca^{2+} signaling.

Materials and Methods

RyR RT-PCR

Total RNA was isolated from various tissues and cells with TRIzol reagent according to the manufacturer's protocol (GIBCO-Life Technologies, Grand Island, NY). The RNA was used for RT-PCR and 5′-RACE. For RT-PCR, total RNA was treated with DNase and reverse-transcribed by using a first strand cDNA synthesis kit according to the manufacturer's instructions (GIBCO). Rat RyR_1-specific primers were designed on the basis of comparative sequence of novel mouse brain RyR_1 and the partial sequence of rat skeletal muscle RyR_1 that was provided by Dr. Deborah Bennett (Cambridge University, UK) (Zhang et al., 1997). The sequences of the sense (+) and antisense (−) primer pairs used for specific amplification of rat type I, II, III ryanodine receptors were: RyR_1 carboxyl-terminal end (+) 5′-GGTGGCCTTCAACTTCTTCC-3′ and (−) 5′-ACTTGCTCTTGTTGGTCTCG-3′ (287 bp); RyR_1 amino-terminal end (+) 5′-TCTGATTGAGAGTCCTGAGGTG-3′ and (−) 5′-CATCACCTCGAAGTACCACTTG-3′ (301 bp); RyR_2 (+) 5′-GAGAACTATCCTGTCCTCAG-3′ and 5′-CTTGCTCTTGTTGGTCTCTG-3′ (380 bp); RyR_3 (+) 5′-GTTGCAACCTGTGGAACTC-3′ and 5′-CTACTGGGCTAAAGTCAAGG-3′ (430 bp). Mouse primers for RyR_1 (+) 5′-GAGAGGAGAGATAGTGTGTG-3′ and (−) 5′-ACTGTGGTGCCTGAGTCCTC-3′; RyR_2 (+) 5′-TCGTGAGGATGCTCAGCCTG-3′, and (−) 5′-CCTCTTTCGCCTTCTGCTCC-3′; RyR_3 (+) 5′-GATGATGACGAGGAAGAAGC-3′, and (−) 5′-CCAGGCAAGGTAGAGAAAGG-3′. Using these primers, RyR cDNAs were amplified by PCR with a thermocycler (Model 2400, Perkin Elmer or Hybaid PCR Sprint) for 35–45 cycles with 1 unit of Pfu, Taq or Platinum Pfx polymerase. Reactions were carried out as follows: denaturation for 1 min at 95°C, annealing for 2 min at 60°C, and extension for 2.5 min at 74°C. For RyR_1 the reactions consisted of a 2-min initial denaturation, then 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C. An aliquot of each PCR reaction was subjected to electrophoresis through a 1.5% agarose gel, and DNA was visualized by ethidium bromide staining. The DNA products were purified and sequenced in both directions by the CAMBI Nucleic Acid Facility (DNA Sequencing Service, The University at Buffalo).

Rapid Amplification of 5′-cDNA Ends (5′-RACE)

Total RNA (~2.5 μg) isolated from parotid 3-9 cells as described above was used as a template for the 5′-RACE procedure, which was carried out according to the manufacturer's instructions (GIBCO-Life Technologies). Gene-specific cDNAs were purified using spin columns, and then tailed with poly-C using terminal deoxynucleotidyl transferase. After first-strand DNA synthesis, sequences were amplified for 35 cycles using the RyR_1-specific antisense primer 5′-ACTTGCTCTTGTTGGTCTCG-3′, and the universal anchor primer supplied in the kit. Nested gene-specific antisense primer (5′-GACGACCCGGTACAGTTCAT-3′) and nested anchor primer confirmed the sequence. The thermal cycling conditions for the RT-PCR procedure consisted of an initial denaturation step for 2 min at 94°C, followed by 30 sec at 94°C, annealing at 55°C for 30 sec, and extension at 72°C for 2 min. PCR products were analyzed on a 1% agarose gel using 100 bp DNA ladder molecule markers. DNA bands were excised from the gel and sequenced directly as described above. A control reaction was performed with parotid mRNA in the absence of reverse transcriptase to eliminate the possibility of amplification due to genomic DNA contamination.

Western Blot Analysis

An antibody previously shown to recognize the α_1 subunit of muscle and neuronal voltage-gated Ca^{2+} channels (Hell et al., 1993) was used to detect similar proteins in non-muscle cells. Parotid cells were prepared by sequential digestion with trypsin and collagenase of freshly isolated parotid glands from two rats as

described previously (Rubin & Adolf, 1994). A homogenate fraction from these cells was subjected to electrophoresis (5% gel) and subsequently electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After the membrane was blocked with phosphate-buffered saline (PBS) plus 8% non-fat dry milk and 0.2% Tween-20, it was incubated with Ca^{2+} channel antibody anti-α_{1C} (1:1000) (Alomone Labs, Jerusalem, Israel) in PBS plus 8% milk. The primary antibody reaction was carried out at room temperature overnight. After immunoreactivity was visualized using peroxidase-conjugated secondary antibody, the blot was processed for enhanced chemiluminescence (ECL) (Pierce, Rockford IL, USA) and exposed to X-ray film.

Immunofluorescence Analysis of DHPR

Initially 5–10 μm thick frozen sections of rat parotid gland were fixed for 10 min with 2% paraformaldehyde in PBS (pH 7.4), followed by 10 min of permeabilization with 0.1% Triton X-100. After being treated for 30 min with PBS and washed three times with PBS, the sections were blocked for 1 hr with 5% BSA in PBS. For immunofluorescence localization of DHPR, the primary antibody reaction was performed using Ca^{2+} channel antibody α_{1C} (1:200 or 1:2000) in PBS containing 5% BSA plus 0.1% Triton X-100 and incubated for 3 hr at 37°C. The sections were then washed 3 times with PBS containing 0.1% Triton X-100, and reacted in total darkness with secondary antibody (anti-rabbit 1:100) conjugated to fluorescein. After the sections were washed three times with PBS containing 0.1% Triton X-100 and covered with Prolong Antifade Reagent (Bio-Rad), they were initially viewed using epifluorescence optics of a Nikon upright Optiphot microscope. Confocal imaging was then performed with a laser scanning microscopy system (Model MRC-1024; Bio-Rad) configured with a Nikon microscope and a krypton-argon laser (488 nm). A 60× oil immersion objective was first employed to provide an overall view, and then zoom 2.64× optics yielded a high-magnification view. The system was operated by a Compaq pentium 100 computer and photographs were processed using a disublimation printer. Specificity of immunofluorescence was determined by incubating secondary antibody alone before microscopic analysis or by mixing antibody with its corresponding peptide prior to incubation. Immunofluorescence analysis of IP_3R was also carried out by confocal microscopy as previously described using a 1:20 dilution of affinity-purified IP_3R subtype II rabbit polyclonal antisera (Zhang et al., 1999).

Results

Characterization of RyR mRNA in Various Cell Types

In a previous study (Zhang et al., 1997), an RT-PCR product amplified in the 3′-end region of RyR_1 mRNA of rat parotid cells was sequenced and demonstrated by multiple sequence alignment to be similar to the RyR_1 sequence in rabbit, human, and mouse mRNAs. In the present study, RT-PCR analysis provided evidence that rat brain-type RyR_1 mRNA is also expressed in several tissues and cell types. In addition to parotid acinar cells, an RT-PCR product of expected size (287 bp) for the carboxyl-terminal 3′-end of RyR_1 was identified in rat skeletal muscle, cardiac muscle, brain, anterior pituitary (GH_3) cells, islets of Langerhans, RINm5F and

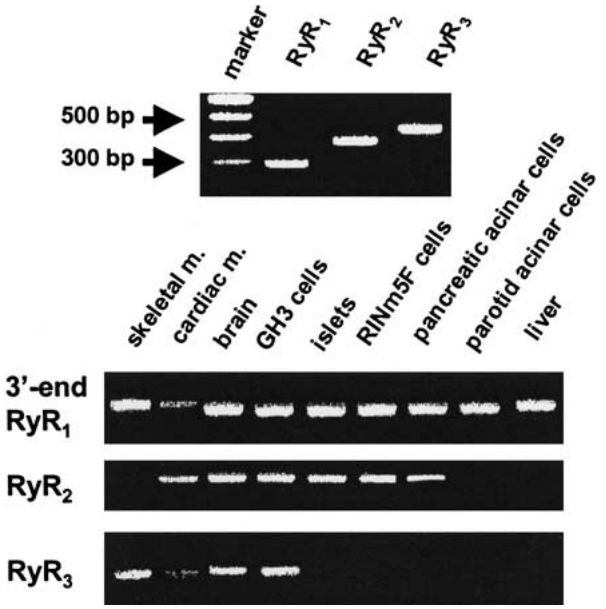

Fig. 1. Expression of RyR mRNAs in various tissue and cell types. Primers to the 3′-end of RyR_1, RyR_2 and RyR_3 were amplified by RT-PCR using RNA isolated from skeletal muscle (*m.*), cardiac muscle, brain, and other tissues or cells as indicated. The relative sizes of the RyR amplified products are shown in the top panel, where the first lane shows nucleotide size markers (*marker*). The results shown are representative of 3 separate experiments.

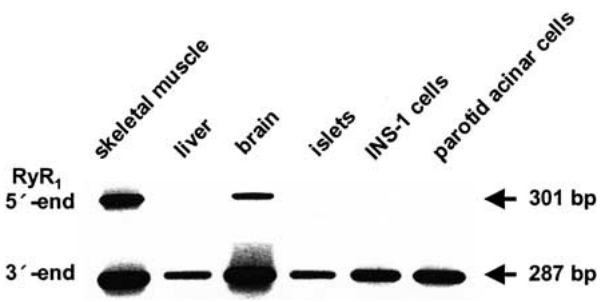

Fig. 2. Unique expression of 3′- and 5′-end RyR_1 mRNA in various cell types. RT-PCR amplification of mRNA was carried out for mRNA extracted from several rat tissues and cells as indicated, including islets of Langerhans (islets), insulinoma (INS-1) cells, and parotid acinar 3-9 cells. PCR primers targeted either the carboxyl-terminus (3′-end) or amino-terminus (5′-end) of rat RyR_1. PCR product was not detected in samples to which no reverse transcriptase was added (*not shown*). The size of the amplified products is indicated at the right hand margin. Similar results were obtained in two to four experiments.

INS-1 insulinoma cells, pancreatic acinar cells, and liver (Figs. 1, 2). In contrast, amplification of a rat skeletal muscle RyR_1 gene sequence located near the amino-terminal 5′-end identified a PCR product of 301 bp in cDNA from rat skeletal muscle and brain. However, the RyR_1 5′-end primers failed to amplify cDNA from parotid acinar 3-9 cells, islets of Langerhans, INS-1 insulinoma cells, and liver (Fig. 2).

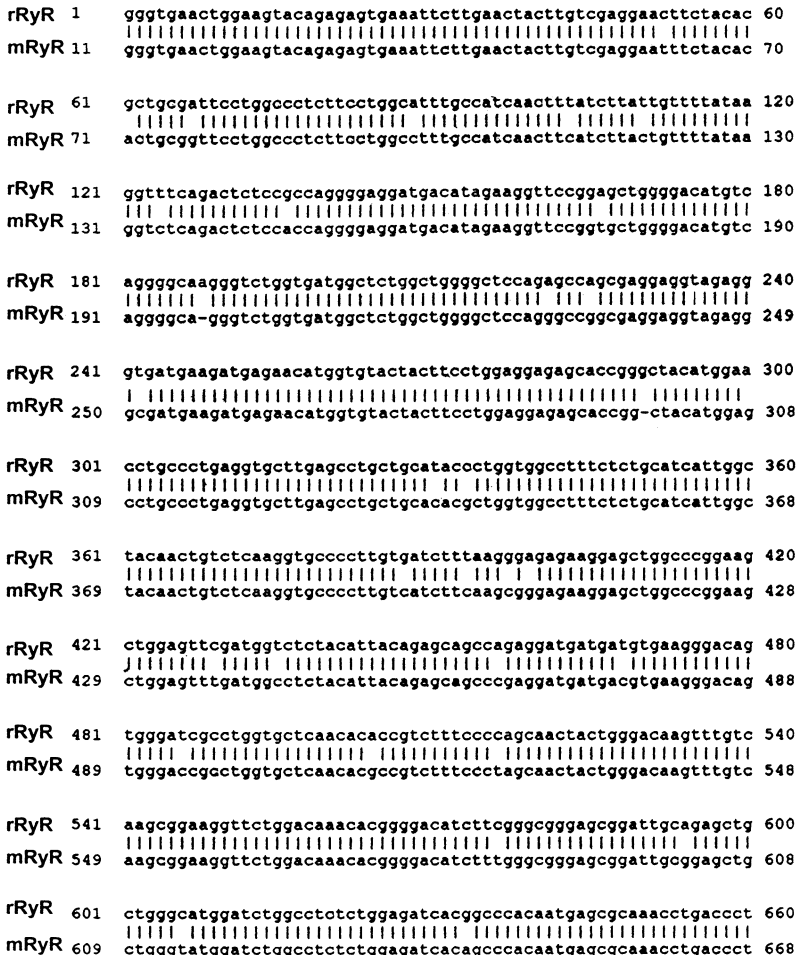

Fig. 3. Comparison of the cDNA nucleotide sequences at the carboxyl-terminus of the RyR_1 for rat (rRyR) and mouse (mRyR). Vertical lines indicate identical nucleotide bases common to the rat and mouse receptor sequences.

The failure to identify a homologous region of the amino-terminal end of the RyR_1 gene in the latter tissues and cells suggests that the 5′-end of a skeletal muscle-type RyR_1 sequence is not common to all tissues, and points to a unique sequence or structure in the amino-terminal region of RyR_1 in some tissues.

Analysis of RyR_2 and RyR_3 mRNA expression was also carried out to determine tissue specificity among these receptor subtypes. RyR_2 mRNA was identified in pancreatic acinar cells and islets and RINm5F cells (Fig. 1), confirming the presence of authentic RyR_2 in these tissues as previously reported (Holz et al., 1999; Leite et al., 1999). RyR_2 mRNA was also identified in GH_3 cells, brain and cardiac muscle (Fig. 1). In contrast, RyR_2 mRNA was not identified in parotid acinar cells, skeletal muscle or liver (Fig. 1). RyR_3 mRNA was identified in GH_3 cells (Fig. 1), which coincided with evidence for RyR_3 in rat anterior pituitary glands (Sundaresan et al., 1997). RyR_3 mRNA was also identified in skeletal muscle, cardiac muscle, and brain, but not in parotid acinar cells, pancreatic acinar cells, pancreatic islets, RINm5F cells, or liver (Fig. 1).

Mouse tissues reflected the observed RyR mRNA expression in rat tissues. As expected, mouse brain expressed skeletal muscle type RyR_1, RyR_2, and RyR_3 mRNAs (*data not shown*). Similar to rat islets and RINm5F cells, murine βHC9 insulinoma cells also expressed the 3′-end of RyR_1 and RyR_2 (*data not shown*).

5′-Race and Sequence Analysis Using Parotid Acinar Cells

The previously published partial sequence of the 3′-end of rat RyR_1 extended only to 293 bp (Zhang et al., 1997). To further determine the sequence of the ubiquitously distributed RyR_1 isoform, 5′-RACE, i.e., rapid amplification of the cDNA 5′-end, was utilized for sequence analysis of cultured parotid (3-9) cells. The continuous epithelial cell line generated by stable transfection of freshly prepared rat parotid acinar cells developed in our laboratory retains the basic structural and functional properties of primary culture epithelial cells after treatment with rat serum (Zhu et al., 1998). Primers were designed to amplify discrete domains of the RyR_1 sequence with increasing distance from the 3′-end following cDNA synthesis from mRNA. The control reactions to rule

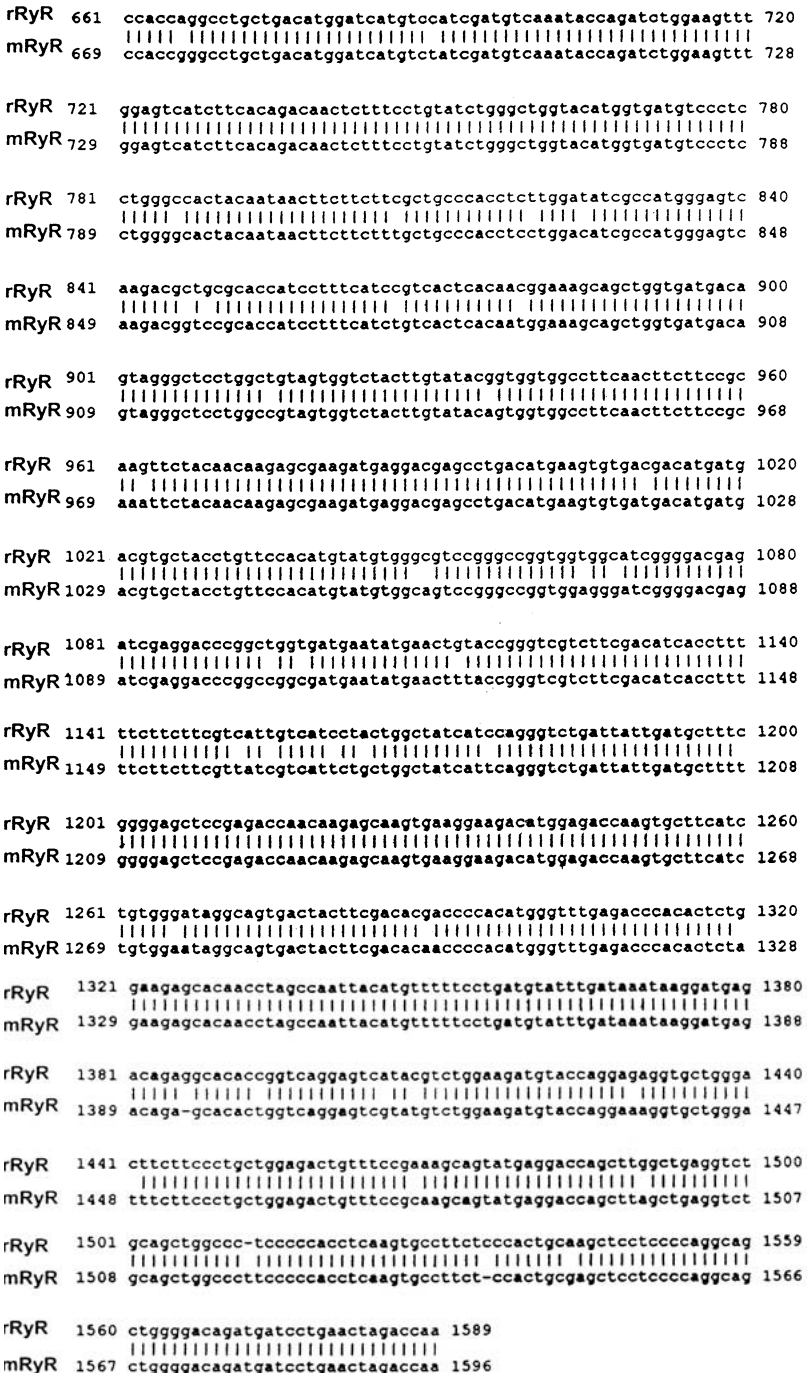

Fig. 3. Continued.

out genomic DNA contamination performed in the absence of reverse transcriptase did not result in amplification.

The authenticity of each of the amplified bands as RyR_1 fragments was confirmed by sequencing analysis. A total of 1589 bases of the rat RyR_1 were amplified using the 5′-RACE strategy in addition to RT-PCR amplification using primers (+) 5′-gggtgaactggaagtacagagagtg-3′, and (−) 5′-ttggtctagtt caggatcatctgtc-3′ to mouse brain type RyR sequence. Attempts to extend the sequence further were not successful. The rat 3-9 cell RyR_1 sequence was 99% homologous with rat brain RyR_1 (GenBank accession no. AF130879) and 95% homologous to mouse RyR_1 (GenBank accession no. X83932) as determined by Blast analysis (Fig. 3). Attempts to identify the rat parotid gland RyR_1 by Western blotting and Northern analysis proved unsuccessful, indicating that the antisera used to identify RyR_1 did not bind to a specific carboxy-terminal epitope on the RyR_1 (*see* Zhang et al., 1999) and that the expression of the RyR_1 mRNA was below detectable levels.

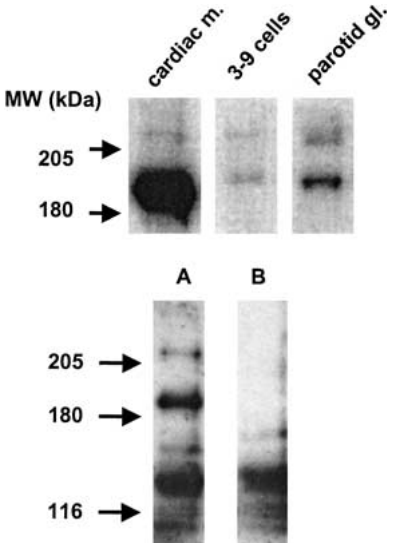

Fig. 4. Expression of VDCCα_1C subunit proteins in various tissues. In the upper panel, homogenates were analyzed for VDCCα_1C subunits by electrophoresis through a 5% SDS-polyacrylamide gel and immunoblot in cardiac muscle (*m.*), parotid cell line (3-9 cells), and freshly prepared dispersed rat parotid acinar cells (*gl.*). A high (~210 kDa) and low (~190 kDa) molecular weight form were detected in all tissues. In the lower panel, the specificity of the antibody representing the C-terminal of the anti-α_{1C} subunit was confirmed by immunoblots of rat brain depicting bands corresponding to the high and low molecular weight forms (*A*), which were abrogated after the antibody was mixed with its corresponding peptide (*B*). The position of molecular size markers is indicated by the arrows. The blots shown are representative of three separate experiments.

Identification and Localization of a Dihydropyridine-sensitive Channel

Because RyR is coexpressed with voltage-activated dihydropyridine (DHP)-sensitive Ca^{2+} channels in cardiac and skeletal muscle, and brain (Franzini-Armstrong & Protasi, 1997; Franzini-Armstrong et al., 1999), we tested the hypothesis that a DHP-sensitive Ca^{2+} channel is present in non-muscle cells in association with RyR. Western blot analysis was carried out on homogenates of freshly prepared parotid acinar cells and clonal parotid 3-9 cells using antibody directed against a peptide that represents the C-terminal of the anti-α_{1C} subunit. This subunit functions as the cation channel of the DHPR (Meir et al., 1999). Like cardiac muscle, the antibody recognized a high and a low molecular weight form, with the higher molecular weight form (210 kDa) being present in lesser amounts than the lower molecular weight form (190 kDa) (Fig. 4, upper panel). These values compare favorably with high and low molecular weight forms in skeletal muscle of 214 and 193 kDa, respectively (DeJongh et al., 1991). Moreover, using rat brain as a positive control (Fig. 4*A*, lower panel), we verified the specificity of the antibody by showing that when it was mixed with its corresponding peptide, bands representing the high and low molecular weight forms were no longer observed (Fig. 4*B*, lower panel). These studies demonstrate that a form of the L-type Ca^{2+} channel is expressed in parotid acinar cells.

To determine the cellular distribution of the DHPR, immunocytochemistry was carried out using frozen sections of parotid glands. Intact acini rather than cultured cells were employed in these experiments in order to localize the fluorescent signal to a specific cytoplasmic component of the acinar cell. Confocal microscopy revealed that acini exposed to anti-α_{1C} subunit antibody displayed a fluorescence that was most prominent in the apical portion of the cell (Fig. 5*A*). A diffuse signal of rather homogeneous intensity was also observed throughout the basal region of the cell; however, the intensity of the fluorescence was clearly weaker than in the apical region. No signal was detected when the primary antibody was preincubated with the antigenic peptide or in the absence of primary antibody (*data not shown*). By comparison, IP_3Rs identified by antiserum specific to subtype II, which is the most abundant subtype (circa 90%) (Zhang et al., 1999), were primarily distributed in the apical pole of the parotid cell (Fig. 5*B*). As previously reported (Zhang et al., 1999), the use of fluorescently labeled BODIPY-ryanodine showed that the highest levels of RyR expression were localized to the basal pole of the cell, and RyR expression was completely absent in the most apical aspect of the cell (Fig. 5*C*). Thus, in parotid cells the DHPR primarily co-localizes with the IP_3R, rather than with the RyR. The localization of the RyR observed in our studies for parotid cells coincides with that reported for exocrine cells of the pancreas in that the RyR was predominantly localized to the basal aspect of the exocrine cell and excluded from the extreme apex of the cell called the trigger zone (Leite et al., 1999; Straub, Giovannucci & Yule, 2000). The trigger zone is the region of the exocrine cell where agonist-induced Ca^{2+} signals are initiated (Tojyo, Tanimura & Matsumoto, 1997; Straub et al., 2000).

Discussion

Because Ca^{2+} regulates a number of diverse cellular processes, the properties of the Ca^{2+} signal encompass a varied number of Ca^{2+} release events, including those involving the RyR. Our previous work utilizing the exocrine cells of the rat parotid gland tentatively identified a RyR_1 isoform by RT-PCR and described its intracellular distribution by cytochemical analysis (Zhang et al., 1997, 1999). The present study broadens this analysis by using RT-PCR to demonstrate the expression of an RyR_1-like mRNA in a number of other non-muscle cells, in-

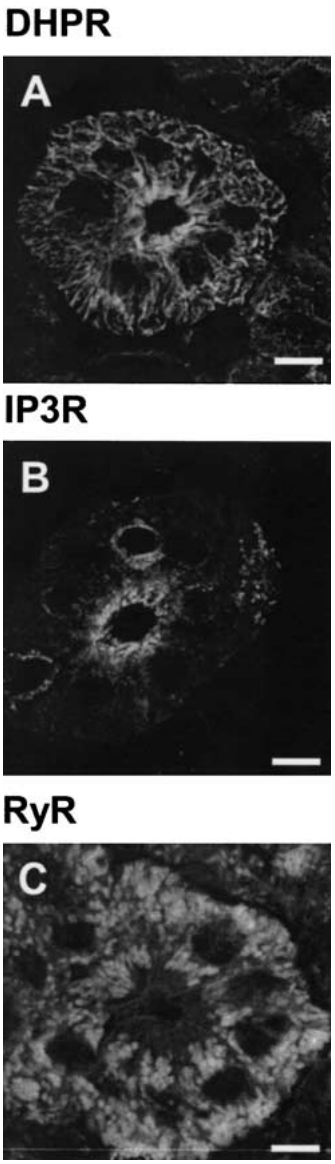

Fig. 5. Confocal images demonstrating the fluorescence localization of DHPR, IP_3R, and RyR in rat parotid gland. Representative images of acini treated with: (*A*) Ca^{2+} channel antibody (anti-α1C); (*B*) 1:20 dilution of affinity-purified polyclonal inositol trisphosphate receptor subtype II (IP_3R) rabbit clonal antisera; and (*C*) 25 μM BODIPY-ryanodine (reproduced with permission from Zhang et al., 1999, Biochem. J. 340, p. 521, Fig. 1*A*. © the Biochemical Society). Scale bar 10 μm. Similar results were obtained in three other experiments.

cluding pancreatic exocrine and endocrine cells, and liver. In another study, an RyR_1 was identified in non-excitable lymphocytes by immunoblotting and molecular biological techniques (Sei, Gallagher & Basile, 1999).

In addition to demonstrating that a brain/skeletal muscle-type RyR_1 mRNA is present in several rat tissues and cell lines, the present study utilized 5′-RACE analysis of parotid cells to extend the sequence of the RyR_1 to 1589 bases. High homology exists between the rat parotid cell sequence and mouse skeletal muscle RyR_1. Unfortunately, perhaps because of secondary structures, GC-rich sequences, or even an absence of sequence, the 5′-RACE analysis was unable to extend the sequence any further than reported.

In order to obtain evidence for a full-length RyR_1 in non-muscle tissues, primers were designed from a sequence recently reported for the 5′-end of rat skeletal muscle RyR_1 (Martin et al., 1999). As expected, the primer sequence in proximity to the 5′-end of RyR_1 amplified skeletal muscle and brain mRNAs. Surprisingly, several tissues that expressed mRNA with high sequence homology to 3′-end rat brain RyR_1 failed to express a sequence near the 5′-end of RyR_1. These data suggest that the 5′-end of RyR_1 in tissues such as parotid cells, pancreatic islet β-cells, and liver was either not present or differed in sequence such that those primers were not recognized. Precedent for the expression of a truncated form of RyR_1 exists in the novel brain-type RyR_1 which contains only ~2000 base pairs as compared to skeletal muscle RyR mRNA which is composed of ~16,000 nucleotides (Takeshima et al., 1993). Although the functional role of this variant has not been characterized, the brain protein contains the C-terminal region which is thought to express the Ca^{2+} channel domain but lacks the "foot" region (Takeshima et al., 1993; Furuichi et al., 1994).

Previous studies from our laboratory suggested that the RyR identified in parotid exocrine cells differs in its functional properties from those of authentic RyR_1 (Zhang et al., 1997, 1999). Unlike classical RyR_1, the parotid cell RyR lacked a sensitivity to caffeine, and ryanodine failed to open the RyR/channel at low concentrations or block the channel in higher concentrations in permeabilized parotid cells (Zhang et al., 1997). Morphologically, the expression of an RyR high-affinity binding site was demonstrated in parotid acini by confocal microscopy using the fluorophore BODIPY-ryanodine and by [^{3}H]ryanodine binding (Zhang et al., 1997, 1999). However, the finding that cADPR and cAMP markedly reduced [^{3}H]ryanodine binding (Zhang et al., 1999) contrasts with studies showing that adenine nucleotides, including cADPR, either enhance, inhibit, or have negligible effects on [^{3}H]ryanodine binding in muscle and nerve (Fruen et al., 1994; Zucchi & Ronca-Testoni, 1997; Shoshan-Barmatz & Ashley, 1998; Hadad et al., 1999). Such evidence portrays a RyR_1 isoform in parotid cells that possesses less complex regulatory mechanisms compared to full-length RyR_1 (*see* Furuichi et al., 1994). The notion favoring the existence of a novel RyR isoform was supported by Western blot analysis of parotid cell membranes using antibodies raised against type -I, -II, and -III RyRs, which failed to demonstrate protein bands that have electrophoretic

mobility patterns similar to that of the RyR (Zhang et al., 1999). It is noteworthy that comparative studies of the channel properties of mammalian RyR isoforms and isoforms expressed in chicken, frog, fish, and lobster muscle have revealed diversity in the gating and activation of the channels (Sutko et al., 1997). This diversity underlies different roles in Ca^{2+} signaling for each RyR isoform, including the parotid RyR_1.

Besides IP_3- and ryanodine-induced Ca^{2+} release, L-type Ca^{2+} channels may also play a functional role in parotid cells. The present study reveals that a form of the α_1 polypeptide associated with voltage-gated Ca^{2+} channels of excitable cells is also expressed in parotid acinar cells. Western blot analysis identified two forms of the peptide, with the lower molecular weight form present in higher levels (*see* Fig. 4). The fact that these two forms of the α_1 subunit of DHPR are of a size comparable to those identified in muscle and nerve (Catterall, 1997) suggests that L-type channels in parotid cells share certain structural and/or functional properties with those in excitable cells. Although pharmacological evidence does not support the presence of a muscle- or nerve-type of voltage-dependent Ca^{2+} channel in exocrine acinar cells (Putney, 1981; Williams & Burnham, 1985), the report that murine erythroleukemia cells express a truncated form of the α_1 subunit of the cardiac voltage-gated Ca^{2+} channel (Ma, Kobrinsky & Marks, 1995) is consistent with the concept that non-excitable cells express a DHPR that is somehow linked to mechanisms regulating Ca^{2+} signaling.

Although the mechanism of coupling between L-type Ca^{2+} channels and RyRs differs in skeletal and cardiac muscle (*see* Introduction), there is tight coupling between L-type channels and RyRs in both tissues. By contrast, in smooth muscle cells L-type channels are loosely coupled to RyRs by virtue of an increase in the effective distance between the Ca^{2+} channel and RyR (Collier, Wang & Kotlikoff, 2000). These findings lead to the conclusion that the interaction between RyR and DHPR is tissue-specific (*see* also Bhat et al., 1997b). The mechanism of Ca^{2+} gating in the parotid acinar cell may primarily involve coupling of the L-type channels to IP_3Rs in the apical pole where both proteins predominate. The fact that the RyR and DHPR are not colocalized to a large extent in the parotid cell is in accord with our other major finding that the parotid cell (as well as certain other cells/tissues) possess a variant of the RyR_1 that may be devoid of at least a portion of the foot region (5′-region). The foot region is a component of the RyR/channel responsible for linking the RyR to the DHPR (Franzini-Armstrong & Protasi, 1997; Franzini-Armstrong et al., 1999). On the other hand, our results do not preclude the possibility that the relatively weak fluorescent signal in the basal region of the cell may reflect colocalization of the DHPR with the RyR. In this case, the immunoreactivity observed in both the apical and to a lesser extent in the basal portion of the cell may represent the low and high molecular weight forms, respectively, of the α_{1C} subunit, which are differentially localized in the parotid acinar cell and perform diverse functions. Several lines of evidence favor the concept that DHPRs can regulate the activity of IP_3Rs (Mackrill, 1999). On the basis of the structural similarity in the C-terminal (channel) region of the IP_3Rs and RyRs (Takeshima, 1993), as well as functional studies, Spat, Rohacs & Hunyady (1994) postulated that IP_3Rs interact with DHPRs in the endoplasmic reticulum of rat adrenal glomerulosa cells in a manner analogous to that occurring between the RyR and DHPR in skeletal muscle. Spat et al. (1996) also theorized that, since the L-type channel serves as a voltage sensor in skeletal muscle, in a non-excitable cell (one not activated by depolarization), IP_3R function must be regulated by a variant of the L-type channel. In this connection, Zhang and O'Neil (1996) demonstrated a Ca^{2+} channel in the apical membrane of rabbit renal epithelial cells that is DHP-sensitive and activated by membrane depolarization. However, compared with a typical L-type channel in excitable cells, the renal channel manifested certain diverse properties, including a relatively small channel size (Zhang & O'Neil, 1996). Thus a variant of the typical L-type channel may also operate in parotid cells to modulate IP_3-induced Ca^{2+} mobilization or store-operated Ca^{2+} entry, where Ca^{2+} influx is regulated by the filling of depleted intracellular Ca^{2+} stores (Parekh & Penner, 1997). Functional studies are planned to address these possible interactions.

The parotid acinar cell is polarized such that the endoplasmic reticulum and nucleus are located in the basal region of the cell, whereas the apical region harbors the zymogen granules. It is interesting that carbachol stimulation of parotid acinar cells results in the initiation of a Ca^{2+} wave at the apical pole, which spreads toward the basal pole despite the fact that agonists that induce IP_3 production exert their primary action on the basal aspect of the acinar cell surface (Tojyo, Tanimura & Matsumoto, 1997; Liu, Scottt & Smith, 1998). In attempting to explain these findings, one may speculate that the DHPR that we have identified in the parotid cell may be a contributory factor to the greater sensitivity to Ca^{2+} release exhibited by IP_3 in the apical region (Tanimura, Matsumoto & Tojyo, 1998).

In conclusion, the present and previous studies have identified what is perhaps a variant of RyR_1, along with three IP_3R isoforms and two DHPR subtypes in parotid acinar cells (Zhang et al., 1997, 1999). The identification of these key elements represents a potentially important step in gaining a deeper understanding of the mechanisms regulating Ca^{2+} signaling in non-excitable cells.

These studies were supported in part by the NIH research grant DE-059654 to RPR and a grant from the American Diabetes Association to SGL. BL was supported by a fellowship from The Juvenile Diabetes Foundation International. We thank Dr. Keshore Bidasee of Indiana University School of Medicine for carrying out multiple sequence analysis of RT-PCR product and Dr. Xuejun Zhang for designing several primers. We also thank Dr. Richard Wojcikiewicz of Upstate Medical University at Syracuse (New York) for generously supplying the IP_3R antiserum specific to subtype II.

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