Multiple isoforms of the ryanodine receptor are expressed in rat pancreatic acinar cells

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Regulation of cytosolic Ca^{2+} is important for a variety of cell functions. The ryanodine receptor (RyR) is a Ca^{2+} channel that conducts Ca^{2+} from internal pools to the cytoplasm. To demonstrate the presence of the RyR in the pancreatic acinar cell, we performed reverse transcriptase (RT)-PCR, Western blot, immunocytochemistry and microscopic Ca^{2+} -release measurements on these cells. RT-PCR showed the presence of mRNA for RyR isoforms 1, 2 and 3 in both rat pancreas and dispersed pancreatic acini. Furthermore, mRNA expression for RyR isoforms 1 and 2 was demonstrated by RT-PCR in individual pancreatic acinar cells selected under the microscope. Western-blot analysis of acinar cell immunoprecipitates, using anti-

bodies against RyR1 and RyR2, showed a high-molecular-mass (> 250 kDa) protein band that was much less intense when immunoprecipitated in the presence of RyR peptide. Functionally, permeabilized acinar cells stimulated with the RyR activator, palmitoyl-CoA, released Ca^{2+} from both basolateral and apical regions. These data show that pancreatic acinar cells express multiple isoforms of the RyR and that there are functional receptors throughout the cell.

Key words: calcium-induced calcium release, intracellular calcium store, palmitoyl-coenzyme A.

INTRODUCTION

Cytosolic Ca^{2+} is an important regulator of many cell functions. Increases in cytosolic Ca^{2+} occur by release of Ca^{2+} from intracellular stores located in the endoplasmic reticulum. There are at least two known types of Ca^{2+} channel that regulate release from Ca^{2+} stores: an $\text{Ins}(1,4,5)P_3$ -sensitive channel [$\text{Ins}(1,4,5)P_3$ receptor] and a ryanodine-sensitive channel (ryanodine receptor, RyR). As its name implies, $\text{Ins}(1,4,5)P_3$ opens the $\text{Ins}(1,4,5)P_3$ receptor and is used as a second messenger in many cells to release Ca^{2+} . The RyR is responsible for calcium-induced calcium release [1].

Ryanodine, a plant-derived insecticide, binds to the Ca^{2+} -induced- Ca^{2+} -release channel [2,3] and causes Ca^{2+} release from skeletal-muscle sarcoplasmic reticulum [4]. At higher doses, ryanodine can also cause channel blockade. RyRs have been cloned from skeletal muscle [5,6], cardiac muscle [7] and brain [8] (isoforms 1, 2 and 3, respectively). The drug caffeine also causes Ca^{2+} release through the RyR; thus, these two pharmacological agents (ryanodine and caffeine) are used to demonstrate the presence and study the behaviour of the RyR in muscle and other cell types.

There have been conflicting data on the presence and role of RyRs in pancreatic acinar cells. Kasai et al. [9] found Ca^{2+} -induced Ca^{2+} release in the presence of the $\text{Ins}(1,4,5)P_3$ antagonist heparin, suggesting a non- $\text{Ins}(1,4,5)P_3$ -regulated store. Another study found that acinar cells had slower-moving Ca^{2+} waves following hormone stimulation when pretreated with ryanodine and caffeine [10]. cADP-ribose, which may act through the RyR,

was shown to release intracellular Ca^{2+} when microinjected into these cells [11]. We have shown previously that palmitoyl-CoA causes Ca^{2+} release from a heparin-insensitive store, and that release is potentiated by low-dose ryanodine and caffeine [12]. These studies imply a functional non- $\text{Ins}(1,4,5)P_3$ -receptor Ca^{2+} channel with characteristics typical of RyRs. However, RyR was not identified in the pancreas by reverse transcriptase (RT)-PCR screening of pig tissues, although each of the three RyR isoforms was found in various other tissues [13]. Another study reported immunostaining for RyR in salivary gland, but failed to demonstrate it in pancreatic acinar cells [14]. On the other hand, a recent report [15] demonstrated the expression of RyR2, but not RyR1 or RyR3, in rat pancreatic acini.

To resolve these discrepancies we performed RyR-isoform-specific RT-PCR, Western-blot analysis with different antibodies and microscopic Ca^{2+} -release measurements with an activator of RyR, palmitoyl-CoA [16,17]. The results show that multiple forms of RyR are expressed in rat pancreatic acinar cells.

MATERIALS AND METHODS

Isolation of pancreatic acini and single acinar cells

Rat pancreatic acini were isolated by collagenase digestion, as described previously [18]. Single rat pancreatic acinar cells were isolated using the method developed by Bruzzone et al. [19]. For RT-PCR analysis on individually identified acinar cells, single cells were suspended in 199 medium + penicillin (100 units/ml) and streptomycin (0.1 mg/ml), viewed by phase-contrast microscopy, and drawn into a Hamilton syringe. Approx. 100 cells

Abbreviations used: RyR, ryanodine receptor; RT, reverse transcriptase; VEGF-R1, type-1 receptor for vascular-endothelial growth factor; PECAM-1, platelet endothelial cell adhesion molecule 1.

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Table 1 PCR primer sequences

mRNA	Forward primer	Reverse primer	Expected product size (bp)	GenBank accession number
RyR, consensus	NNF: 5'-CAACAACCTCTTITTIGICG-3' NYW: 5'-AACTACTGGGACAAITTTGT-3'	CFI: 5'-CCATTCACAGATGAAGCA-3'	468 741	—
RyR1	S1: 5'-GAAGGTTCTGGACAAACACGGG-3'	AS1: 5'-TCGCTCTTGTGTAGAATTGCGG-3'	435	X83932
RyR2	S2: 5'-GAATCAGTGAGTTACTGGGCATGG-3'	AS2: 5'-CTGGTCTCTGAGTTCTCCAAAAGC-3'	635	X83933
RyR3	S3: 5'-CCTTCGCTATCAATTCATCTGC-3'	AS3: 5'-TCTTCTACTGGGCTAAAGTCAAGG-3'	505	X83934
VEGF-R1 (Flt-1)	5'-CTTTCTTAGGGGGTTCTCC-3'	5'-CAGCTCAGGGTCGTCTTCATC-3'	664	D28498
PECAM-1	5'-AGGAAGCAAGGCCAAACAG-3'	5'-CATTAAAGGAGCCTCCGTTCT-3'	334	L06039

Figure 1 The expression of RyR mRNA in rat pancreatic tissue and dispersed acini

(A) Total RNA was extracted from pancreatic tissue and dispersed acini, reverse transcribed and amplified using two consensus primer pairs for the RyR (Table 1): NNF + CFI (lanes a) and NYW + CFI (lanes b). (B, C) RT-PCR products amplified with consensus primer pairs were gel-purified and subjected to restriction-digest analysis with endonucleases specific for RyR isoforms. Shown are the predicted fragment sizes (in bp) based on mouse and rat RyR sequences (see the Materials and methods section). Uncut RT-PCR product is shown in lanes 1.

were collected in this way, pelleted, washed with PBS and then RNA was extracted, reverse transcribed and amplified as described below.

Detection of mRNA for RyRs by RT-PCR

Total RNA was extracted and reverse transcribed from normal rat pancreatic tissue and from isolated acinar cells as described previously [20]. Five sets of primers were used for PCR amplification of RyR mRNAs (Table 1): the consensus primer pairs NYW + CFI or NNF + CFI and the RYR1-, RYR2- and RYR3-specific primer pairs S1 + AS1, S2 + AS2 and S3 + AS3, respectively. The primer sequences were based on mouse [21] and rat (GenBank® accession numbers AF011788, U95157, AF011789 and AF130881) cDNAs for RyR types 1, 2 and 3, and all cross at least one intron in the genomic DNA.

The primer pairs NNF + CFI and NYW + CFI were based on cDNA sequences common to all three known types of RyR. The

amplification conditions for these primers were 94 °C for 30 s, 53 °C for 1 min and 72 °C for 2 min, for 35 cycles. With the primer pairs specific for RyR isoforms, the amplification conditions were 94 °C for 45 s, 60 °C for 1 min and 72 °C for 1.5 min, for 32–36 cycles. Negative controls were performed by omitting the cDNA template from the PCR reactions or by omission of the RT step. To verify their identity, PCR products were separated on an agarose gel stained with ethidium bromide, purified with GeneClean II kit (Bio 101), and subjected to restriction-digest analysis. The restriction endonucleases used (see Figures 1 and 2) were chosen based on the mouse and rat RyR sequences as referred to above.

A potential source of contamination in the preparations of pancreatic acini and acinar cells could be remnants of endothelial cells from the pancreas. To exclude such contamination in our RNA samples, we performed similar RT-PCR analysis for two markers specific for endothelial cells: type-1 receptor (Flt-1) for vascular endothelial growth factor (VEGF-R1 [22]) and platelet

Figure 2 The expression of RyR-isoform-specific mRNAs in rat pancreatic tissue and dispersed acini

(A) Total RNA was extracted from pancreatic tissue and dispersed acini, reverse transcribed and amplified using primers specific for RyRs, 1, 2 and 3 (Table 1). The data shown for RyR3 in acini are the result of two rounds of PCR; the product of the first round was visible but faint. (B) RyR-isoform-specific RT-PCR products were gel-purified and subjected to restriction-digest analysis with endonucleases specific for each RyR isoform. Shown are the predicted fragment sizes. Uncut RT-PCR products are shown in lanes 1, 5 and 9. *, In the mouse the predicted sizes were 533 and 102 bp, see the Results section for details.

endothelial cell adhesion molecule 1 (PECAM-1 [23]). The sequences of the primers used and expected product sizes are presented in Table 1. The amplification conditions for these primers were 94 °C for 30 s, 56 °C for 45 s and 72 °C for 1.5 min.

Immunoprecipitation and Western-blot analysis

Whole-cell protein from dispersed pancreatic acini or from isolated acinar cells was extracted and immunoprecipitated as described previously [20] except that the extracts were precleared by incubation for 40 min at 4 °C with Protein A–Sepharose. Primary antibodies (mouse monoclonal antibody XA7B6 or rabbit polyclonal antibody 2149) were used at a 1:120 dilution. The final immunoprecipitate was resuspended and boiled for 3 min in Tris/glycine/SDS sample buffer containing 10% (v/v) 2-mercaptoethanol. Samples were electrophoresed on 4–20% Tris/glycine gels and transferred to poly(vinylidene difluoride) membranes in a buffer containing 12 mM Tris, 96 mM glycine (pH 8.2) and 0.01% SDS. Membranes were subjected to Western-blot analysis as described previously [20] using anti-RyR anti-

bodies (general RyR antibodies XA7B6 and 2149 or subtype-specific rabbit polyclonal antibodies 2141 and 2143 to RyR1 and RyR2, respectively) at a 1:1000 dilution. Goat anti-mouse or goat anti-rabbit horseradish peroxidase conjugates (1:1000; BioRad, Hercules, CA, U.S.A.) were used as secondary antibodies. Antibody complexes were visualized with X-ray film using chemiluminescence.

Immunocytochemistry of the RyR

Pancreatic acini were isolated as described above, rinsed in PBS, then fixed in 2% formalin for 3 min. Acini were incubated for 5 min in PBS+50 mM glycine, for 20 min in blocking buffer (PBS+50 mM glycine/1% BSA/1% gelatin/1% normal goat serum) and for 5 min in working buffer (PBS+50 mM glycine/1% BSA/1% gelatin/0.1% normal goat serum), all at room temperature. Then, the acini were incubated at 4 °C overnight in working buffer+1 mg/ml saponin with rabbit polyclonal antibody 2149 (1:1000 dilution). The acini were washed three times for 5 min with working buffer, then incubated for 2 h with secondary antibody (1:50, FITC-conjugated goat

anti-rabbit; American Qualex, San Clemente, CA, U.S.A.). Acini were pelleted, resuspended in 5 μ l of anti-fade medium, and viewed on a Carl Zeiss 310 confocal laser-scanning microscope equipped with argon ion and helium/neon lasers and a 100 $\times$ fl.3 Pan Neofluor lens. Images of control slides stained only with the secondary antibody were collected using the same contrast and brightness settings as for the experimental images.

Ca²⁺ imaging

Acinar cells were washed once and resuspended in a 1:1 mixture of Krebs–Ringer buffer with Hepes (135 mM NaCl, 12.5 mM Hepes, 5 mM NaHCO₃, 5 mM glucose, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1 mM CaCl₂, pH 7.2) and 199 medium with 0.1% BSA. Resuspended cells were plated on to poly-D-lysine-coated coverslips and allowed to attach for at least 15 min. Just prior to the experiment, the coverslips were washed with 2 ml of buffer A (100 mM KCl, 20 mM NaCl, 20 mM Hepes, 1 mM MgCl₂, 1 mM EGTA and 0.33 mM CaCl₂, pH 7.2; free Ca²⁺ $\approx$ 100 nM) and permeabilized for 5 min at room temperature in buffer A containing 0.1 units/ml streptolysin O (Murex Diagnostics) and a protease-inhibitor cocktail (5 μ g/ml each pepstatin, leupeptin, chymostatin, antipain and aprotinin). Coverslips were then incubated for 5 min at room temperature with 50 μ M C₁₈-Calcium Green in buffer B (buffer A + 3 mM ATP, 10 mM creatine phosphate, 10 units/ml creatine phosphokinase, 10 μ M oligomycin, protease-inhibitor cocktail, 0.01 mM EGTA and 3.3 μ M CaCl₂; free Ca²⁺ $\approx$ 100 nM). Coverslips were next washed with 2 ml of buffer B and mounted on to a small chamber with 0.25 ml of buffer B bathing the permeabilized cells. Confocal microscopy was performed using a Carl Zeiss LSM 410 inverted microscope equipped with an argon/neon laser and a 100 $\times$ fl.4 fluor objective. Excitation wavelength was set to 488 nm and the emission cut-off filter to > 510 nm. Calcium-release experiments were carried out at room temperature (18 °C). Palmitoyl-CoA was added as a 25 $\times$ concentrated solution in buffer B to give a concentration of 100 μ M.

Materials

BSA was from Boehringer Mannheim (Indianapolis, IN, U.S.A.); non-fat dried milk was from Bio-Rad; sample buffer, standards and precast gels were from Novex (San Diego, CA, U.S.A.); 199 medium, TRIzol reagent and Superscript II Preamplification System were from Gibco BRL (Grand Island, NY, U.S.A.); the GeneClean II kit was from Bio 101 (La Jolla, CA, U.S.A.); DNA restriction enzymes and buffers were from New England Biolabs (Beverly, MA, U.S.A.) or Boehringer Mannheim; and the Protein A–Sephadex and enhanced chemiluminescence kit were from Pierce (Rockford, IL, U.S.A.). Anti-RyR mouse monoclonal antibody XA7B6 was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-peptide rabbit polyclonal antibody 2149 recognizing all three RyR isoforms, and antibodies 2141 and 2143 specific for RyR1 or RyR2 were prepared by F. A. Lai. Secondary antibodies were from American Qualex. C₁₈-Calcium Green was from Molecular Probes (Eugene, OR, U.S.A.) and the other chemicals were from Sigma (St. Louis, MO, U.S.A.).

RESULTS

RyR mRNA expression

To determine whether RyR mRNA is present in rat pancreas and, in particular, pancreatic acinar cells, we isolated total RNA and performed RT-PCR as described in the Materials and methods section. We used both consensus primers and RyR-

isoform-specific primers. As shown in Figure 1(A), RT-PCR products of expected sizes were amplified from both pancreatic tissues and dispersed pancreatic acini with two different consensus RyR primer pairs (NNF + CFI and NYW + CFI; see Table 1). To test whether the RT-PCR products contained different RyR isoforms, both of the 'consensus' RT-PCR products were gel-purified and digested with endonucleases specific for each isoform. Figures 1(B) and 1(C) demonstrate that all three RyR isoforms were present in each of the RT-PCR products amplified with the NNF + CFI or NYW + CFI consensus primer pairs. This type of analysis, under conditions of complete digestion, gives an estimate of the relative abundance of each isoform. For example, several enzymes specific for RyR1 digested the consensus RT-PCR products to a large degree (Figures 1B and 1C), suggesting that RyR1 is the more abundant isoform in rat pancreatic acini. In contrast, digestion with endonucleases specific for RyR2 was much less pronounced. However, such an estimate is not quantitative because different sequences are amplified with differing efficiency (even with the same primers), which in itself depends on the primers used. Indeed, based on digestion with the same *Sfi*I endonuclease, RyR3 appeared less abundant in the consensus RT-PCR product obtained with the NNF + CFI primer pair (Figure 1B) than in that obtained with NYW + CFI primers (Figure 1C).

We then used primers specific for individual RyR isoforms (Table 1) to directly demonstrate mRNA expression of all three isoforms in both pancreatic tissue and dispersed acini (Figure 2A). The RT-PCR products amplified with RyR-isoform-specific primers were gel-purified and their identity verified by restriction-digest analysis. Endonucleases that cut only once in the target region were chosen, and all endonucleases, except one, generated fragments of the predicted sizes (Figure 2B). No cleavage of the RyR2-specific RT-PCR product occurred with *Eco*RI (Figure 2B, lane 6), which is predicted to yield 102 and 533 bp fragments in the mouse RyR2 sequence. Indeed, a recently submitted partial sequence of rat RyR2 (GenBank accession number U95157) reveals a point mutation that eliminated the *Eco*RI site in this species.

To demonstrate that the RyR mRNA is from the acinar cell and not from another contaminating cell type, we performed RT-PCR for RyR isoforms on individual acinar cells collected under the microscope using a microsyringe. One or two rounds of PCR with the RyR1-specific primers amplified a product of the expected size from total RNA isolated and reverse transcribed from about 100 microscopically selected acinar cells (Figure 3A). Two successive rounds of PCR were required to produce a visible product with RyR2-specific primers (Figure 3A). Cell-free medium collected from the same preparation did not give any RT-PCR product, indicating the absence of contamination with extracellular RNA.

In another control experiment, RT-PCR with primers specific for the endothelial-cell marker VEGF-R1 failed to generate any product even after two rounds of PCR on RNA obtained from the microscopically selected acinar cells (Figure 3B). These same primers did amplify a fragment of the expected size with RNA from pancreatic tissue. As compared with the whole pancreas, a very faint RT-PCR product for VEGF-R1 was obtained from dispersed pancreatic acini (Figure 3B, lanes 1 and 2). Similar results were obtained from mRNA expression of another endothelial marker, PECAM-1 (results not shown).

The data presented in Figures 1–3, and in particular the results obtained on microscopically selected cells, demonstrate that mRNAs for both RyR isoforms 1 and 2 are expressed in the pancreatic acinar cell. Our data also show the expression of RyR3 mRNA in dispersed acini.

Figure 3 RyR-isoform-specific mRNA expression in microscopically selected rat pancreatic acinar cells

(A) RT-PCR of RyR1 and RyR2 using RNA from individually collected acinar cells identified under the microscope. In each gel, lanes 1 and 2 represent the first and second successive rounds of PCR, respectively. Results shown are representative of three experiments on different cell preparations, which all gave identical results. (B) RT-PCR of the endothelial marker VEGF-R1 showing the absence of contamination in the preparation of microscopically selected pancreatic acinar cells. Lane 1, pancreatic tissue; lane 2, dispersed pancreatic acini; lanes 3 and 4, microscopically selected acinar cells, two successive rounds of PCR.

Figure 4 Western blot of the RyR in pancreatic acinar cells

(A) RyR was immunoprecipitated from dispersed pancreatic acini with a general anti-RyR antibody (2149; left-hand lane) or with the antibody preabsorbed with the immunizing RyR peptide (right-hand lane). Proteins were separated on 4–20% Tris/glycine gels, blotted on to poly(vinylidene difluoride) membrane, and probed with a second anti-RyR antibody (XA7B6). (B) RyR immunoblot using isoform-specific antibodies; anti-RyR1 (2141, left-hand lane) and anti-RyR2 (2143, right-hand lane). (C) The specificity of the RyR1-specific antibody 2141 was confirmed in immunoblots on skeletal- versus cardiac-muscle sarcoplasmic reticulum. The data in panels A–C represent the entire blot.

Western-blot analysis of the RyR

To determine the presence of the RyR protein in isolated acini, we used immunoprecipitation followed by Western-blot analysis. Figure 4(A) shows that a general anti-RyR antibody (2149) immunoprecipitates a protein with high molecular mass (> 250 kDa). This band was much less intense when immunoprecipitated in the presence of competing peptide to which the antibody was raised (Figure 4A). The immunoblot was performed with a second RyR antibody (XA7B6) as a further confirmation

Figure 5 Immunocytochemistry of the RyR in pancreatic acinar cells

Confocal fluorescent image (A) and DIC (differential interference contrast) image (B) of dispersed pancreatic acini stained with secondary antibody only. Fluorescent image (C) and corresponding DIC image (D) of acini stained with anti-RyR antibody 2149. Staining with anti-RyR antibody shows diffuse 'reticular' staining over most of the cytoplasmic surface. Bars indicate 10 μ m.

of the band's identity. Immunoblotting with isoform-specific antibodies for RyR1 (2141) and RyR2 (2143) showed that both types are present in pancreatic acini (Figure 4B). We verified the specificity of 2141 anti-RyR antibody by showing (Figure 4C) that it cross-reacted with sarcoplasmic reticulum from skeletal (RyR1-containing) but not cardiac (not containing RyR1) muscle.

Thus the high-molecular-mass protein band in Figures 4(A) and 4(B) was immunoprecipitated or stained by four different anti-RyR antibodies. The lower-molecular-mass bands are most likely degradation products as seen in other reports [24]. Using the isoform-specific antibodies, we also immunoprecipitated RyR from smooth and rough endoplasmic reticulum fractions that represent agonist-sensitive Ca^{2+} stores [25]. These results showed that both RyR1 and RyR2 are present in all fractions of the endoplasmic reticulum (not shown).

Immunocytochemistry of the RyR

To localize the RyR we stained isolated acini with the general anti-RyR antibody 2149 that demonstrated a specific band in Western blotting. The pattern of staining was reticular and occurred throughout most of the cell cytoplasm, but excluded the nucleus (Figure 5C). Staining with the secondary antibody alone was much less intense, demonstrating specificity (Figure 5A). Similar results were obtained with the mouse monoclonal antibody XA7B6 (results not shown).

Localization of Ca^{2+} release

To localize the functional response of the RyR in pancreatic acini, we measured Ca^{2+} changes with confocal microscopy in permeabilized acinar cells. The cells were loaded with the Ca^{2+} -indicator dye, C_{18} -Calcium Green, which partitions into membranes, thus providing a Ca^{2+} measurement in localized areas

Figure 6 Ca^{2+} release from the acinar cell

Fluorescence of C_{18} -Calcium Green in permeabilized pancreatic acinar cells at the time (0 s) of 100 μ M palmitoyl-CoA (PCoA) addition (A), and at 1.7-s intervals after addition (B–E). The apical (a) and basolateral (b) regions of the individual acinar cells are clearly visible. For unknown reasons C_{18} -Calcium Green failed to stain the middle portion of these cells. (F) Fluorescence (arbitrary units) against time tracings of boxed regions of the cell in (A). Results are representative of five similar experiments.

within the cell [26,27]. We used palmitoyl-CoA (an activator of Ca^{2+} release from the RyR [16,17]) to activate Ca^{2+} release through RyR. We found Ca^{2+} release throughout the acinar cell (Figure 6). This release can be seen in the basolateral areas simultaneously or immediately prior to Ca^{2+} release in the apical region (one of five similar experiments).

DISCUSSION

The present study (preliminary results of which have been reported in abstract form [28]) demonstrates that mRNAs for all three known RyR isoforms are expressed in pancreatic acinar cells. We found that the levels of RyR-isoform-specific mRNA expression were similar in dispersed acini and pancreatic tissue (Figure 2A). Moreover, both RyR1 and RyR2 RT-PCR products were amplified from microscopically selected individual acinar cells, with RyR1 product being abundant even after the first round of PCR. In contrast, RT-PCR products for endothelial-cell-specific mRNAs, a potential contaminating source, decreased and finally disappeared as the proportion of acinar cells increased within each of three preparations; pancreatic tissue $\rightarrow$ dispersed acini $\rightarrow$ microscopically selected acinar cells.

Western-blot analysis, using antibodies common for all RyRs and those specific for isoforms 1 and 2, demonstrated that RyR1 and RyR2 proteins are expressed in pancreatic acinar cells. We also showed the presence of isoforms 1 and 2 in subcellular fractions of both rough and smooth endoplasmic reticulum. Stimulation of permeabilized acinar cells with palmitoyl-CoA, an activator of RyR, caused Ca^{2+} release throughout the cell.

The failure of one of the previous PCR studies [13] to detect RyR in pancreas could be due to a poor cDNA library. Our initial attempts failed to amplify RyR products from a commercially available cDNA library. We cannot know exactly why the previous immunohistochemistry studies [14] failed, but it could be due to the different antibodies used. Immuno-

precipitation and Western blot with one of the antibodies (from Calbiochem) used in that study failed to find any protein (results not shown). With respect to RyR2, our observations are in agreement with those of Leite et al. [15]. However, in contrast to our findings, that study failed to detect RyR1 or RyR3 by RT-PCR in pancreatic acini. This could be due to the strategy employed by the authors that did not use RyR-isoform-specific primers, but instead relied on restriction-enzyme digestion of the PCR product generated by degenerate primers common to all three RyR isoforms. Only one restriction endonuclease was used to identify each RyR isoform. If the degenerate primers used in [15] preferentially amplified RyR2 sequences, or if the sites for the one restriction endonuclease used to identify RyR1 or RyR3 were mutated within the rat PCR product (as we found with *EcoRI* in Figure 2B), this type of analysis would give a false negative result. Of note is that the one restriction endonuclease used by Leite et al. to identify RyR2 digested only $\sim 50\%$ of the consensus PCR product (see Figure 2 in [15]), suggesting the presence of other RyR isoforms.

There is conflicting functional evidence for RyR in the pancreatic acinar cell. Ryanodine, caffeine and cADP-ribose have been used on the acinar cell, but these pharmacological agents have had mixed results. Schmid et al. [29], using a patch clamp of an endoplasmic reticulum Ca^{2+} channel, found an increased probability of the channel being open with caffeine (10 mM), but no effect of ryanodine (10 μ M or 300 μ M). Dehlinger-Kremer et al. [30] also found no effect with ryanodine, but 20 mM caffeine induced a slow Ca^{2+} release. Wakui et al. [31] found that 1 mM caffeine caused a Ca^{2+} increase, but 20 mM had no effect. Thorn et al. [11] found that 10 mM caffeine increased Ca^{2+} , and that ryanodine could both potentiate and block Ca^{2+} release. In our hands, ryanodine and caffeine added to permeabilized acini had little effect on their own, but potentiated the effect of palmitoyl-CoA on Ca^{2+} release [12]. On the other hand, we found that cADP-ribose had little effect on Ca^{2+}

release, in contrast to the study by Thorn et al. [11]. The discrepancies mentioned above could be due to differences in methods of cell preparation or the presence or absence of various endogenous modulators.

In the present study, we show that multiple RyR isoforms are present in pancreatic acinar cells. RyR1 appears to be more highly expressed at the mRNA level than the other two isoforms. Multiple RyR isoforms are frequently co-expressed in different cell types [32]; however, the functional significance of multiple isoform expression remains largely unknown. One possibility is that co-expression of different isoforms serves to amplify Ca^{2+} release mediated by another RyR isoform. For example, RyR3, which is expressed at a very low level in skeletal muscle compared with RyR1, has been shown to play an important role in muscle contraction in neonatal mice [33]. In the pancreatic acinar cell, amplification of intracellular Ca^{2+} release by interaction with multiple RyR isoforms could be important in digestive enzyme secretion. Ito et al. [34] found that micromolar spikes of intracellular $[\text{Ca}^{2+}]$ were necessary for inducing exocytosis of zymogen granules. Our previous results suggest that Ca^{2+} stores gated by RyRs account for more than 50 % of the total releasable intracellular Ca^{2+} [12]. Therefore, multiple RyR isoforms in the pancreatic acinar cell may serve to amplify agonist-mediated Ca^{2+} release and raise intracellular $[\text{Ca}^{2+}]$ high enough and fast enough to cause the secretory response.

This work was supported by the Department of Veterans Affairs and Public Health Service grant NIDDK DK 33010. This article is partial fulfilment of a Ph.D. degree for T.J.F. in the Biomedical Sciences Graduate Program from the University of California at San Diego.

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