# Expression and subcellular localization of the ryanodine receptor in rat pancreatic acinar cells

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The ryanodine receptor (RyR) is the principal $Ca^{2+}$-release channel in excitable cells, whereas the inositol 1,4,5-trisphosphate ($Ins P_3$) receptor ($Ins P_3R$) is primarily responsible for $Ca^{2+}$ release in non-excitable cells, including epithelia. RyR also is expressed in a number of non-excitable cell types, but is thought to serve as an auxiliary or alternative $Ca^{2+}$-release pathway in those cells. Here we use reverse transcription PCR to show that a polarized epithelium, the pancreatic acinar cell, expresses the type 2, but not the type 1 or 3, isoform of RyR. We furthermore use immunochemistry to demonstrate that the type 2 RyR is distributed throughout the basolateral and, to a lesser extent, the apical region of the acinar cell, but is excluded from the trigger zone, where cytosolic $Ca^{2+}$ signals originate in this cell type. Since propagation of $Ca^{2+}$ waves in acinar cells is sensitive to ryanodine, caffeine and $Ca^{2+}$, these findings suggest that $Ca^{2+}$ waves in this cell type result from the co-ordinated release of $Ca^{2+}$, first from $Ins P_3Rs$ in the trigger zone, then from RyRs elsewhere in the cell. RyR may play a fundamental role in $Ca^{2+}$ signalling in polarized epithelia, including for $Ca^{2+}$ signals initiated by $Ins P_3$.

Key words: calcium, calcium-release channels, cyclic ADP-ribose, inositol trisphosphate.

## INTRODUCTION

Cytosolic $Ca^{2+}$ ($Ca_i^{2+}$) regulates a wide range of cell functions [1,2] and plays a particularly important role in controlling secretion in polarized epithelia [3]. Spatial patterns of $Ca_i^{2+}$, including polarized $Ca_i^{2+}$ waves and other types of $Ca_i^{2+}$ gradients, may be essential for the proper regulation of epithelial secretion [4]. Since the effective range of diffusion of $Ca^{2+}$ is highly limited in cytosol [5], these spatial patterns of $Ca_i^{2+}$ signals likely depend upon the subcellular distribution of $Ca_i^{2+}$-release channels. The two major channels that regulate release of $Ca^{2+}$ from intracellular stores are the ryanodine receptor (RyR) and the $Ins(1,4,5)P_3$ ($Ins P_3$) receptor ($Ins P_3R$) [1]. $Ins P_3R$ is thought to be primarily responsible for $Ca_i^{2+}$ signals in epithelia [1], since increases in $Ca_i^{2+}$ originate at the site of this receptor [6–8] and since $Ins P_3R$ antagonists block $Ca_i^{2+}$ signalling in these cells [9,10]. However, $Ins P_3R$ is restricted to the extreme apex in epithelial cells [6–8], which suggests that RyR may be expressed in other regions of these cells and supports propagation of $Ca_i^{2+}$ waves to those regions. The goal of the present study was to determine the expression and subcellular location of RyR in pancreatic acinar cells. RyR was localized in acinar cells because other features of $Ca_i^{2+}$ signalling have been characterized extensively in this model polarized epithelium [4,6,9–12].

## MATERIALS AND METHODS

### Reverse transcription (RT) and PCR amplification

Rat pancreatic acini were prepared as described in [6], and total RNA was isolated by standard methods [13,14]. A 1 $\mu$g sample of RNA was used in 20 $\mu$l of cDNA-synthesis reaction mixture. The reaction was carried out using an RNA PCR kit (Perkin Elmer, Indianapolis, IN, U.S.A.). First-strand cDNA was synthesized using oligo-$d(T)_{16}$ and Moloney-murine-leukaemia-virus reverse transcriptase. Two control cDNA reactions were carried out. In one, RNA, but no reverse transcriptase, was added (RNA control), while in the other no RNA was added (DNA control). PCR amplification was performed in a PTC-100 automated thermocycler (MJ Research, Watertown, MA, U.S.A.) using 2 $\mu$l of the first-strand cDNA reaction, 150 pmol of each degenerate primer, 50 $\mu$M dNTPs, 2.5 units of AmpliTaq DNA polymerase in 10 mM Tris/HCl, pH 8.3, containing 50 mM KCl and 2.5 mM $MgCl_2$ in a total volume of 100 $\mu$l. After a hot start (2 min at 94 °C), the samples were subjected to 30 cycles of 45 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C. This was followed by a final extension at 72 °C for 10 min. The degenerate primers were designed to amplify a 530 bp product from the 3′ region of all three known RyRs [15–17]. The forward primer was 5′-CA(C/T)(C/T)T-(A/C/G/T)(C/T)T(A/C/G/T)GA(C/T)AT(A/C/T)GCIATG-GG-3′ and the reverse primer was A(A/G/T)(A/G)TA(A/G)-TT(A/C/G/T)GCIA(A/G)(A/G)TT(A/G)TG(C/T)-TC-3′. The resulting PCR product was analysed by agarose-gel electrophoresis.

### Restriction-enzyme analysis of the RT-PCR product

The restriction enzymes *Sac*I, *Bgl*II and *Eae*I were used to determine which RyR isoforms were amplified [16,17]. *Sac*I only cuts type 1 RyR (RyR-1), into fragments of $\approx$ 395 bp and 135 bp, *Bgl*II cuts only type 2 RyR (RyR-2), into

Abbreviations used: RyR, ryanodine receptor; RyR-1, type 1 ryanodine receptor (etc.); $Ins P_3$, $Ins(1,4,5)P_3$; $Ins P_3R$, $Ins P_3$ receptor; $Ca_i^{2+}$, cytosolic $Ca^{2+}$; RT, reverse-transcription; cADPR, cyclic ADP-ribose.

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fragments of ≈ 295 bp and 235 bp, whereas *Eae*I cuts RyR-3 only, into 350 bp and 180 bp fragments. Digestion products were analysed on a 2 %-agarose gel.

### Cloning and sequencing of RT-PCR products

The PCR products were separated electrophoretically on a 1.5 %-agarose gel and revealed under UV light. A single band, of predicted size, was cut out of the gel and purified using a DNA purification kit (American Bioanalytical, Natick, MA, U.S.A.). The purified DNA was cloned into a TA cloning vector (In-Vitrogen, San Diego, CA, U.S.A.), according to the manufacturer's instructions. DNA from 20 colonies was purified, and subjected to restriction-enzyme analysis with *Eco*RI to identify positive clones. The clone containing an insert of the expected size was sequenced in both directions and aligned with previously published RyR sequences to determine which isoform was present.

### Western-blot analysis

Immunoblotting was performed to demonstrate protein expression of RyR-2 in the acinar cells, to confirm lack of expression of RyR-1 and RyR-3 in the acinar cells, and to confirm specificity of immunochemical labelling for RyR-2 in pancreas. Immunoblotting of acinar-cell and cardiac lysates was performed using mouse monoclonal antibody C3-33 directed against the canine cardiac RyR-2 [18]. Immunoblotting of acinar-cell and diaphragmatic lysates was performed using mouse monoclonal antibody XA7 B6, directed against the rabbit skeletal-muscle RyR-1 [19], or using an affinity-purified polyclonal antibody directed against a peptide sequence specific to RyR-3 [20]. Diaphragm was used as a positive control for both RyR-1 and RyR-3, since that tissue contains both of these isoforms [21]. Acinar cells and cardiac and diaphragmatic lysates were diluted in Laemmli reducing sample buffer [22], and boiled for 5 min. The samples were then electrophoresed through a SDS/polyacrylamide 4–20 % gradient gel, electrophoretically transferred on to an Immobilon polyvinylidene membrane, and incubated with anti-RyR antibody. Immunoreactive bands were detected by incubation with protein A–horseradish peroxidase conjugate followed by revelation with enhanced chemiluminescence (ECL®, Amersham) reagents.

### Confocal immunofluorescence histochemistry

To determine the subcellular distribution of RyR, sections of rat pancreas were labelled with anti-RyR-2 antibody C3-33 [18]. Specimens were co-labelled with Texas Red–phalloidin, because this stain facilitates identification of the apical and basolateral poles of pancreatic acinar cells [23]. Negative controls for RyR labelling were stained with secondary antibodies alone, along with Texas Red–phalloidin. Sections of rat heart were fixed and labelled with anti-RyR-2 antibodies as a positive control, since the immunohistochemistry of RyR-2 in myocardium has been well described [18]. Separate sections of rat pancreas were labelled with a mouse monoclonal antibody directed against the N-terminal end of the human type 3 Ins$P_3$R (Transduction Laboratories, Lexington, KY, U.S.A.), to compare the distribution of Ins$P_3$R-3 with that of RyR-2.

Immunochemistry was performed on 4-$\mu$m-thick frozen sections of rat pancreas. For RyR-2 staining, tissue was fixed by perfusion with 2 % (w/v) paraformaldehyde in 0.075 M sodium phosphate, pH 7.3, cryopreserved overnight in 15 % (w/v) sucrose, and frozen in isopentane/liquid nitrogen. After quenching with 50 mM $NH_4Cl$ and 3 % (v/v) goat serum in PBS, the sections were labelled with a 1 : 5 dilution of anti-RyR-2 antibody overnight, then washed and incubated with Alexa 488 (a fluorescent dye)-conjugated anti-mouse secondary antibodies (Molecular Probes, Eugene, OR, U.S.A.), along with Texas Red-conjugated phalloidin [23,24]. Negative controls were stained with Alexa 488-conjugated anti-mouse secondary antibodies alone, along with Texas Red-conjugated phalloidin. Perfusion fixation plus all washes and incubations included pepstatin (2 $\mu$M), PMSF (0.2 mM),and benzamidine (0.5 mM) to inhibit proteolysis [6]. Specimens were examined with a Bio-Rad MRC-600 confocal microscope equipped with a krypton/argon mixed-gas laser [6]. To ensure specificity of RyR-2 staining, images were obtained using confocal machine settings (i.e., aperture, gain and black level) at which no fluorescence was detectable in negative control samples labelled with secondary antibody alone. Double-labelled specimens were serially excited at 488 nm and observed at $>$ 515 nm to detect Alexa 488, then excited at 568 nm and observed at $>$ 585 nm to detect Texas Red. This approach eliminated bleed-through of Alexa 488 fluorescence into the Texas Red channel [6].

In order to localize the type 3 Ins$P_3$R [6], pancreas was cryopreserved in sucrose, then fixed in ice-cold acetone for 10 min, blocked with 1 % BSA, labelled with a 1 : 100 dilution of the mouse monoclonal anti-Ins$P_3$R-3 antibody, and counterstained with Alexa 488-conjugated anti-mouse secondary antibodies. Labelled tissue was examined by confocal fluorescence microscopy as described above.

Immunochemistry was performed on 8-$\mu$m-thick sections of cardiac tissue, which were frozen unfixed, then fixed with ice-cold methanol for 10 min immediately prior to staining. The sections were blocked with PBS solution containing 1 % BSA, stained with the anti-RyR-2 antibody, counterstained with FITC-goat anti-mouse secondary antibody, and examined by confocal fluorescence microscopy as described above.

## RESULTS

### Expression of RyR in pancreatic acinar cells

RT-PCR was used to amplify RyR cDNA from rat pancreatic-acinar-cell RNA using degenerate primers from two sequences common to each of the three known RyR isoforms [15,16]. A single 530 bp PCR product was identified (Figure 1), which was then digested using the restriction enzymes *Sac*I, *Bgl*II and *Eae*I to look for RyR-1, RyR-2 and RyR-3 respectively [16]. *Bgl*II digestion resulted in two fragments, whereas *Sac*I and *Eae*I had

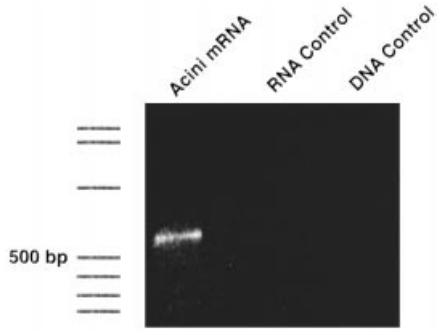


**Figure 1 RT-PCR detection of RyR in pancreatic acinar cells**

Using isoform-specific primers, a single 530 bp product was identified in pancreatic acinar mRNA, but not in RNA- or DNA-negative controls.

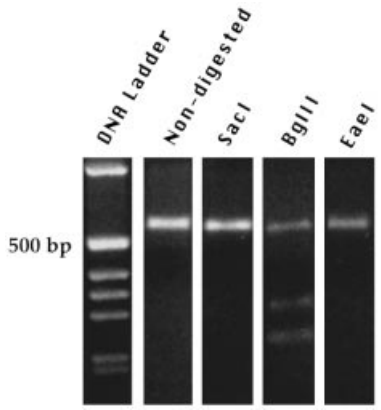


**Figure 2 Restriction-enzyme analysis of RyR RT-PCR product in pancreatic acinar cells**

The 530 bp PCR product shown in Figure 1 was digested using *Sac*I, *Bgl*II, and *Eae*I to detect RyR-1, RyR-2 and RyR-3 respectively. *Bgl*II digestion revealed two fragments, whereas *Sac*I and *Eae*I had no effect.

no effect on the PCR product (Figure 2). The original 530 bp PCR product was then sequenced and found to be 94% identical with RyR-2 of *Mus musculus* (house mouse) (Figure 3) and 88% identical with rabbit RyR-2. These sequencing data demonstrate that the small amount of PCR product remaining after *Bgl*II digestion (Figure 2) was due to incomplete digestion rather than to RyR-1 or RyR-3 that was present as well. Thus these findings show that pancreatic acinar cells express the RyR-2, but not RyR-1 or RyR-3.

### Detection of the RyR-2 in pancreas by Western blotting.

Immunoblot analysis was used to test for the presence of RyR isoforms in rat pancreatic lysates (Figure 4). Blots probed with anti-RyR-2 antibody C3-33 showed a single band of immunoreactivity with an apparent molecular mass of ≈ 565 kDa (Figure 4A). A single band of immunoreactivity with the same molecular mass was identified in lysates from rat heart (Figure 4A), which

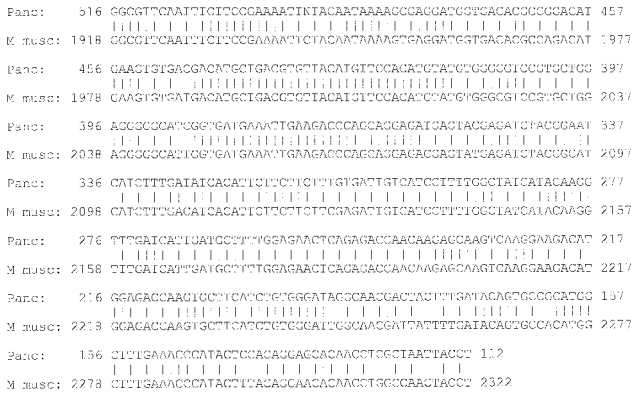


**Figure 3 The RT-PCR product of pancreatic acinar cells shows high sequence similarity to RyR-2**

The sequence of the RT-PCR product (Panc) was determined and compared with the sequence of the corresponding portion of RyR-2 message of *M. musculus* (M musc). The two sequences are 94% identical at the nucleotide level.

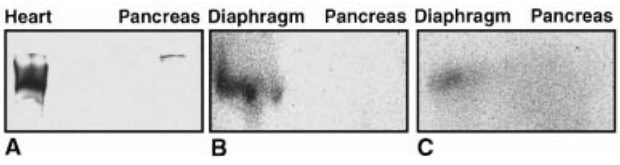


**Figure 4 Pancreatic acinar cells express RyR-2, but not RyR-1 or RyR-3 protein by immunoblot**

(**A**) Western analysis using RyR-2 monoclonal antibody C3-33 identifies a single ≈ 565 kDa band in lysates from both heart and pancreas. Expression in heart is intense, as expected. (**B**) Western analysis using anti-RyR-1 monoclonal antibody XA7 B6 identifies a single band in lysate from diaphragm (which expresses both RyR-1 and RyR-3), but not from pancreatic lysate. (**C**) Similarly, Western analysis using an affinity-purified polyclonal antibody directed against RyR-3 identifies a single band in lysate from diaphragm but not from pancreas.

is known to heavily express RyR-2. In contrast, blots probed with antibody directed against RyR-1 (Figure 4B) or RyR-3 (Figure 4C) showed no immunoreactivity. These results demonstrate that the RyR-2, but not RyR-1 or RyR-3, is expressed in rat pancreas, and confirm the specificity of antibody C3-33 for labelling RyR-2.

### Subcellular localization of the RyR-2 by confocal microscopy.

To localize RyR within pancreatic acinar cells, slices of rat pancreas were double-labelled with Texas Red-conjugated phalloidin and with anti-RyR-2 antibody C3-33, and then detected with fluorescently labelled secondary antibodies (Figures 5A–5C). The extreme apex of acinar cells, where the trigger zone is located [6–8], was identified by intense phalloidin labelling of actin along the apical membrane (Figure 5A), as previously reported [23]. RyR-2 staining labelled acinar cells much more diffusely (Figure 5B). Double-label images revealed that RyR-2 staining was largely in the basolateral region of the acinar cells and was specifically excluded from the extreme apical region of these cells (Figure 5C). Slices of rat heart stained with anti-RyR-2 antibody (Figure 5D) revealed an intracellular pattern that corresponds to the sarcoplasmic reticulum, along which RyR-2 is known to be distributed in myocytes [18]. To compare the distribution of RyR-2 and Ins$P_3$R more directly, separate slices of rat pancreas were labelled with an antibody directed against type 3 Ins$P_3$R (Figure 5E). In contrast with RyR-2 staining, that of Ins$P_3$R was localized to the extreme apex, in a pattern very similar to that of Texas Red–phalloidin and consistent with previous reports [6–8]. These results confirm that pancreatic acinar cells express RyR-2 and demonstrate that the receptor is distributed throughout the basolateral, and to a lesser extent the apical, region in such cells, but is excluded from the region of Ins$P_3$R.

## DISCUSSION

Here we describe the expression and subcellular location of RyR-2 in pancreatic acinar cells. The central role of RyR in $Ca_i^{2+}$ signalling in muscle and nerve cells is well known [15,25,26]. More recently, RyR expression has been described in a number of non-excitable cell types as well [15,16]. Expression of the various RyR isoforms has been described in several types of epithelia in particular, including RyR-2 in kidney cells and cell lines [27], RyR-2 and RyR-3 in the T84 colonic cell line [17], and RyR-1 and RyR-3 in parotid acinar cells [28,29]. Studies that show [$^3$H]ryanodine binding to microsomes and effects of ryanodine on $Ca_i^{2+}$ signalling in these and other epithelia suggest

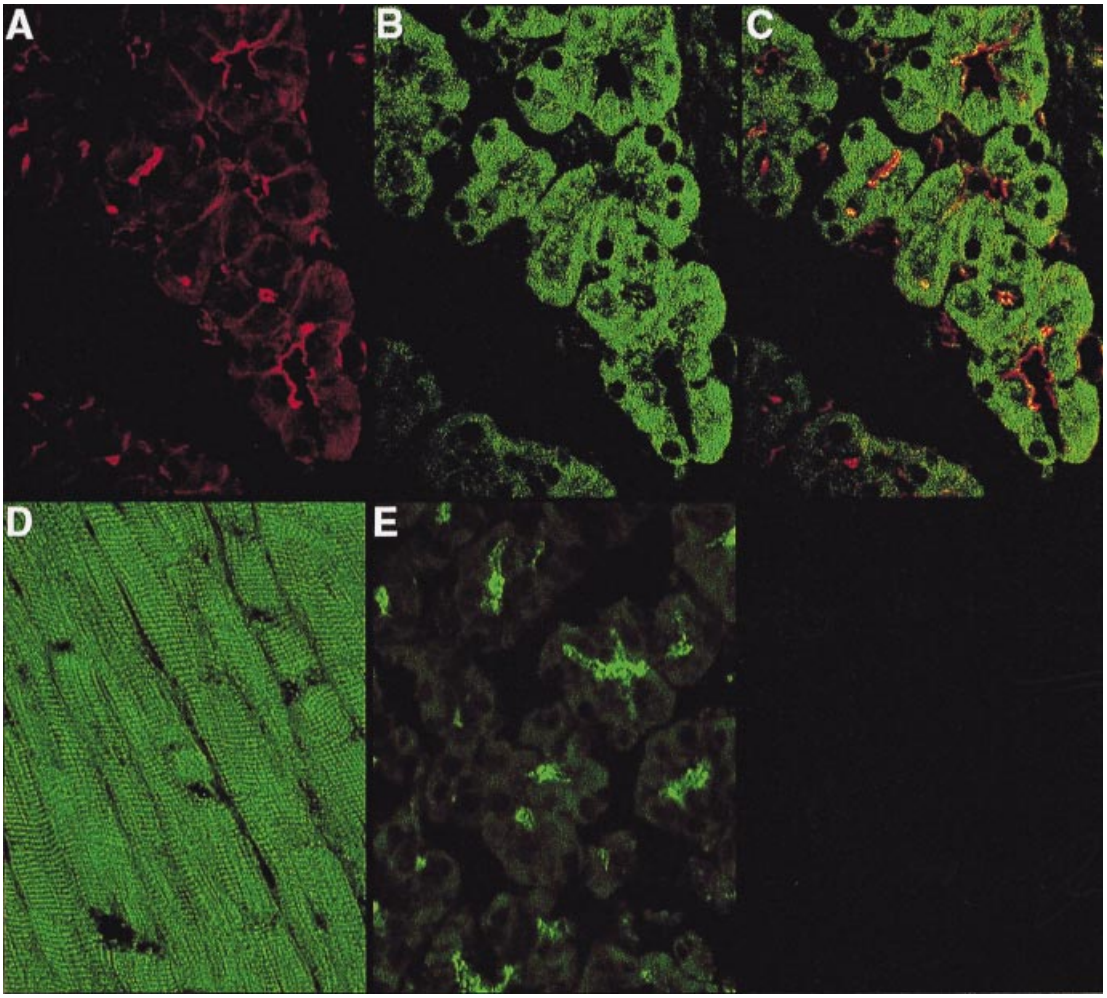


**Figure 5 Comparison of the subcellular localization of the type 2 RyR and the type 3 Ins$P_3$R in pancreas, as determined by confocal immunofluorescence microscopy**

(**A**) Texas Red–phalloidin labelling of pancreas identifies the plasma membranes of the acinar cells. Labelling is most intense along the apical membranes, the site of the trigger zone where Ins$P_3$R is most concentrated. (**B**) Distribution of RyR-2, labelled with anti-RyR-2 antibody C3-33. (**C**) Superimposed images (**A**) and (**B**), demonstrating the polarized distribution of RyR-2 in acinar cells. Receptor labelling is diffusely distributed, but is most concentrated in the basolateral region and is excluded from the trigger zone. (**D**) Staining of cardiac tissue using anti-RyR-2 antibody C3-33. Labelling of the sarcoplasmic reticulum by RyR antibody is evident. (**E**) Distribution of the type 3 Ins$P_3$R in a separate section of pancreas, labelled with a monoclonal antibody directed against the N-terminal region of the human Ins$P_3$R-3. In contrast with RyR-2, Ins$P_3$R-3 is concentrated in the trigger zone, similar to the distribution of Texas Red–phalloidin.

a functional role for epithelial RyRs [17,27–29]. However, the precise role of RyR in epithelial $Ca_i^{2+}$ signalling has not been completely defined, in part because the subcellular location of this receptor in such cells was unknown. Recently, fluorescently labelled ryanodine was used to observe that ryanodine binding occurs diffusely in the cytosol of parotid acinar cells [29]. However, the polarity of the distribution of the fluorescent tag was not examined. In addition, ryanodine-binding sites do not always reflect the location of RyR itself, as evidenced by the fact that hepatocyte microsomes contain ryanodine-binding sites [30,31], yet hepatocytes do not express RyR [15]. In another recent study, immunochemistry was used to examine RyR expression in pancreatic acini, but no labelling was detected [8]. This negative finding could in part reflect the fact that anti-RyR antibodies used in that study were not specific for RyR-2 [8]. Here, molecular techniques were used to demonstrate the presence of RyR-2 message in pancreatic acinar cells, and immunoblotting was used to demonstrate both RyR-2 protein expression in the acinar cells and the specificity of the particular anti-RyR-2 antibody. Immunochemistry was then used and it showed that RyR-2 is expressed in a polarized fashion in pancreatic acinar cells: it is diffusely distributed across the basolateral region and excluded from the extreme apex.

The current findings have important implications for the mechanism of $Ca_i^{2+}$ wave propagation in pancreatic acinar cells. Agonist-induced $Ca_i^{2+}$ signals in these cells begin in a highly localized region in the apex which has been called the 'trigger zone' [10]. Increases in $Ca_i^{2+}$ then may either remain as localized apical $Ca_i^{2+}$ pulses, or else spread as a non-diminishing $Ca_i^{2+}$ wave across the remainder of the cell [9–12]. Initial increases in $Ca_i^{2+}$ are mediated by Ins$P_3$ [9,10], and Ins$P_3$R is localized to the region of the trigger zone [6–8], so this distribution of the receptor likely accounts for the polarized fashion in which $Ca_i^{2+}$ signals are initiated in this cell type. $Ca_i^{2+}$ waves then spread

from the trigger zone in a manner that is sensitive to ryanodine and caffeine, both of which are known to modulate RyR activity [32]. In addition, direct microinjection of $Ca^{2+}$ into pancreatic acinar cells causes $Ca^{2+}$-induced $Ca^{2+}$ release, which originates in a region distinct from the trigger zone [10]. Finally, increases in $Ca_i^{2+}$ in pancreatic acinar cells can be initiated by the RyR agonist cyclic ADP-ribose (cADPR), and these cADPR-induced $Ca_i^{2+}$ signals are sensitive to ryanodine [33]. Together, these findings suggest that the spread of $Ca_i^{2+}$ waves away from the trigger zone is mediated by $Ca^{2+}$ release from RyR. The present study provides direct support for this hypothesis by demonstrating that RyR is present in pancreatic acinar cells, and that this receptor is distributed in regions of the acinar cell that would account for the pattern of $Ca_i^{2+}$ waves that is experimentally observed. In addition, RyR present in pancreatic acinar cells is the type 2 isoform, and this isoform is thought to be sensitive to cADPR [34,35], which also is consistent with previous observations in acinar cells [33].

These findings may have more general implications for cellular $Ca_i^{2+}$ signalling mechanisms as well. RyR is of primary importance for $Ca^{2+}$ release in excitable cells [26,36,37], while Ins$P_3$R had been thought to govern $Ca^{2+}$ release in non-excitable cells [1]. However, Ins$P_3$R also is expressed in excitable cells [38,39], while RyR also is expressed in non-excitable cells [15,16]. Until now, an independent and somewhat limited role had been suggested for each of these receptors in cells in which they are not thought to be of primary importance for $Ca_i^{2+}$ signalling. For example, in cardiac myocytes, RyR is found along sarcoplasmic reticulum throughout the cell, and $Ca_i^{2+}$ sparks in the cytosol trigger $Ca^{2+}$-induced $Ca^{2+}$ release via RyR, inducing excitation–contraction coupling [37]. In contrast, Ins$P_3$R is concentrated at the intercalated discs [38] and is thought to represent a back-up mechanism for initiating $Ca_i^{2+}$ signals in disease states in which RyR function may be impaired [40]. In sea-urchin eggs, both Ins$P_3$R and RyR are distributed throughout the cell, and either Ins$P_3$ or cADPR can induce transcellular $Ca_i^{2+}$ waves [41]. In those cells, it is postulated that the two receptors regulate redundant signalling pathways [41]. In lacrimal acinar cells, both acetylcholine and phenylephrine increase $Ca_i^{2+}$, but stimulation of muscarinic receptors increases $Ca_i^{2+}$ via Ins$P_3$ and its receptor, while $\alpha$-adrenergic stimulation instead increases $Ca_i^{2+}$ via cADPR and RyR [42,43]. These disparate examples each demonstrate the assumption that Ins$P_3$R and RyR are activated by independent, albeit alternative, signalling pathways. However, in light of previous findings in acinar cells [10,32,33], the present study provides evidence that Ins$P_3$R-mediated and RyR-mediated $Ca^{2+}$ release instead can be activated in an integrated, co-ordinated, and sequential fashion. Further work will be needed to demonstrate more directly that RyR and Ins$P_3$R interact in order to organize the subcellular pattern of $Ca_i^{2+}$ signalling in pancreatic acini and in other types of cells.

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