Calcium Wave Propagation in Pancreatic Acinar Cells

Functional Interaction of Inositol 1,4,5-Trisphosphate Receptors, Ryanodine Receptors, and Mitochondria

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ABSTRACT In pancreatic acinar cells, inositol 1,4,5-trisphosphate (InsP3)–dependent cytosolic calcium ([Ca2+]i) increases resulting from agonist stimulation are initiated in an apical “trigger zone,” where the vast majority of InsP3 receptors (InsP3R) are localized. At threshold stimulation, [Ca2+]i signals are confined to this region, whereas at concentrations of agonists that optimally evoke secretion, a global Ca2+ wave results. Simple diffusion of Ca2+ from the trigger zone is unlikely to account for a global [Ca2+]i elevation. Furthermore, mitochondrial import has been reported to limit Ca2+ diffusion from the trigger zone. As such, there is no consensus as to how local [Ca2+]i signals become global responses. This study therefore investigated the mechanism responsible for these events. Agonist-evoked [Ca2+]i oscillations were converted to sustained [Ca2+]i increases after inhibition of mitochondrial Ca2+ import. These [Ca2+]i increases were dependent on Ca2+ release from the endoplasmic reticulum and were blocked by 100 μM ryanodine. Similarly, “uncaging” of physiological [Ca2+]i levels in whole-cell patch-clamped cells resulted in rapid activation of a Ca2+-activated current, the recovery of which was prolonged by inhibition of mitochondrial import. This effect was also abolished by ryanodine receptor (RyR) blockade. Photolysis of n-myo InsP3, P1(5)-1-(2-nitrophenyl)-ethyl ester (caged InsP3) produced either apically localized or global [Ca2+]i increases in a dose-dependent manner, as visualized by digital imaging. Mitochondrial inhibition permitted apically localized increases to propagate throughout the cell as a wave, but this propagation was inhibited by ryanodine and was not seen for minimal control responses resembling [Ca2+]i puffs. Global [Ca2+]i rises initiated by InsP3 were also reduced by ryanodine, limiting the increase to a region slightly larger than the trigger zone. These data suggest that, while Ca2+ release is initially triggered through InsP3R, release by RyRs is the dominant mechanism for propagating global waves. In addition, mitochondrial Ca2+ import controls the spread of Ca2+ throughout acinar cells by modulating RyR activation.

KEY WORDS: calcium dynamics • intracellular signaling • exocrine cells • flash photolysis • digital imaging

INTRODUCTION

Ca2+ release from inositol 1,4,5-trisphosphate (InsP3)–sensitive stores exerts control over a wide variety of physiological processes including secretion, gene transcription, and apoptosis (Berridge, 1993; Clapham, 1995). InsP3-induced Ca2+ release often takes the form of [Ca2+]i oscillations, the frequency and amplitude of which are dependent on InsP3 concentration (Ito et al., 1999). In pancreatic acinar cells, the majority of InsP3 receptors (InsP3R) have been shown, through immunohistochemical analysis, to be tightly localized near the luminal membrane (Nathanson et al., 1994, Lee et al., 1997; Yule et al., 1997). In these cells, the agonist-evoked, InsP3–dependent [Ca2+]i increase has two fates: (a) it can remain localized to the apical pole of the cell at threshold agonist concentrations (Kasai et al., 1993), or (b) it can travel through the cell as a global wave at peak secretory agonist concentrations (Kasai et al., 1993; Yule and Williams, 1994). Physiologically, this global [Ca2+]i increase plays pivotal roles in both the exocytosis of secretory granules and the activation of Ca2+-dependent ion channels on the apical and basal membranes that are responsible for driving fluid secretion from the acinar cell (Kasai and Augustine, 1990; Kasai et al., 1993; Yule and Williams, 1994).

Since it is known that the initial trigger for global [Ca2+]i elevations is Ca2+ release from InsP3R in the apical trigger zone (Kasai and Augustine, 1990; Toescu et al., 1992; Ito et al., 1999), it has been suggested that global [Ca2+]i elevations may be due to activation of
heterogeneous populations of InsP₃R that differ in their sensitivity to InsP₃ and Ca²⁺ (Kasai et al., 1993). An alternative hypothesis is that Ca²⁺-induced–Ca²⁺ release (CICR) through ryanodine receptors (RyRs) plays a role in the propagation of [Ca²⁺] waves (Nathanson et al., 1992). The role of RyR has been well characterized in the exocrine cells of the submandibular (Lee et al., 1997) and parotid (DiJulio et al., 1997; Zhang et al., 1997, 1999) salivary glands. However, the presence of RyR in the exocrine pancreas remains controversial (Lee et al., 1997). Nevertheless, a recent report using biochemical and molecular methods (Leite et al., 1999) has demonstrated the presence of type-2 RyR in pancreatic acinar cells, although their potential role in Ca²⁺ signaling is not yet fully understood.

Much recent work has suggested that mitochondrial Ca²⁺ import (for review, see Gunter et al., 1994; Duchen, 1999; Nicholls and Budd, 2000) plays a role in regulating the spread of [Ca²⁺], through the cytoplasm. This may be relevant in pancreatic acinar cells since inhibition of mitochondrial Ca²⁺ import was reported to convert apically localized [Ca²⁺], signals to global [Ca²⁺], increases (Tinel et al., 1999). The mechanism by which this occurs, however, is not clear, given the limited “range” of Ca²⁺ diffusion within the cytoplasm (Allbritton et al., 1992). It would seem unlikely that released Ca²⁺, in the absence of mitochondrial buffering, could diffuse the 10–20-μm distance required to traverse the cell. Thus, regenerative Ca²⁺ release is a likely mechanism by which an apical [Ca²⁺], increase could result in a global [Ca²⁺], response.

The focus of this study was to investigate the mechanisms by which an initial, apically localized Ca²⁺ release event is subsequently propagated throughout the cell and the possible modulatory role of mitochondria in this process. We show that, after Ca²⁺ release from InsP₃R, the propagation of Ca²⁺ waves is modulated by a functional interaction between RyR and mitochondria. This indicates that CICR from RyR is likely the mechanism by which an apical [Ca²⁺], signal triggers a global [Ca²⁺], wave.

**Methods**

**Materials**

Purified collagenase (CLSPA grade) was purchased from Worthington Biochemicals. Fura-2 AM was purchased from Teflabs. Oregon green 488 Bapta-2 (OGB-2), tetramethylrhodamine ethyl ester (TMRE) perchlorate, benzothiazole coumarin, N-myo-InsP₃, P(1,5,6)-1-(2-nitrophenyl)-ethyl ester (caged InsP₃), 1-nitrophosphoryl EGTA (NP-EGTA), Mitotracker red, and BODIPY-ryanodine were purchased from Molecular Probes. Dulbecco’s Modified Eagles Medium (DMEM) was purchased from GIBCO BRL. All other materials were obtained from Sigma-Aldrich.

**Preparation of Pancreatic Acini**

Mouse pancreatic acini were prepared essentially as described previously (Williams et al., 1978). In brief, after CO₂ asphyxiation and cervical dislocation, pancreata were removed from freely fed male NIH-Swiss mice (25 g). The tissue was enzymatically digested with purified collagenase in DMEM with 0.1% BSA and 0.1 mg/ml soybean trypsin inhibitor for 30 min, followed by gentle tituration. Acini were then filtered through 100-μm nylon mesh, centrifuged at 75 g through 1% BSA in DMEM, and resuspended in 1% BSA in DMEM.

**Measurement of [Ca²⁺] and Flash Photolysis of Caged Compounds**

Isolated acinar cells were incubated with 2 μM fura-2 AM at 25°C for 30 min, followed by washing and resuspension in physiological salt solution (PSS) containing (mM): 127 NaCl, 0.56 MgCl₂, 4.7 KCl, 0.55 Na₂HPO₄, 1.28 CaCl₂, 10 HEPES-NaOH, 11 n-glucose, pH 7.4. Nominal Ca²⁺ containing external solution contained the above without Ca²⁺ and addition of 0.1 mM EGTA. For [Ca²⁺], measurement, fura-2-loaded cells were placed in a closed recording chamber and mounted on the stage of an Eclipse TE2000 microscope (Nikon) equipped with a Nikon Super Fluor 40×, 1.3 NA oil immersion objective. Acini were superfused at 1 ml/min with PSS and rapid solution changes were accomplished by use of a valve attached to a multi-chambered reservoir. Determination of [Ca²⁺], was performed using digital imaging microscopy with a monochromator-based system and high speed CCD camera (T.I.L.L.-Photonics). Cells were alternately excited at 340/380 ± 15 nm and the fluorescence emission collected through a 510 ± 25-nm band pass filter (Chroma). Images were acquired at a rate of 1 Hz. Mean gray values in user-defined areas of interest were used to compute 340/380 ratios. Calibration of fluorescent ratio signals to [Ca²⁺] was performed using the equation of Grynkwicz et al. (1985) by comparing the fluorescence of fura-2-containing buffers of known [Ca²⁺]. For measurement of [Ca²⁺], during whole-cell patch-clamp experiments, cells were loaded with 75 μM OGB-2 by dialysis through the patch pipette. Cells were excited at 488 ± 15 nm and fluorescence emission collected through a 525 ± 25-nm band pass filter (Chroma). Images were acquired at 33 Hz without additional binning and displayed as ΔF/ΔF₀, where ΔF/ΔF₀ = 100[(F – F₀)/F₀]. F is the recorded fluorescence, and F₀ was obtained from the average of 15 sequential frames after equilibration with the patch pipette solution and before stimulation (Kidd et al., 1999). ΔF/ΔF₀ images were scaled to 275 levels of gray from a 12-bit scale, between minimum and maximum values. A [Ca²⁺], increase was defined as an increase in fluorescence of 25 levels of gray above the minimum scale value. The spread of the wave was determined by measuring the distance between the pixel (pixel size, 0.025 μm) that first increased (corresponding to the trigger zone) and the furthest point at which the [Ca²⁺], increased to the previously described threshold value. The wave speed was calculated from the time needed for [Ca²⁺], to increase over the specified area and thus represents the mean velocity. Photolytic release of NP-EGTA (caged Ca²⁺) and caged InsP₃, were achieved using a pulsed xenon arc lamp fed to a dual port epifluorescence condenser using a fiber-optic guide (T.I.L.L.-Photonics). An 80-J, 0.5-ms flash of UV light (360 ± 7.5 nm) was reflected onto the plane of focus using a DM400 dichroic mirror and Super Fluor 40×, 1.3 NA oil immersion objective.

**Whole-Cell Patch-clamp Recordings**

Ca²⁺-activated Cl⁻ and nonspecific cation currents were recorded at a sampling rate of 1 kHz using an Axopatch 200A patch clamp amplifier (Axon Instruments, Inc.), Instrutech digital interface, and IGOR PRO/Pulse Control XOP software (Herrington and Bookman, 1994), using the whole-cell patch-clamp technique. Cells were superfused with an extracellular recording solution
that contained (mM): 140 NaCl, 10 HEPES-NaOH, 4.7 KCl, 1.13 MgCl₂, 1 CaCl₂, 10 d-glucose, pH 7.5, which was equilibrated with 100% O₂ gas. After achieving a high resistance seal (>20 GΩ), cells were superfused with the above solution supplemented with 1% BSA, which was present for patch rupture as well as throughout the experiment. Whole-cell series resistances of 8–15 MΩ were achieved after patch rupture. Intervals of 3 min were maintained after patch rupture and between stimuli to allow for equilibration with the patch pipette solution. Cells were maintained at a holding potential of ∼30 mV. For photolytic release of caged InsP₃, the intracellular recording solution contained (mM): 140 KCl, 10 HEPES-NaOH, 1.13 MgCl₂, 2 Mg-ATP, 1 α-hydroxethyl-ethylenediaminetriacetic acid, 0.001–0.002 caged InsP₃, pH 7.3. For photolytic release of caged Ca²⁺, the intracellular recording solution contained (mM): 130 KCl, 10 HEPES-NaOH, 10 3-nitrophenyl EGTA, 5 CaCl₂, 2 Mg-ATP, 1.2 MgCl₂, pH 7.2. Using this intracellular solution, the resting [Ca²⁺], and free [Mg²⁺] were calculated to be 175 nM and 1 mM, respectively.

Determination of the Subcellular Localization of Mitochondria and Ryanodine Receptors

For RvR staining, isolated acinar cells and small clusters were incubated with 0.1 μM BODIPY-ryanodine for 2 h in PSS supplemented with 0.5% BSA. At the end of the incubation, the cells were gently pelleted and washed three times by resuspension in buffer. Localization was examined by laser scanning confocal microscopy using a Noran Oz system. BODIPY-ryanodine was excited at 488 nm and emission was recorded using a 525-nm long pass filter. In some experiments, cells were incubated with 5 μM ryanodine 30 min before incubation with fluorescent probe to determine nonspecific fluorescence. In this case, using identical laser power settings, no measurable signal was detected. For the detection of mitochondrial staining, cells were incubated with 0.1 μM mitotracker red for 2 min, followed by identical wash steps. Mitochondria were visualized by confocal microscopy after excitation of the dye at 565 nm with emission collected above 600 nm.

Estimation of Changes in Mitochondrial Membrane Potential

Isolated acinar cells were loaded with 100 nM TMRE perchlorate for 15 min at room temperature in PSS. 100 nM TMRE was also included in all solutions used for superfusion. Cells loaded with dye were excited at 545 ± 15 nm and fluorescence emission collected using a 565-nm-long pass filter. Fluorescence distribution was similar to the distribution of fluorescence in cells that were loaded with Mitotracker red. Fluorescence traces were generated from mean gray values using a user-defined region of interest corresponding to regions of high TMRE fluorescence.

Statistical Analysis

For caged Ca²⁺ experiments, ionic currents were analyzed to determine peak current (Iₚₑᵃᵏ), total charge (Qₚₑᵃᵏ), and time to steady state recovery (Tᵣₑｃ) using IGOR PRO software. Iₚₑᵃᵏ was defined as the maximum current of the record after photolysis of NP-EGTA and was determined after baseline current subtraction. Qₑᵃᵏ was determined by integrating current traces from the time of current activation until recovery of current to baseline or, in some cases, new steady state levels. Tᵣₑｃ was defined as the time between Iₑᵃᵏ and recovery to baseline or steady state. Data within groups (control vs. treated) were analyzed using a Wilcoxon test for paired data and between experimental groups (±ryanodine) using a Mann-Whitney test for unpaired, nonparametric data with Graph Pad Prism software. In other experiments, data was analyzed using an appropriate Student’s t test. All data is represented as mean ± SEM.

RESULTS

Effect of Mitochondrial Depolarization on Agonist-induced [Ca²⁺], Oscillations

Stimulation of acinar cells with low doses of the muscarinic agonist carbachol (CCh) (50–250 nM) produced oscillations in [Ca²⁺], with a regular frequency (three to six per minute) and amplitude (100–300 nM) in nominally Ca²⁺-free external solution. The oscillations were generally maintained for up to 6 min (Yule and Gallacher, 1988). Treatment with 0.5 μM FCCP, to reduce the driving force for mitochondrial import, resulted in an enhanced and sustained rise in [Ca²⁺], lasting several hundred seconds that averaged 207 ± 90 nM above levels reached with agonist (Fig. 1A, n = 10, and see Tinel et al., 1999). After washout of FCCP, some cells resumed oscillations in the continued presence of agonist (data not shown). Similar results were obtained using a range of FCCP concentrations (0.15–0.5 μM). Since inhibition of mitochondria could potentially result in local ATP depletion, additional experiments were performed combining mitochondrial inhibitors with the ATP synthase inhibitor oligomycin. This maneuver was designed to prevent the consumption of ATP by the synthase acting in reverse after dissipation of the mitochondrial membrane potential. As shown in Fig. 1B, treatment with oligomycin (0.5 μM) did not alter the increase in [Ca²⁺], evoked by FCCP (n = 3). Moreover, the ability of FCCP to convert an oscillatory into sustained [Ca²⁺], increase was not agonist-specific, as the rise was observed in cells stimulated with either CCh (Fig. 1A) or cholecystokinin (B). Additionally, treatment with the respiratory chain inhibitor antimycin (0.5 μM) in combination with 0.5 μM oligomycin resulted in a sustained [Ca²⁺], rise to 273 ± 33 nM after CCh stimulation (Fig. 1C, n = 8). Treatment with FCCP resulted in mitochondrial depolarization, as indicated by the mitochondrial membrane potential-sensitive dye, TMRE. TMRE fluorescence was concentrated in a well-defined region surrounding the apical pole. In cells loaded with 100 nM TMRE, a rapid decrease in fluorescence was observed after treatment with 0.5 μM FCCP (Fig. 1, inset, n = 3).

Because simple diffusion of Ca²⁺ in the absence of mitochondrial import is unlikely to account for the global Ca²⁺ rise evoked by low agonist concentrations, we investigated whether a regenerative Ca²⁺ release mechanism, dependent on internal stores, was involved. Treatment of furin-2–loaded acinar cells with the SERCA pump inhibitor cyclopiazonic acid after stimulation with an oscillatory dose of CCh resulted in a slow release of Ca²⁺ from endoplasmic reticulum (ER) stores, followed by inhibition of oscillations and a re-
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turn of $[\text{Ca}^{2+}]_i$ to basal levels (Fig. 2 A). Subsequent application of FCCP to depolarize mitochondria resulted in only a small increase in $[\text{Ca}^{2+}]_i$, when compared with the increase obtained in the presence of an intact ER store (10 ± 6 vs. 207 ± 90 nM; $n = 6$). Similarly, stimulation with 0.1 mM CCh in nominally Ca$^{2+}$-free bath solution resulted in a large increase in $[\text{Ca}^{2+}]_i$, that returned to basal levels over the course of several minutes (Fig. 2 B). CCh was removed, and then reapplied to ensure that the ER store was depleted. Subsequent depolarization of mitochondria resulted in only a small $[\text{Ca}^{2+}]_i$ increase (~16 nM; $n = 2$). This data suggests that the enhanced Ca$^{2+}$ release after mitochondrial depolarization was due predominantly to Ca$^{2+}$ release from ER stores, and not from the mitochondria.

Figure 1. Effect of mitochondrial depolarization on agonist-induced $[\text{Ca}^{2+}]_i$ oscillations. (A) $[\text{Ca}^{2+}]_i$ oscillations elicited by CCh were converted to an enhanced, sustained rise in $[\text{Ca}^{2+}]_i$ after treatment with FCCP in fura-2-loaded acinar cells. As shown in the inset, treatment of TMRE-loaded acinar cells with FCCP caused a rapid decrease in TMRE fluorescence, indicative of mitochondrial depolarization. (B) Oscillations initiated by cholecystokinin were likewise converted to a sustained rise in $[\text{Ca}^{2+}]_i$ after treatment with FCCP and the ATP synthase inhibitor oligomycin. (C) The respiratory chain inhibitor antimycin in combination with oligomycin similarly resulted in the conversion of oscillations to a sustained rise in $[\text{Ca}^{2+}]_i$. In all experiments, cells were maintained in nominally Ca$^{2+}$-free bath solution throughout.

Subcellular Localization of Ryanodine Receptors and Their Role in Agonist-induced $[\text{Ca}^{2+}]_i$ Signaling

Work by Leite et al. (1999) demonstrated the presence of type 2 RyR in acinar cells, consistent with the possibility that the global $[\text{Ca}^{2+}]_i$ increase observed after mitochondrial depolarization was dependent on RyR. We therefore investigated whether RyR and mitochondria might be morphologically and/or functionally linked. Confocal microscopy revealed that the BODIPY-ryanodine fluorescence was concentrated in a region surrounding the apical pole of the cell (Fig. 3, A and B; $n = 5$ preparations). More diffuse fluorescence was seen throughout the apical and basal regions, while no fluorescence was seen when cells were incubated with a 50-fold excess of nonfluorescent ryanodine before in-
cubation with BODIPY-ryanodine \((n = 2\) preparations). Mitochondrial distribution was investigated using the mitochondrial-specific dye Mitotracker red. Mitochondria were found to be concentrated in a similar region, surrounding the apical zymogen granule-containing region (Fig. 3, C and D, and see Tinel et al., 1999). This distribution was similar to that observed with TMRE. The distribution of RyR and mitochondria thus overlaps in acinar cells.

To determine the functional significance of this colocalization, the contribution of RyR to the FCCP-induced \([\text{Ca}^{2+}]\) increase was investigated. After incubation with CCh, cells were superfused with 100 \(\mu\)M ryanodine, a concentration that has been reported to block RyR in a nonconducting state (Lai et al., 1989; McGrew et al., 1989; for review see Shoshan-Barmatz and Ashley, 1998). Ryanodine treatment significantly decreased the amplitude of CCh-induced oscillations from 157 ± 25 to 96 ± 21 nM above basal (Fig. 4, \(n = 5\), \(P = 0.003\)), but did not significantly alter the frequency of oscillations. Moreover, in the presence of ryanodine, FCCP treatment failed to evoke an additional \([\text{Ca}^{2+}]\) rise. Measurement of TMRE fluorescence showed that treatment with ryanodine did not affect the ability of FCCP to depolarize mitochondria (Fig. 4, inset, \(n = 5\)). These data indicate that ryanodine treatment disrupts a functional interaction between mitochondria and ryanodine-sensitive \([\text{Ca}^{2+}]\) stores.

**Activation of CICR through Photolytic Release of \([\text{Ca}^{2+}]\)**

The aforementioned experiments reveal that \([\text{Ca}^{2+}]\) release through ryanodine-sensitive stores can be evoked after mitochondrial depolarization, and further suggest that this occurs as a result of colocalization. To determine whether \([\text{Ca}^{2+}]\) in the vicinity of mitochondria could influence RyR activity, we evoked transient global elevations in \([\text{Ca}^{2+}]\), through photolytic release of caged \([\text{Ca}^{2+}]\) (Ellis-Davies and Kaplan, 1994; Takahashi et al., 1999; Zahradnikova et al., 1999). Changes in \([\text{Ca}^{2+}]\), were monitored in whole-cell patch-clamped acinar cells using a \([\text{Ca}^{2+}]\)-activated \(\text{Cl}^-\) current. This current has been extensively used to report changes in \([\text{Ca}^{2+}]\), in this cell type (Thorn et al., 1993; Cancela et al., 1999; Kidd et al., 1999; Park et al., 1999; Xu et al., 1999). Photolysis was achieved by a 0.5-ms UV flash.
min after obtaining a stable whole-cell configuration, a time sufficient to allow equilibration between the pipette solution and cell. Repetitive uncagings at regular intervals evoked reproducible increases in the Ca$^{2+}$-activated Cl$^{-}$ current (Fig. 5 A). Using the low-affinity Ca$^{2+}$-sensitive dye benzothiazole coumarin, an in situ calibration (Ito et al., 1997) estimated that, on average, photolysis of NP-EGTA produced an increase in [Ca$^{2+}$], of 7 µM, a level within the range of [Ca$^{2+}$], increases in the apical region typically associated with high doses of agonist (Ito et al., 1997). Currents were evoked before and after FCCP treatment and the total charge, recovery time, and peak current values were respectively compared. After treatment with FCCP for 3 min, photolysis resulted in a current significantly enhanced with respect to the total charge passed ($Q_{\text{tot}}$) ($34,685 \pm 12,070$ pC vs. $8,735 \pm 3,554$ pC, Fig. 5 B; $n = 7$, $P = 0.016$). This increase in $Q_{\text{tot}}$ was the result of a significant, nearly fourfold increase in the time to steady state recovery ($T_{\text{rec}}$) ($214 \pm 61$ vs. $54 \pm 12$ s, $n = 7$, $P = 0.022$), since no significant increase in the peak current ($I_{\text{peak}}$) was observed ($612 \pm 104$ vs. $455 \pm 59$ pA, $n = 7$, $P = 0.156$). No measurable current change was produced by FCCP treatment in the absence of photolysis. Control currents evoked in the presence or absence of ryanodine in the pipette solution were not significantly different ($Q_{\text{tot}} = 8,814 \pm 2,346$ vs. $8,735 \pm 3,554$ pC, $P = 0.628$; $T_{\text{rec}} = 39 \pm 6$ vs. $53 \pm 12$ s, $P = 0.234$; $n = 7$ control, $n = 6$ ryanodine). In contrast, in the presence of ryanodine, the evoked currents in FCCP were significantly altered compared with FCCP treatment alone following subtraction of the control currents ($5,353 \pm 2,751$ vs. $25,950 \pm 8,987$ pC, $P = 0.008$; $17 \pm 6$ vs. $160 \pm 52$ s, $P = 0.022$). The ability of ryanodine to abolish the prolonged $T_{\text{rec}}$ induced by FCCP suggests that the slow recovery time was due, at least in part, to persistent Ca$^{2+}$ release from a Ca$^{2+}$- and ryanodine-sensitive store.

**Physiological Role of CICR from Ryanodine Receptors**

Since pharmacological inhibition of mitochondria revealed what appeared to be CICR from a ryanodine-sensitive store, we investigated whether this Ca$^{2+}$ release was physiologically relevant to [Ca$^{2+}$], wave propagation in acinar cells. Photolytic release of low levels of InsP$_3$ was used to evoke [Ca$^{2+}$], increases that remained largely confined to the apical region of whole-cell patch clamped cells. The spatial characteristics of the [Ca$^{2+}$], signal were monitored by digital imaging of OGB-2 fluorescence. This approach allowed the effects of mitochondrial depolarization and RyR blockade on the properties of a propagating [Ca$^{2+}$], wave to be directly investigated. On average, a single UV flash generated a reproducible, local [Ca$^{2+}$], signal that traveled $7.7 \pm 0.8$ µm from the site of initiation at a speed of $16.0 \pm 0.1$ µm/s (Fig. 6 A, $n = 8$). This type of "contained" signal transiently raised [Ca$^{2+}$], within the apical half of the cell (cell diameter = $16.5 \pm 0.4$ µm; $n = 36$). As shown in Fig. 6 B, I and III, photolysis of 1 µM caged InsP$_3$ evoked a localized [Ca$^{2+}$], increase that, after treatment with FCCP, still initiated at the trigger zone, but subsequently spread throughout the cell. On average, the [Ca$^{2+}$], signal now traveled $15.6 \pm 1.3$ µm (Fig. 6 B, II and IV, $n = 4$), consistent with the hypothesis that mitochondrial buffering is important for restricting this type of [Ca$^{2+}$], signal. Next, we repeated these experiments after treatment with ryanodine to test the hypothesis that Ca$^{2+}$ release from RyR contributed to the propagation of a [Ca$^{2+}$], wave throughout the cell. Photolysis of 1 µM caged InsP$_3$ in the presence of 100 µM ryanodine evoked a localized [Ca$^{2+}$], increase, similar to that produced by the control uncaging in the absence of ryanodine (Fig. 6 C, I and III). However, a subsequent flash after treatment with FCCP now failed to evoke a global elevation in [Ca$^{2+}$], the signal propagating only $6.3 \pm 2.1$ µm, significantly different to the dis-
tance observed in FCCP alone (Fig. 6 C, II and IV, \( n = 3, P = 0.008 \)). The observation that \( \text{Ca}^{2+} \) did not spread throughout the cell after mitochondrial depolarization when RyRs were inhibited suggests that RyRs play a central role in propagating \([\text{Ca}^{2+}]\text{i}\) increases throughout the cell and points to a potential role for mitochondria in modulating RyR activation.

In cells where photolytic release of InsP\(_3\) caused \( \text{Ca}^{2+} \) release resembling a \([\text{Ca}^{2+}]\text{i}\) “puff” (i.e., a highly localized, small, and transient response), mitochondrial depolarization had no apparent effect on the propagation of the signal (Fig. 7, \( n = 3 \)). In these experiments, \([\text{Ca}^{2+}]\text{i}\) remained apically confined, despite mitochondrial depolarization, suggesting that \([\text{Ca}^{2+}]\text{i}\) increases of this magnitude were not sufficient to recruit participation of mitochondrial \( \text{Ca}^{2+} \) import, as has been suggested by Tinel et al. (1999), and were not sufficient to cause activation of RyR.

To investigate whether CICR occurred during normal signaling events, and not solely when mitochondrial \( \text{Ca}^{2+} \) import was inhibited, a higher concentration of caged InsP\(_3\) (2 \( \mu \)M) was used to photolytically induce global \([\text{Ca}^{2+}]\text{i}\) increases. This allowed for the direct assessment of the role of RyR on the propagation of global \( \text{Ca}^{2+} \) waves. Using this paradigm, global \([\text{Ca}^{2+}]\text{i}\) increases could be reproducibly evoked (Fig. 8, D and E). After release of InsP\(_3\) under control conditions, 100 \( \mu \)M ryanodine was added to the external bath solution. In the continued presence of ryanodine, sequentially evoked \([\text{Ca}^{2+}]\text{i}\) responses became progressively more confined to the apical region of the cell compared with control (Fig. 8, A and B, \( n = 7 \)). The addition of ryanodine not only prevented the \([\text{Ca}^{2+}]\text{i}\) increase throughout the basal region, but also caused the signal to retreat well into the apical region, giving increases in a region only slightly more diffuse than the trigger zone. By the fourth uncaging, the \([\text{Ca}^{2+}]\text{i}\) wave propagated 11.2 \( \pm \) 1.8 \( \mu \)m (\( n = 7 \)) in the presence of ryanodine, compared with 15.7 \( \pm \) 1.3 \( \mu \)m in time-matched controls (Fig. 8 C, \( n = 10, P = 0.006 \)). This decrease in propagation represented a reduction in the distance traveled across the cell from 95.5 \( \pm \) 2.9% of the diameter of the cell to 63.9 \( \pm \) 10.9% in the presence of ryanodine. In addition, the rate of propagation was significantly slowed in the presence of ryanodine, traveling at 21.2 \( \pm \) 6 \( \mu \)m/s (\( n = 10 \)) in the absence of and 9.5 \( \pm \) 2.3 \( \mu \)m/s (\( n = 7 \)) after incubation in ryanodine for 12 min (\( P = 0.03 \)). The latency after the flash to the initiation of the
Figure 6

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signal was not significantly altered by treatment with ryanodine (192 ± 5 vs. 175 ± 2 ms, control vs. treated after 12 min, n = 4), as expected for a signal initiated through InsP₃R. Interestingly, the spatially limiting effects of RyR blockade could be overcome at higher agonist concentrations (Fig. 8, A V and B V).

**DISCUSSION**

In pancreatic acinar cells, Ca²⁺ signaling is initiated by the binding of InsP₃ to its receptor on specialized portions of the ER present in the extreme apical regions of the cell, the so-called trigger-zone (Kasai and Augustine, 1990; Kasai et al., 1993; Thorn et al., 1993). At threshold agonist concentrations, the [Ca²⁺], increase can be confined to this region (Kasai et al., 1993; Thorn et al., 1993); however, under peak secretory conditions, the [Ca²⁺], signal is propagated throughout the cell in the form of a wave (Kasai and Augustine, 1990; Kasai et al., 1993; Yule and Williams, 1994). A preponderance of evidence indicates that the abundance of InsP₃R in the trigger zone is responsible for the initiation of the [Ca²⁺] signal (Lee et al., 1997; Nathanson et al., 1994; Yule et al., 1997). In addition, an added complexity is the presence of a band of mitochondria surrounding the trigger zone, which has been suggested to apically limit [Ca²⁺], signals by buffering the initial spread of the wave (Tinel et al., 1999). In contrast to these initial events, little consensus exists as to the manner by which the signal becomes a “global” wave, presumably overcoming the mitochondrial barrier in doing so. Data presented in this study indicates that the propagation of a global wave is largely dependent on RyR and intriguingly indicates that their localization, in relative abundance close to mitochondria at the apical-basal boundary, may play a key role in regulating the transition from a local to a global signal.

Numerous studies in a wide variety of cell types have demonstrated that mitochondria play an important role in buffering changes in [Ca²⁺], under physiological as well as pathological situations (for review, see Duchen, 1999). This is accomplished due to the privileged localization of mitochondria in the vicinity of Ca²⁺ release or influx channels, which allows mitochondria to sense microdomains of high [Ca²⁺], (Rizzuto et al., 1998; Csordas et al., 1999). Although mitochondria in pancreatic acinar cells are not ideally positioned to sense a [Ca²⁺], microdomain released by InsP₃R, the localization of mitochondria does overlap with the distribution of the greatest abundance of RyR. While RyR are expressed diffusely through the cytoplasm, the highest density are found in a band bordering the apical region of the cell. The expression of RyR has been difficult to demonstrate in pancreatic acinar cells; however, both this study and Leite et al. (1999) have now positively identified RyR in this cell type. The localization reported here is generally similar to that previously reported; the greatest abundance of receptors being excluded from the apical pole, a localization similar to that found in salivary acinar cells (Lee et al., 1997; Zhang et al., 1999).

Several pieces of experimental evidence indicate that the activity of the RyR is influenced by the ability of mitochondria to buffer [Ca²⁺], in their immediate vicinity.
Figure 8. Inhibition of RyRs diminishes global \([\text{Ca}^{2+}]\), increases resulting from photolysis of caged InsP₃. (A I and B I) Photolysis of 2 µM caged IP₃ under control conditions evokes a global \([\text{Ca}^{2+}]\), increase that initiates at the apical trigger zone and spreads as a wave throughout the cell, as shown by the \([\text{Ca}^{2+}]\), traces in A and OGB-2 fluorescence images in B. \([\text{Ca}^{2+}]\), traces were generated from the apical and basal regions depicted in the brightfield image. (A II-IV, and B II-IV) After addition of ryanodine to the bath solution, the propagation of the \([\text{Ca}^{2+}]\), increase into the basal region decreases with each subsequent uncaging, such that the increase in \([\text{Ca}^{2+}]\), is only evident within the granule-containing region of the cell. (A V and B V) Stimulation with a high dose of CCh is able to overcome the effects of blocking RyR, resulting in a global \([\text{Ca}^{2+}]\), increase. (C) Pooled data showing distance \([\text{Ca}^{2+}]\), wave propagated, plotted as a percentage of the first flash value, for control and ryanodine-treated cells. For control, \(n = 9\) for ryanodine treated, \(n = 7\). *Statistically significant, \(P < 0.04\). (D and E) Control uncaging of 2 µM caged InsP₃. InsP₃ was photolyzed every 3 min. Traces and fluorescence images correspond to time points I and IV in A, respectively, illustrating no significant run down of the response over this time period.
First, during stimulation with physiological concentrations of agonist, mitochondrial depolarization results in a large $[\text{Ca}^{2+}]$, increase. However, this $[\text{Ca}^{2+}]$, increase may not simply be the result of decreased mitochondrial buffering and release of stored mitochondrial Ca$^{2+}$. Since the $[\text{Ca}^{2+}]$, increase was largely blocked by either emptying ER Ca$^{2+}$ stores or by inhibition of RyR, this initial observation may indicate that Ca$^{2+}$ release from the ER is the important event after limited release of mitochondrial Ca$^{2+}$ as the initial trigger. The attenuation of the response by ryanodine is indicative of a CICR event mediated by RyR. An alternative explanation is that, under conditions of physiological stimulation, mitochondria preferentially import Ca$^{2+}$ released by RyR. Thus, treatment with ryanodine would be expected to lead to decreased mitochondrial sequestration of Ca$^{2+}$ and the resultant refractory response to FCCP. This interpretation would also support the notion of a microdomain of mitochondria and ER expressing RyR and is consistent with the view that mitochondria preferentially sense rapid, oscillatory changes in $[\text{Ca}^{2+}]$, (Hajnoczky et al., 1999; Szalai et al., 2000).

Mitochondrial depolarization after photolytically induced global $[\text{Ca}^{2+}]$, increases resulted in a significant augmentation of $[\text{Ca}^{2+}]$, as evidenced by the delayed recovery of Ca$^{2+}$-activated currents in the presence of FCCP. Similar effects have been observed in sympathetic neurons after depolarization-induced Ca$^{2+}$ influx (Colegrove et al., 2000). In the present study, this increase in $[\text{Ca}^{2+}]$, reflects not only a decrease in Ca$^{2+}$ clearance, but also activation of CICR, since the prolonged recovery was inhibited by ryanodine. An interpretation of this data is that, when mitochondrial buffering is active, little CICR occurs, seemingly as RyR are not exposed to activating levels of Ca$^{2+}$. In contrast, when mitochondrial buffering is compromised, the altered kinetics of Ca$^{2+}$ clearance allows CICR to occur. An alternate and perhaps more likely scenario is that CICR through RyR is occurring under these conditions, but the signal is limited by active mitochondrial buffering. Depolarization of mitochondria would therefore result in this Ca$^{2+}$ release being “unmasked,” as the release is no longer effectively sequestered. The later idea is consistent with reports from smooth and cardiac muscle, which demonstrate that mitochondria actively buffer release from RyR (Nassar and Simpson, 2000; Szalai et al., 2000). This type of localized regulatory effect of mitochondria has also recently been demonstrated in hepatocytes, where it was shown that mitochondrial Ca$^{2+}$ import caused a suppression of Ca$^{2+}$ feedback effects on InsP$_3$R (Hajnoczky et al., 1999).

Uncaging threshold concentrations of InsP$_3$, induced apically localized Ca$^{2+}$ signals; a Ca$^{2+}$ wave was generated that was restricted to the zymogen granule-containing region and did not invade the basal region of the cell. Mitochondrial depolarization resulted in the transition from a spatially limited response to a global Ca$^{2+}$ signal. The global increase was largely attenuated by treatment with ryanodine, suggesting that the transition from a local to global Ca$^{2+}$ signal following mitochondrial depolarization was dependent on a CICR event. It also follows that the signal is normally limited in the presence of mitochondria and as a result of buffered or limited RyR activation. In some experiments, the smallest and most transient signals were unaffected by mitochondrial depolarization. These data would indicate that the smallest signals appear limited by processes such as clearance of Ca$^{2+}$ by plasma membrane and ER ATPases, cytoplasmic buffering, as well as the metabolism of InsP$_3$, and not by mitochondrial buffering.

Data showing that inhibitory concentrations of ryanodine result in a global $[\text{Ca}^{2+}]$, signal being spatially limited to the apical region of the cell is strong evidence that RyR play an important role in wave propagation out of the trigger zone under physiological conditions, and not simply when mitochondrial import is compromised. The observation that the wave speed of the residual apical signal was slowed significantly in the presence of ryanodine also indicates that CICR plays a role in propagating the signal from the initial InsP$_3$-induced release. This is consistent with the reduction in wave speed noted in the presence of ryanodine by Nathanson et al. (1992). Since the $[\text{Ca}^{2+}]$, signal was reduced essentially to a region limited by mitochondria, an implication of these data is that RyR activation and subsequent CICR is an important event leading to the barrier posed by mitochondria being breached. An attractive hypothesis is that the transition from spatially limited to global Ca$^{2+}$ signaling is governed by an interaction between mitochondria and RyR. On stimulation with sufficient InsP$_3$, and presumably with peak secretory doses of agonist, a signal of sufficient intensity arrives at the boundary of apical and basal portions of the cell and the amplification of the Ca$^{2+}$ signal through CICR plays a major role in essentially overwhelming the local buffering capacity of mitochondria. In this way, the mitochondria could function to provide a “set point” in the transition from a local to global Ca$^{2+}$ signal.

Ca$^{2+}$ signals are apparently capable of propagating across cells in the absence of CICR through RyR, as indicated by the observation that high concentrations of agonist were able to overcome inhibition by ryanodine, resulting in an apical-to-basal global Ca$^{2+}$ wave. These data are consistent with the demonstration that low levels of InsP$_3$R are expressed throughout the cytoplasm of acinar cells (Lee et al., 1997), and thus a wave can be propagated as a result of the intrinsic synergistic activity of Ca$^{2+}$ on the InsP$_3$R. This observation raises the possibility that InsP$_3$-induced Ca$^{2+}$ release outside the trigger zone may also synergize at RyR to provide a stimulus for CICR.
In conclusion, the present study indicates a continuum of events that determine whether a [Ca\textsuperscript{2+}] signal is localized to the apical region or spreads as a wave throughout the cell. The initial common trigger appears to be InsP\textsubscript{3}-induced Ca\textsuperscript{2+} release from the apical trigger zone. At all but the lowest concentrations of agonist, apically localized signals are confined to this region by the buffering capacity of mitochondria. At greater stimulus strength, the buffering capacity of mitochondria is overcome as CICR through RyR becomes the dominant mechanism for propagating a global Ca\textsuperscript{2+} wave.

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