Ca\textsuperscript{2+} release dynamics in parotid and pancreatic exocrine acinar cells evoked by spatially limited flash photolysis

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Won JH, Cottrell WJ, Foster TH, Yule DL. Ca\textsuperscript{2+} release dynamics in parotid and pancreatic exocrine acinar cells evoked by spatially limited flash photolysis. Am J Physiol Gastrointest Liver Physiol 293: G1166–G1177, 2007. First published September 27, 2007; doi:10.1152/ajpgi.00352.2007.—Intracellular calcium concentration ([Ca\textsuperscript{2+}]i) signals are central to the mechanisms underlying fluid and protein secretion in parotid and pancreatic acinar cells. Calcium release was studied in natively buffered cells following focal laser photolysis of caged molecules. Focal photolysis of caged-inositol 1,4,5 trisphosphate (InsP\textsubscript{3}) in the apical region resulted in Ca\textsuperscript{2+} release from the apical trigger zone and, after a latent period, the initiation of an apical-to-basal Ca\textsuperscript{2+} wave. The latency was longer and the wave speed significantly slower in pancreatic compared with parotid cells. Focal photolysis in basal regions evoked only limited Ca\textsuperscript{2+} release at the photolysis site and never resulted in a propagating wave. Instead, an apical-to-basal wave was initiated following a latent period. Again, the latent period was significantly longer under all conditions in pancreas than parotid. Although slower in pancreas than parotid, once initiated, the apical-to-basal wave speed was constant in a particular cell type. Photo release of caged-Ca\textsuperscript{2+} failed to evoke a propagating Ca\textsuperscript{2+} wave in either cell type. However, the kinetics of the Ca\textsuperscript{2+} signal evoked following photolysis of caged-InsP\textsubscript{3} were significantly dampened by ryanodine in parotid but not pancreas, indicating a more prominent functional role for ryanodine receptor (RyR) following InsP\textsubscript{3} receptor (InsP\textsubscript{3}R) activation. These data suggest that differing expression levels of InsP\textsubscript{3}R, RyR, and possibly cellular buffering capacity may contribute to the fast kinetics of Ca\textsuperscript{2+} signals in parotid compared with pancreas. These properties may represent a specialization of the cell type to effectively stimulate Ca\textsuperscript{2+}-dependent effectors important for the differing primary physiological role of each gland.

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STIMULATION OF EXOCRINE CELLS derived from the pancreas or salivary glands with Goq-coupled secretagogues results in an elevation in the cytosolic calcium concentration ([Ca\textsuperscript{2+}]i) (30, 49). This event is a tightly controlled consequence of Ca\textsuperscript{2+} release from intracellular stores mediated primarily by the action of inositol 1,4,5 trisphosphate (InsP\textsubscript{3}) on InsP\textsubscript{3} receptor (InsP\textsubscript{3}R), followed by Ca\textsuperscript{2+} influx from the extracellular space (2, 3, 6, 32, 33, 40, 41, 50). Both phases of the Ca\textsuperscript{2+} signal are absolutely required for the appropriate activation of effectors important for the primary physiological roles of the tissue, namely, fluid secretion from salivary glands and exocytotic protein secretion from pancreatic acinar cells (29, 46).

The Ca\textsuperscript{2+} signal stimulated in cells derived from each gland exhibits characteristic temporal and spatial properties. Superficially, these properties and the underlying mechanisms are similar: stimulation of either cell type with secretagogue or global uniform release of InsP\textsubscript{3} from a caged precursor results in a signal, which invariably initiates in the apical pole of either cell, and then can spread throughout the cytoplasm to generate a global Ca\textsuperscript{2+} signal (18, 21, 24, 34, 39, 42, 44, 45). The initiation of the Ca\textsuperscript{2+} signal is believed to occur largely because of the predominant localization of InsP\textsubscript{3}Rs, which are tightly apposed to the luminal plasma membrane (23, 24, 34, 43, 52). However, the localization of secretagogue receptors themselves, and thus presumably production of InsP\textsubscript{3}, has been reported close to the junctional complexes (27, 37), and this may contribute to the initiation of the Ca\textsuperscript{2+} signal in an agonist-specific manner. Alternatively, it has been suggested that activation of basal secretagogue receptors and subsequent diffusion of InsP\textsubscript{3} to apical InsP\textsubscript{3}R can fully account for the spatial properties of the signal (4). In either case the nonuniform generation of InsP\textsubscript{3} acting on a specific distribution of InsP\textsubscript{3}R is likely to underlie the mechanism, accounting for the Ca\textsuperscript{2+} signals observed in both cell types.

Although the general characteristics of the Ca\textsuperscript{2+} signal in each cell type are similar, some important differences have been reported. These characteristics appear generally consistent with the particular specialized exocrine function of each gland. In particular, the machinery in parotid acinar cells appears dedicated to yield rapid, very large, global Ca\textsuperscript{2+} signals compared with pancreatic acinar cells (17, 23, 50). These Ca\textsuperscript{2+} signals appear ideally suited to activating the spatially separated ion channel effectors necessary for fluid secretion. For example, a recent study from our laboratory using total internal reflection microscopy to measure membrane-delimited Ca\textsuperscript{2+} signals has demonstrated that the magnitude of Ca\textsuperscript{2+} influx is vastly greater in parotid acinar cells compared with cells derived from the exocrine pancreas (50). This difference is most striking at low, presumably physiological, concentrations of secretagogues.

In addition, studies using both confocal and wide-field microscopy have reported that in pancreatic cells standing gradients of Ca\textsuperscript{2+} can be established locally in the apical domain of the cell (21, 44). These signals occur exclusively following exposure to threshold stimulation. This phenomenon is particularly marked when cells are exposed to a low, spatially uniform InsP\textsubscript{3} concentration ([InsP\textsubscript{3}]), following flash photolysis, or when InsP\textsubscript{3} is presented via a whole cell, patch-clamp pipette (17, 20, 39, 44). The majority of studies have, however, not observed local apically limited Ca\textsuperscript{2+} sig-
nals in acinar cells derived from salivary glands (but see Ref. 18 performed in submandibular cells). Important caveats, which apply to this experimental paradigm in general, are that dialysis from the patch pipette will disrupt the native buffering of the cytoplasm and thus may influence the spatial and temporal properties of the signal. In addition, as noted previously, cells are unlikely to experience physiologically a spatially uniform increase in InsP3. Following more intense stimulation, the Ca2+ signals can propagate in a manner typical of a true “wave” toward the basal aspects of the cell; i.e., constant wave speed typical of an “all or nothing” phenomenon dependent on Ca2+-induced Ca2+ release (CICR) [17, 24, 25, 27, 35, 45]. CICR can occur through either InsP3R or through the classical CICR channel, the ryanodine receptor (RyR), which is also expressed in exocrine cells (14, 26, 39, 53). In pancreatic acinar cells, two general models have been proposed to account for the propagation of the Ca2+ wave. One model suggests that following initiation in the apical trigger zone, the wave is propagated as a function of the sequential activation of both InsP3R and RyR in the extra apical region (5, 25, 35). The second model does not require a prominent role of RyR but is dependent on the activation of InsP3R present throughout the extra-apical endoplasmic reticulum (ER) at lower density than in the trigger zone. The sequential activation of these InsP3Rs occurs as the wave front progresses as a function of the coagonist activity of Ca2+ at the InsP3R [21]. In parotid acinar cells, global, uniform InsP3 exposure results in a signal, which is very rapidly initiated in the apical portion of the cell but, in contrast to pancreas, invariably globalizes to the basal regions, regardless of the [InsP3] (17). At high [InsP3] the kinetics of the globalization are not consistent with properties of a true wave and appear to result in what is termed a “cinematic” Ca2+ wave, initiation in the apical region followed by simultaneous Ca2+ release, taking place essentially autonomously and independently in neighboring subcellular regions as a function of activation of InsP3R and/or RyR.

This study was, therefore, designed to compare and probe the mechanism(s) underlying the Ca2+ signal globalization in these individual exocrine cell types. To these ends, we have designed and constructed a relatively simple means of locally releasing chemically caged molecules using high powered, focal laser photolysis. This method, combined with the use of cell permeant forms of caged-InsP3 and caged-Ca2+, has most importantly allowed these data to be obtained in intact and thus more natively buffered acinar cells. In particular, by monitoring the spatial and temporal characteristics of the Ca2+ signals evoked by InsP3, we have functionally defined the relative sensitivities of various subcellular regions of both pancreatic and parotid acinar cells to InsP3 and investigated the role of RyR to the signals observed. The study demonstrates that these exocrine cells are not sensitive to focal InsP3 liberation in any cellular region apart from the extreme apical region. Moreover, localized InsP3 generation in both apical and basal regions of either acinar cells results in an apical-to-basal Ca2+ wave with kinetics consistent with the apical region of parotid cells, exhibiting enhanced sensitivity to InsP3 compared with pancreas. Furthermore, following initiation of an apical-to-basal wave, the cytoplasmic region of parotid acinar cells appears to constitute a more “excitable” medium compared with pancreas. This excitability appears to be attributed in the main to the activity of InsP3R, although RyRs also appear to contribute to the fast kinetics of Ca2+ signals in parotid acinar cells.

MATERIALS AND METHODS

Materials. Fluo-4-AM was purchased from Teflabs. O-nitrophenyl ethylenediaminetetraacetic acid (NP-EGTA) was from Invitrogen. Caged-isopropylidene inositol 1,4,5 trisphosphate pentoxy methyl ester (Ci-InsP3/PM) (cell permeable caged-InsP3) was from Axxora. In isopropylidene inositol 1,4,5 trisphosphate (i-InsP3) the 2-3 hydroxyls of the inositol ring are protected by an isopropylidene group (12). This molecule is a full agonist at the InsP3R. Dulbecco’s modified Eagle medium (DMEM) was purchased from GIBCO. All other materials were obtained from Sigma.

Preparation of pancreatic acini. These experiments were reviewed, approved by, and carried out in compliance with policies of the University Committee on Animal Resources at the University of Rochester. The investigation conforms to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publ. No. 85-23, revised 1996). Mouse pancreatic acini were prepared essentially as described previously (48). Briefly, following CO2 asphyxiation and cervical dislocation, pancreata were removed from freely fed male NIH-Swiss mice (25 g). The tissue was enzymatically digested with type-II collagenase in DMEM with 0.1% bovine serum albumin (BSA) and 1 mg/ml soybean trypsin inhibitor for 30 min, followed by gentle titration. 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.

Preparation of parotid acinar cells. Single cells or small clusters of parotid acinar cells were isolated from freshly dissected parotid glands from NIH-Swiss mice (25 g) by sequential digestion with (single cells) or without (cell clusters) trypsin, followed by collagenase as previously described (13). Following isolation, cells were resuspended in 1% BSA, containing BME supplemented with 2 mM glucose, penicillin-streptomycin, incubated at 37°C, and gassed with 5% CO2 and 95% O2 until ready for use.

Design and characterization of focal laser photolysis instrumenta-
tion. Focal intracellular photolysis was performed using a UV laser (Innova Enterprise; Coherent, Santa Clara, CA), operating at 363.8 nm and coupled into a Nikon TE300 microscope through a dual-port UV flash condenser (Till-Photronics, Pleasanton, CA) as shown in Fig. 1A. The laser output was directed through a neutral density filter wheel (FW102; Thorlabs, Newton, NJ) and a shutter before being coupled into a single mode silica fiber (SM400N; Coastal Conne-
A sigmoidal function of the form

$$\text{ESF}(x) = v_e \text{erf}(v_h x + v_i) + v_x x + v_y$$

was used to fit the ESF data using least squares, where $v_e$, $v_h$, $v_x$, $v_y$, and $v_z$ are fit constants, erf is the Gaussian error function, and $x$ is the edge translation through the beam. Representative ESF data and the corresponding sigmoid fit of Eq. 1 are shown in Fig. 1C. The first derivative of the ESF is the LSF, the Fourier transform of which is equivalent to a slice through the two-dimensional Fourier transform of the PSF, whose FWHM defines the maximum spot size of the photolysis system. At best focus, the PSF-FWHM measured 0.65 and 0.74 µm in the $x$- and $y$-axes, respectively. Modeling of the optical system in Code V (Optical Research Associates, Pasadena, CA) indicated a best-focus $z$-axis FWHM of ~2.1 µm. The computed in-plane FWHM was 0.33 µm. The discrepancy between the calculated and measured widths is likely the result of aberrations of the imaging objective at the excitation wavelength and is equivalent to approximately one-quarter wave of spherical aberration. These results suggest that ~70% of the optical power is contained inside a spot with 1-µm diameter, allowing very targeted molecular uncaging within a well-defined intense focal volume.

Imaging. Before experimentation, aliquots of cell suspensions from pancreas or parotid were loaded by incubation with 4 µM Fluo-4-AM, and either Cp-InsP3/PM (cell permeable InsP3) or NP-EGTA-AM (cell permeable caged-Ca$^{2+}$) as indicated in a HEPES-buffered physiological saline solution (HEPES-PS) containing in mM 5.5 glucose, 137 NaCl, 0.56 MgCl$_2$, 4.7 KCl, 1 Na$_2$HPO$_4$, 10 HEPES (pH 7.4), and 1.2 CaCl$_2$, after which they were resuspended in HEPES-PS and kept at 4°C until ready for use. Before experiments, acini were seeded on coverslips, which formed the base of a superfusion chamber, and mounted on the stage of a Nikon TE300 inverted microscope. Solution changes were accomplished by selecting flow from a multichambered, valve-controlled, gravity-fed reservoir. [Ca$^{2+}$] imaging was performed essentially as described previously (39, 47, 50) by using an inverted epifluorescence Nikon microscope with a ×40 oil immersion objective lens (numerical aperture, 1.3). Cells were excited at 488 ± 10 nm using a monochrometer (TILL Photonics). Fluorescence images were captured and digitized with a digital camera driven by TILL Photonics Vision software. Images were captured every ~40–80 ms with an exposure of 10 ms and 2 × 2 binning. Changes in Fluo-4 fluorescence are expressed as $\Delta F/F_0$, where $F$ is the fluorescence captured at a particular time, and $F_0$ is the mean of the initial 10 fluorescence images captured.

Laser photolysis. Before initiating experiments each day, the $x$-$y$ position of the laser spot in relation to the field of view was assessed by illumination of a drop of fura-2 placed on the coverslip as shown in (Fig. 2A and graphical representation in 2B). Similarly, the beam...
Fig. 3. Photolysis of caged-isopropylidene inositol 1,4,5 trisphosphate (Ci-InsP3) in pancreatic acinar cells. A: a series of images at the times indicated from a small pancreatic acini shown in brightfield (top, left) incubated with 5 μM Ci-InsP3 and the Ca2+ indicator Fluo-4. The laser was positioned in the extreme apical region of one cell (as indicated by the lightning bolt) and exposed to laser illumination to release Ci-InsP3. The images show the initiation of the Ca2+ signal in the apical domain followed by propagation to the basal region. The corresponding kinetic recorded from an apical (blue box) or basal (red box) are shown in a. B (images) and b (kinetic): a typical experiment is shown following photolysis toward the center of a doublet of acinar cells. A limited increase in intracellular calcium concentration ([Ca2+]i) is observed at the photolysis site, which is then followed after a significant latency by initiation and propagation of an apical-to-basal Ca2+ wave. C (images) and c (kinetic): a typical experiment where photolysis was performed in the extreme basal aspects of a small pancreatic acinus. A limited Ca2+ signal is observed at the photolysis site, which is followed by an extended latency before initiation of an apical-to-basal wave. Local apically delimited signals were never observed under these conditions. Changes in Fluo-4 fluorescence are expressed as ΔF/ΔF0, where F is the fluorescence captured at a particular time, and F0 is the mean of the initial 10 fluorescence images captured.
was focused in the z plane to attain the brightest spot, and this position was fixed by means of a motorized z drive. The camera chip coordinates of the brightest pixel were recorded and regarded as the center of the spot illumination. Small acini were positioned using a motorized stage such that the laser beam illuminated the desired region of the cell. Photolysis of caged compounds was accomplished following a brief (50 ms) shutter opening. In initial experiments it was determined empirically that a power density of 16 μW·μm⁻² was sufficient to effectively uncage both NP-EGTA and Ci-InsP₃. The energy resulting from this laser exposure did not have any obvious deleterious effects on [Ca²⁺], in cells not incubated with dye but loaded with dye and was therefore used throughout the study.

RESULTS

**Focal uncaging of Ci-InsP₃ in pancreatic acinar cells.** The apical pole of acinar cells has been documented to be preferentially sensitive to the effects of InsP₃ compared with other regions of exocrine cells. Consistent with these earlier observations, as shown in the images in Fig. 3A and the corresponding kinetic in Fig. 3a, brief focal release of i-InsP₃ close to the luminal plasma membrane in pancreatic acini incubated with 5 μM Ci-InsP₃/PM resulted in a transient increase in [Ca²⁺], at the photolysis site. This initial Ca²⁺ signal was followed after a marked latency by initiation of a Ca²⁺ wave in the extreme apical pole of the cell. Of note, despite spatially limited photolysis, the Ca²⁺ signal always subsequently propagated toward the basal aspects of the cell. On average the latency between photolysis and initiation of the propagating wave was 0.83 ± 0.2 s (defined as time between the laser exposure and an increase in fluorescence at the apical pole of >5% above baseline). The latency was not significantly shortened by increasing the laser power to maximum values (24 μW·μm⁻²) or by increasing the incubated Ci-InsP₃/PM concentration ([Ci-InsP₃/PM]) to 10 μM. Thus this time appears to approach an absolute latency for initiation of the Ca²⁺ signal. In contrast, the latency increased significantly following photolysis in cells incubated with lower concentrations of Ci-InsP₃/PM. For example, in cells incubated with 1 μM Ci-InsP₃/PM the latency increased dramatically to 7.04 ± 0.51 s (see pooled kinetic data in Fig. 5, A and E). Once initiated, the Ca²⁺ wave invariably propagated throughout the cell with a constant velocity of 10.90 ± 0.54 μm/s (5 μM Ci-InsP₃; n = 21 cells). Despite drastically lengthening the latency, the Ca²⁺ wave velocity was not significantly different in cells incubated with 1 μM Ci-IP₃/PM (12.17 ± 0.48 μm/s; n = 11), consistent with initiation of an “all or nothing” process.

To probe the InsP₃ sensitivity of regions outside the apical pole of the cell, the effects of photo release of i-InsP₃ toward the middle and basal aspects of the acinar cells were next investigated. Figure 3, B (images) and c (kinetic), shows a representative experiment in which photolysis of Ci-InsP₃ was achieved toward the middle of a small acinus. Again, a limited increase in fluorescence was generated at the photolysis site. Notably, this initial increase in [Ca²⁺]; never resulted in a propagating Ca²⁺ signal. Instead, following a marked latency of, on average, 3.53 ± 0.39 s (5 μM Ci-InsP₃; n = 20), a Ca²⁺ wave was initiated from the trigger zone Ci²⁺ release sites. Once initiated, the Ca²⁺ wave propagated at a velocity not significantly different from that following apical photolysis of Ci-InsP₃ (11.60 ± 0.90 μm/s; n = 21 cells; P = 0.538). In a similar fashion, photolysis of Ci-InsP₃ close to the basal plasma membrane never resulted in a Ca²⁺ signal propagating from the photolysis site as seen in Fig. 3, C (images) and c (kinetic), and pooled data in Fig. 5B. This observation held even when cells were incubated with higher [Ci-InsP₃/PM] or when the laser power was increased to 24 μW·μm⁻². Again, following an extended latency, which was on average 4.87 ± 0.91 s (5 μM; Ci-InsP₃/PM n = 9) and even longer, averaging 16.91 ± 0.89s at 1 μM Ci-InsP₃/PM (pooled data, Fig. 5E), a Ca²⁺ wave was initiated from the trigger zone and then subsequently propagated toward the basal region of the cell at a velocity of 9.40 ± 1.60 μm/s (n = 4). This wave speed was not significantly different from that initiated by apical focal photolysis (P = 0.181). It should also be noted that local Ca²⁺ release limited to the apical region following photolysis in any cellular region was never observed; if a Ca²⁺ signal was initiated in the apical region, it invariably propagated to the basal region. In total, these data confirm that the apical domain of pancreatic acinar cells is most sensitive to InsP₃ but indicate, somewhat surprisingly, that other regions of the cell are practically so insensitive to InsP₃ that they are unable to support sufficient Ca²⁺ release to initiate a Ca²⁺ wave from the photolysis site. Instead, propagation always occurs from the apical trigger zone to the basal aspects of the cell following a variable latency. These data, however, do not exclude the possibility that following initiation, the propagation of the Ca²⁺ signal is largely independent of the InsP₃R and instead relies on CICR through RyR.

**Focal uncaging in parotid acinar cells.** Previous reports have shown that the apical region of salivary gland acinar cells also constitutes a trigger zone where Ca²⁺ signals are initiated following exposure to InsP₃ or agonist stimulation. In contrast to pancreatic acinar cells, where signals at threshold stimulation can be localized, in parotid or submandibular salivary glands the Ca²⁺ signals invariably globalize in a manner that is consistent, especially at higher [InsP₃], with largely autonomously Ca²⁺ release from extra-apical sites and not a true Ca²⁺ wave (17, 38). A prediction from these data is that the extracellular regions of parotid acinar cells are more sensitive to InsP₃ and may support at least limited, regenerative Ca²⁺ release. This idea was tested by focal photolysis at sites throughout small parotid acini. When the laser was positioned at the apical pole of the cell, photolysis of Ci-InsP₃ resulted in Ca²⁺ release at the spot site followed rapidly by extensive Ca²⁺ release from the trigger zone, as shown in Fig. 4, A (images) and a (kinetic), and pooled data (Fig. 5C). On average the latency in cells incubated with 5 μM Ci-InsP₃/PM was 0.15 ± 0.02 s (n = 21) and longer at 1 μM Ci-InsP₃/PM (0.37 ± 0.03 s; n = 10) and was not significantly shortened by increasing the laser power to 24 μW·μm⁻². The latency was significantly shorter than that observed under similar conditions in pancreatic acinar cells and consistent with findings reported following global InsP₃ photolysis (17). In a similar fashion to pancreatic acinar cells, following initiation the Ca²⁺ wave propagated throughout the parotid acinar cell in a manner consistent with a true Ca²⁺ wave. Notably, the wave proceeded with a faster velocity of 27.81 ± 2.62 μm/s (5 μM Ci-InsP₃/PM) than observed in pancreatic acinar cells but again was not altered significantly as a function of [Ci-InsP₃/PM] (Ca²⁺ wave velocity 26.29 ± 3.01 μm/s; n = 17; 1 μM Ci-InsP₃/PM). Next, photolysis of Ci-InsP₃ was performed in the mid and basal regions of parotid cells. As shown in Fig. 4, B (images) and b (kinetics), photo
Fig. 4. Photolysis of Ci-InsP$_3$ in parotid acinar cells. A: a series of images at the times indicated from a small parotid acini shown in brightfield (top, left) incubated with 5 μM Ci-InsP$_3$ and the Ca$^{2+}$ indicator Fluo-4. The laser was positioned in the extreme apical region of one cell (as indicated by the lightning bolt) and exposed to laser illumination to release Ci-InsP$_3$. The images show the initiation of the Ca$^{2+}$ signal in the apical domain followed by propagation to the basal region. The corresponding kinetic recorded from an apical (blue box) or basal (red box) are shown in a. Apical photolysis results in the rapid initiation of an apical-to-basal wave.

B (images) and b (kinetics): photolysis is performed in a more central location in a small parotid acinus. A limited increase in [Ca$^{2+}$]$_i$ is observed at the photolysis site, which is then followed after short latency by initiation and propagation of an apical-to-basal Ca$^{2+}$ wave. C (images) and c (kinetics): a typical experiment where photolysis was performed in the extreme basal aspects of a small pancreatic acinus. A limited Ca$^{2+}$ signal is observed at the photolysis site, which is followed by a latent period before initiation of an apical-to-basal wave. Note the much shorter latencies in parotid vs. pancreas.
release in the mid region resulted in modest Ca\textsuperscript{2+} release at the site of laser exposure but did not result in a propagating wave from the photolysis site. Increasing laser power or [Ci-InsP\textsubscript{3}/PM] similarly did not result in a wave propagating from the photolysis spot. These data suggest that this cytoplasmic region of the parotid acinar cell does not express sufficient density of InsP\textsubscript{3}R to support the initiation of a propagating wave. Again, following a latency of 0.21 ± 0.02 s, a Ca\textsuperscript{2+} wave was initiated at the trigger zone which propagated at a velocity of 24.20 ± 1.57 μm/s toward the basal pole of the cell. This wave speed was significantly faster than observed in pancreas, although not different from that initiated following photolysis in other regions of parotid cells. No regenerative Ca\textsuperscript{2+} wave occurred at the initial photolysis site when laser exposure was targeted in the extreme basal pole as shown in Fig. 4, C (images) and c (kinetics), and pooled data in Fig. 5D. Instead, an apical-basal Ca\textsuperscript{2+} wave propagating at a velocity of 21.91 ± 1.62 μm/s was initiated after a latency of 0.38 ± 0.05 s (pooled data in Fig. 5).

In summary, focal production of InsP\textsubscript{3} resulted in Ca\textsuperscript{2+} signals in either cell type with similar spatial characteristics; production of InsP\textsubscript{3} in any region of the cell resulted only in a modest transient Ca\textsuperscript{2+} signal at the photolysis site followed by initiation of a Ca\textsuperscript{2+} wave from the extreme luminal pole of the cell, traveling at a fixed velocity in each particular cell type. The latent period was closely related to the distance between the photolysis site and the trigger zone. Despite these similarities, the Ca\textsuperscript{2+} signals in each cell type exhibited very different kinetic profiles, including markedly shorter latent periods prior to wave initiation and faster wave speeds when initiated in parotid acinar cells.
Focal release of Ca\textsuperscript{2+} in exocrine acinar cells. Next, experiments were performed to explore the possible role of classical CICR in the propagation of acinar cell Ca\textsuperscript{2+} signals. Acinar cells were incubated with caged-Ca\textsuperscript{2+} (25 μM NP-EGTA for 30 min) during the loading with Fluo-4. Photolysis was accomplished in either the apical or basal region of each acinar cell type. Fig. 6, A (images) and a (kinetic), shows a representative example of uncaging Ca\textsuperscript{2+} in the apical region of a small pancreatic acinus and illustrates that a limited Ca\textsuperscript{2+} signal could be observed, which decayed within ~400 ms and never propagated away. B: a similar representative experiment is shown performed in parotid cells; B shows images, and b shows the kinetics. Again, a limited, nonpropagating increase in [Ca\textsuperscript{2+}] was observed at the photolysis site. C: shows the pooled data showing the maximum amplitude of the [Ca\textsuperscript{2+}] signal attained from both cell types following photolysis in either the apical or basal region. Qualitatively similar spatial data was observed following basal photolysis; the Ca\textsuperscript{2+} signal did not propagate. Significantly larger limited Ca\textsuperscript{2+} signals were observed following basal photolysis, which were not significantly different when comparing pancreas and parotid.
expanding significantly from the initial photolysis site \((n = 10)\). Similar spatial characteristics were observed when \(Ca^{2+}\) was released by photolysis in the basal pole of pancreatic acinar cells; specifically, no significant propagation of the signal away from the photolysis site occurred \((n = 12)\), although the magnitude of the \(Ca^{2+}\) signals realized were significantly larger \([1.01 \pm 0.22 \text{ vs. } 3.60 \pm 0.50 \Delta F/F_0 \text{ units (apical vs. basal), respectively}]\) as seen in Fig. 6C. As shown in Fig. 6, \(B\) (images) \(b\) (kinetics), qualitatively and quantitatively similar data was obtained following photo release of \(Ca^{2+}\) in parotid acinar cells; photolysis of NP-EGTA resulted in a limited \(Ca^{2+}\) rise, which failed to propagate. In addition, the magnitude of the \(Ca^{2+}\) signals were not significantly different when parotid and pancreas were compared, as shown in Fig. 6C. These data indicate that under these conditions, raising \(Ca^{2+}\) can itself not support significant CICR in exocrine cells and further suggests that RyR activation, at least in isolation, plays a limited role in \(Ca^{2+}\) wave propagation in exocrine cells.

To further investigate the role of RyR in \(Ca^{2+}\) signal initiation and propagation, we next compared the spatial and temporal characteristics of signals generated by focal uncaging in the presence or absence of a blocking concentration of ryanodine \((25, 35, 39)\). Small acini were incubated in 200 \(\mu\text{M}\) ryanodine for 10 min prior to photolysis of \(\text{CI-InsP}_3\). As shown in Fig. 7A, blockade of RyR failed to markedly alter the kinetic characteristics of \(Ca^{2+}\) signals evoked by apical uncaging of \(\text{CI-InsP}_3\) in pancreatic acini. For example, following photo release in cells incubated with 5 \(\mu\text{M}\) \(\text{CI-InsP}_3/\text{PM}\) \((n = 17)\), the latency was \(1.08 \pm 0.15 \text{ s vs. } 0.825 \pm 0.21 \text{ s (no ryanodine)}\), wave speed was \(10.43 \pm 0.75 \text{ \mu m/s vs. } 10.97 \pm 0.53 \text{ \mu m/s (no ryanodine)}\), and time to half peak \(t_{1/2}\) for the rise to peak in the apical region was \(2.45 \pm 0.27 \text{ s vs. } 2.26 \pm 0.25 \text{ s (no ryanodine)}\). These parameters were not significantly different from those obtained in the absence of ryanodine. In contrast, in parotid acini \((n = 16)\), ryanodine incubation increased the latency for the initiation of a \(Ca^{2+}\) signal following laser exposure from \(0.15 \pm 0.1 \text{ s to } 0.3 \pm 0.1 \text{ s (P = 0.001)}\), significantly slowed the wave speed to \(20.5 \pm 2.0 \text{ from 27.7} \pm 2.59 \text{ (P = 0.001)}\), and increased the \(t_{1/2}\) to peak from \(0.75 \pm 0.02 \text{ to } 1.08 \pm 0.11 \text{ s (P = 0.001)}\). Pooled data is shown in Fig. 7B. These data suggest that in parotid acinar cells, in contrast to pancreatic cells, RyRs play a more prominent role in \(Ca^{2+}\) wave propagation following activation of \(\text{InsP}_3\)R.

**DISCUSSION**

We have designed and developed a system to realize a spatially limited spot of intense laser illumination through a high numerical aperture objective on the stage of an inverted microscope. The equipment facilitated experiments to locally photo release cell permeant caged second messengers, allowing a comparison of the spatial and temporal characteristics of \(Ca^{2+}\)-signaling events in two distinct but related polarized exocrine cells. The design of the apparatus is optically straightforward and was relatively inexpensive to construct and could be duplicated by other investigators with similar experimental needs. The experimental paradigm was designed primarily to investigate signaling events in natively buffered intact cells and secondarily to more closely approximate local production of messengers likely to occur under physiological stimulation of the gland in situ. These studies revealed that the individual cell types exhibit both similar and divergent characteristics, which appear to occur largely as a consequence of utilizing common pathways.

**Role of classical CICR.** Photo-production of \(Ca^{2+}\) never resulted in a propagating wave in either cell type, indicating that classical CICR through RyR in isolation does not appear to be a dominant mechanism in these exocrine cells. These data are consistent with a recent study showing that photo release of \(Ca^{2+}\) in the absence of a global increase in [\(\text{InsP}_3\)] failed to evoke regenerative release \((5)\). In pancreatic acinar cells, little effect was observed of blocking RyR, even on \(Ca^{2+}\) signals initiated by \(\text{CI-InsP}_3\). These data contrast to some earlier reports that RyRs contribute to the propagation of \(Ca^{2+}\) waves in pancreatic acinar cells \((25, 35, 39)\) (but see Ref. 15) and are thus most consistent with \(Ca^{2+}\) wave propagation involving sequential activation of \(\text{InsP}_3\)R by \(\text{InsP}_3\) and \(Ca^{2+}\). A caveat that applies to this experimental paradigm is that these data may reflect the fact that blockade of RyR is generally thought to be use dependent and often requires repetitive stimulation in the presence of ryanodine to observe functional effects. Thus, in the present experiments, which were designed to monitor...
signals following acute exposure to relatively low InsP₃, some effects previously attributed to RyR may be underestimated. Notwithstanding this idea, significant effects of RyR blockade were observed in parotid acinar cells, including slowing latency, rise time, and wave propagation velocity. These data could indicate a comparably more significant role of RyR in parotid acinar cells and is consistent with the higher functional sensitivity of parotid to RyR agonists (24). Following RyR blockade, the Ca²⁺ signaling characteristics in parotid and pancreas were, however, still significantly different, indicating that increased utilization of RyR by parotid acinar cells cannot totally account for the differences observed between pancreas and parotid acinar cells.

Spatial characteristics of the Ca²⁺ wave. Following focal generation of i-InsP₃, the spatial aspects of the Ca²⁺ signal in pancreas and parotid were remarkably similar. Local generation of i-InsP₃ in any region other than in the extreme apical domain also invariably resulted in only limited Ca²⁺ release at the photolysis site. Propagation away from sites outside the apical domain was never observed. Photolysis, regardless of the cellular location, always resulted in initiation of substantial Ca²⁺ release from the apical trigger zone and subsequent entrainment of an apical-to-basal Ca²⁺ wave. In an earlier study by Fogarty and colleagues (15), in which caged-InsP₃ was introduced by microinjection, generally similar conclusions were made regarding the enhanced sensitivity of the apical domain of pancreatic acinar cells over other regions of the cell. However, in a minority of cells in this study, photolysis in more basally localized regions resulted in a Ca²⁺ wave propagating away from the photolysis site. Although the reasons for these seemingly disparate results are presently unclear, a possible contributing factor is that the spot size achieved in the previous study was approximately threefold larger than in the present study (FWHM ~2 μm vs. 0.65 μm this study), giving an effective photolysis spot of ~4 μm. This less spatially limited spot would be predicted to activate many more InsP₃R during laser exposure and possibly, in turn, generate sufficient Ca²⁺ release to facilitate initiation of a wave. This might occur especially if extra-apical photolysis engaged InsP₃R, known to be in relative abundance close to lateral plasma membranes at cell-to-cell borders (23, 24, 52).

Although qualitatively similar in both cell types and at all [Ci-InsP₃] tested, the exclusive activation of the apical-to-basal wave was most striking following photolysis of lower [Ci-InsP₃] limited to the extreme basal region. Under these conditions, a particularly extended latent period (~10 s; Fig. 5B) was observed before initiation of the Ca²⁺ wave in pancreatic acinar cells. In parotid acinar, although this latent period was markedly shorter (~350 ms, Fig. 5, C–E), a clear refractory period following the initial Ca²⁺ release was always observed before initiation of a wave. A conclusion from these experiments is that the density of InsP₃R in both the extreme apical or parotid is not sufficient to initiate and then support regenerative CICR. This is consistent with numerous immunocytochemical studies, which show a low abundance of InsP₃R in all regions of acinar cells compared with the extreme apical domain (24, 34, 43, 52). Presumably the lack of responsiveness of extra-apical InsP₃R occurs because the receptor is not exposed to sufficiently elevated Ca²⁺ required for coactivation of InsP₃-induced Ca²⁺ release until the apical release sites are engaged (8, 16). Physiological stimulation is unlikely to result in uniform InsP₃ generation and would be predicted to result in locally high [InsP₃], which subsequently dissipates by diffusion and metabolism. This might occur, for example, at sites of synapsing nerves. The inability of InsP₃ in isolation to activate extra-apical Ca²⁺ release may ensure the consistent initiation of an apical-to-basal wave, an event thought to be fundamentally important for the appropriate activation of effectors that underlie the physiological activation of secretion in exocrine cells. In addition, the exclusive apical initiation would also be rendered independent of the localization of secretagogue receptors and primary sites of InsP₃ production.

Ca²⁺ wave initiation. The initiation of a Ca²⁺ wave is proposed to rely on the progressive recruitment of elemental Ca²⁺ release events through clusters of InsP₃R, which sum to reach a critical level and thus provide sufficient diffusing Ca²⁺ for activation of neighboring Ca²⁺ release sites (9, 10). According to this generally accepted model, the latent period following i-InsP₃ equilibration would represent the time required to engage sufficient InsP₃ in the apical region to provide the trigger for Ca²⁺ wave propagation. In parotid acinar cells, this time approximates the sum of the time for InsP₃ to diffuse [estimate of the diffusion rate: 200 μm²/s (1)] and the shortest latent period observed following apical uncaging. In contrast, in pancreas under identical conditions, markedly extended latent periods were observed. These times were significantly greater than required for diffusion and may reflect the increased time to recruit a productive Ca²⁺ release event from lower densities or less sensitive InsP₃R in the apical region of pancreas compared with parotid. These ideas are consistent with the known sensitivities of the predominant InsP₃R expressed and the relative abundance of total InsP₃R, given the assumption that the increase in InsP₃R number previously noted is predominately expressed in the apical region of each tissue (19, 24, 52).

Ca²⁺ wave propagation. Following initiation of the apical Ca²⁺ signal, the Ca²⁺ wave velocity was remarkably constant under the various conditions in a particular cell type; for example, the wave velocity was not influenced either by [Cl-I-InsP₃/PM] or the site of i-InsP₃ generation in either cell type. These observations are consistent with activation of an “all or none” process, a primary characteristic of a true propagating wave, which relies on the sequential recruitment of release sites throughout the cytoplasm. In pancreatic acinar cells, we have previously reported similar conclusions based on observations under conditions that resulted in a global and uniform photolysis of InsP₃ (17). In this study, over a wide range of [InsP₃], a relatively constant wave speed was generated. In contrast, in parotid acinar cells, wave speed was observed to increase dramatically with [InsP₃] and be more consistent with global Ca²⁺ signals underpinned by largely autonomous Ca²⁺ release from areas of ER outside the trigger zone, rather than a true propagating wave (17). It should be noted, however, that in the present study, it is unlikely that the high global [InsP₃] achieved following uniform photolysis is ever achieved following focal uncaging. Based on an estimate of the diffusion rate of InsP₃, the latency observed in each cell type would be sufficient for [Cl-I-InsP₃] to equilibrate before entrainment of the apical-to-basal wave (cell diameter ~15 μm) (1). This would, however, be predicted to result in a relatively low global [i-InsP₃]. These present experiments,
therefore, more closely approximate the situation following uniform photolysis of low [InsP3] and perhaps physiological activation of cells. Under these conditions, in the earlier study, Ca\(^{2+}\) signals with properties more consistent with a propagating wave were also elicited in parotid acinar cells following uniform photolysis. Interestingly, the wave velocity of secretagogue-induced Ca\(^{2+}\) waves in pancreatic acinar cells has been reported to be dependent on agonist concentration (37). Thus although the present data provide information indicative of an InsP3-induced wave produced in isolation, secretagogue stimulation results in a more complex series of signal transduction events, which are capable of modulating the properties of the wave. These events could potentially include agonist-stimulated production of other calcium-mobilizing second messengers (51) or activation of kinases that regulate the Ca\(^{2+}\)-handling machinery (47).

Although the apical-to-basal Ca\(^{2+}\) wave velocity was constant in each cell type under various [Ci-InsP3] and following stimulation was achieved through dialysis of solutions from a whole majority of previous studies by our group and others, buffering characteristics of the signals observed in each cell type. In parotid vs. parotid could conceivably contribute to the distinct temporal characteristics of the Ca\(^{2+}\) buffering properties in pancreas and parotid share many morphological and functional features, parotid acinar cells exhibit Ca\(^{2+}\) signaling kinetics, which are more rapid than pancreatic cells. This appears to occur in the main by using generally similar pathways. The enhanced kinetics are consistent with the differing primary effectors for the Ca\(^{2+}\) signals in the individual cells. In pancreas, Ca\(^{2+}\) is required primarily in the apical domain to satisfy the requirement for the relatively slow process of exocytosis. In contrast, the rapid kinetics in parotid acinar cells appear to reflect fine coordination for the appropriate activation of spatially separated ion channels necessary for the initiation of fluid secretion.

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