Modulation of $[Ca^{2+}]_i$ Signaling Dynamics and Metabolism by Perinuclear Mitochondria in Mouse Parotid Acinar Cells*

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Parotid acinar cells exhibit rapid cytosolic calcium signals ($[Ca^{2+}]_i$) that initiate in the apical region but rapidly become global in nature. These characteristic $[Ca^{2+}]_i$ signals are important for effective fluid secretion, which critically depends on a synchronized activation of spatially separated ion fluxes. Apically restricted $[Ca^{2+}]_i$ signals were never observed in parotid acinar cells. This is in marked contrast to the related pancreatic acinar cells, where the distribution of mitochondria has been suggested to contribute to restricting $[Ca^{2+}]_i$ signals to the apical region. Therefore, the aim of this study was to determine the mitochondrial distribution and the role of mitochondrial Ca$^{2+}$ uptake in shaping the spatial and temporal properties of $[Ca^{2+}]_i$, signaling in parotid acinar cells. Confocal imaging of cells stained with Mitotracker dyes (Mitotracker Green FM or Mitotracker CMXRos) and SYTO dyes (SYTO-16 and SYTO-61) revealed that a majority of mitochondria is localized around the nucleus. Carbachol (CCh) and caged inositol 1,4,5-trisphosphate-evoked $[Ca^{2+}]_i$ signals were delayed as they propagated through the nucleus. This delay in the CCh-evoked nuclear $[Ca^{2+}]_i$ signal was abolished by inhibition of mitochondrial Ca$^{2+}$ uptake with ruthenium red and Ru360. Likewise, simultaneous measurement of $[Ca^{2+}]_i$ with mitochondrial $[Ca^{2+}]_i$ ($[Ca^{2+}]_{mit}$), using fura-2 and rhod-FF, respectively, revealed that mitochondrial Ca$^{2+}$ uptake was also inhibited by ruthenium red and Ru360. Finally, at concentrations of agonist that evoke Ca$^{2+}$ oscillations, mitochondrial Ca$^{2+}$ uptake, and a nuclear $[Ca^{2+}]_i$ delay, CCh also evoked a substantial increase in NADH autofluorescence. This autofluorescence exhibited a predominant perinuclear localization that was also sensitive to mitochondrial inhibitors. These data provide evidence that perinuclear mitochondria and mitochondrial Ca$^{2+}$ uptake may differentially shape nuclear $[Ca^{2+}]_i$ signals but more importantly drive mitochondrial metabolism to generate ATP close to the nucleus. These effects may profoundly affect a variety of nuclear processes in parotid acinar cells while facilitating efficient fluid secretion.

Regulation of both the spatial and temporal properties of intracellular Ca$^{2+}$ ($[Ca^{2+}]_i$) signals is known to underlie the specificity of stimulus-response coupling in a variety of cell types (1, 2). For example, gene transcription in T cells can be differentially regulated by the frequency of Ca$^{2+}$ oscillations (3), and in vascular smooth muscle cells global Ca$^{2+}$ signals control contraction, whereas localized Ca$^{2+}$ release (Ca$^{2+}$ sparks) causes vasodilatation (4). These examples highlight the importance of understanding the mechanisms that underlie the “shaping” of [Ca$^{2+}]_i$ signals and how ultimately different patterns of [Ca$^{2+}]_i$ signals can differentially activate physiological end points.

By comparing the spatial and temporal kinetics of [Ca$^{2+}]_i$ signaling in the morphologically and functionally related exocrine acinar cells of the pancreas and salivary glands, we have demonstrated previously (5) how different [Ca$^{2+}]_i$ signals may be tuned to evoke specific physiological responses in these cells. The major function of pancreatic acinar cells is the exocytosis of zymogen granules that can be activated by apically confined [Ca$^{2+}]_i$, signals (5), suggested to be the major physiological [Ca$^{2+}]_i$, signal evoked by threshold agonist concentrations (7). These apically confined [Ca$^{2+}]_i$, signals are in part because of the distribution of mitochondria, which form a belt around the apically located zymogen granules (8, 9). These perigranular mitochondria likely serve two main functions. First, mitochondrial Ca$^{2+}$ uptake provides a buffer barrier thereby preventing the spread of [Ca$^{2+}]_i$, waves to the basal part of the cell (8–10). Second, the Ca$^{2+}$ taken up by mitochondria drives ATP production locally for the energy-consuming exocytotic process (11). The major function of parotid acinar cells is fluid secretion, and this is most effectively activated by rapid global [Ca$^{2+}]_i$, signals. We proposed that rapid global [Ca$^{2+}]_i$, signals facilitate the almost simultaneous activation of Ca$^{2+}$-dependent Cl$^-$ channels on the apical membrane and Ca$^{2+}$-dependent K$^+$ channels on the basolateral membrane (5). The latter maintains the membrane potential at hyperpolarizing potentials and the driving force for Cl$^-$ efflux, thereby ensuring efficient ion and water movement (12). Interestingly, even at threshold stimulation parotid acinar cells exhibit rapid global [Ca$^{2+}]_i$, signals, and apically confined [Ca$^{2+}]_i$, signals were never observed (5). Given the many similarities between parotid and pancreatic acinar cells, the differences in the spatial and temporal patterns of [Ca$^{2+}]_i$, signaling between these two cell types

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1 The abbreviations used are: [Ca$^{2+}]_i$, intracellular calcium concentration; CCh, carbamylcholine (carbachol); FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; OGB2, Oregon Green BAPTA 2; InsP$_3$, inositol 1,4,5-trisphosphate; caged InsP$_3$, n-myo-InsP$_3$, P-4(5)-1-(2-nitrophenyl)-ethyl ester; CaM, calmodulin; RuRed, ruthenium red; NPC, nuclear pore complex; rhod, rhodamine; TRITC, tetramethylrhodamine isothiocyanate.

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are difficult to reconcile if they have a similar distribution of mitochondria. Therefore, the aim of the present study was to determine the mitochondrial distribution and the role of mitochondrial Ca\textsuperscript{2+} uptake in shaping [Ca\textsuperscript{2+}], signals in parotid acinar cells. The study revealed for the first time that there was an absence of any definitive perigranular mitochondria in parotid cells compared with that observed in pancreatic acinar cells. The observation that mitochondrial Ca\textsuperscript{2+} distribution in parotid cells is therefore likely facilitates the rapid, global [Ca\textsuperscript{2+}], signals observed. In contrast, the mitochondria are localized mainly to a region surrounding the nucleus in parotid acinar cells. In addition, mitochondrial Ca\textsuperscript{2+} uptake in this region differentially shapes nuclear [Ca\textsuperscript{2+}] signals and enhances metabolism and thus ATP generation close to the nucleus. This may serve to differentially modulate a variety of processes within the nucleus.

**EXPERIMENTAL PROCEDURES**

**Isolation of Parotid Acinar Cells**—Single cells or small clusters of parotid acinar cells were isolated from freshly dissected parotid glands from wild type Swiss Black mice by sequential digestion with (single cells) or without (cell clusters) trypsin followed by collagenase as described previously (5, 13). Following isolation, cells were resuspended in 1% bovine serum albumin containing basal modified Eagle’s media supplemented with 2 mM glutamine, penicillin/streptomycin and incubated at 37°C and gassed with 5% CO\textsubscript{2} and 95% O\textsubscript{2} until ready for use. Prior to experimentation, aliquots of cell suspensions were loaded with the appropriate fluorescent dyes in a HEPES-buffered physiological saline solution (HEPES/PSS) containing the following (in mM): 5.5 glucose, 137 NaCl, 0.56 MgCl\textsubscript{2}, 4.7 KCl, 1 Na\textsubscript{2}HPO\textsubscript{4}, 10 HEPES, pH 7.4, 1.2 CaCl\textsubscript{2}, after which they were resuspended in HEPES-PSS and kept at 4°C until ready for use. For measurement of [Ca\textsuperscript{2+}], cells were loaded with 2 μM fura-2/AM for 30 min at room temperature. For the simultaneous measurement of cytosolic and mitochondrial [Ca\textsuperscript{2+}], cells that had been pre-loaded with fura-2/AM were then loaded with 10 μM dihydro-rhod-FF/AM for 10 min at 4°C. Dihydro-rhod-FF was formed by reacting 10 μl of 1 mg/ml NaBH\textsubscript{4} with 40 μl of 1 mM rhod-FF stock solution. In some experiments (to confirm mitochondrial Ca\textsuperscript{2+} uptake) cells were simultaneously loaded with 5 μM rhod-FF and 0.5 μM MitoTracker Green FM for 10 min at 4°C. Pharmacological inhibition of mitochondrial Ca\textsuperscript{2+} uptake was achieved by pre-incubating cells with 30 μM ruthenium red (RuRed) (Sigma) (14) or 10 μM RuS660 (Calbiochem) (15) for 30 min at room temperature. Stock solutions of RuS660 (1 mM) were made up in deoxygenated water immediately prior to use.

**Digital Imaging of Cytosolic and Mitochondrial [Ca\textsuperscript{2+}]**—Cells were allowed to adhere to a glass coverslip and continually perfused with oxygenated HEPES-PSS containing the following (in mM): 5.5 glucose, 137 NaCl, 0.56 MgCl\textsubscript{2}, 4.7 KCl, 1 Na\textsubscript{2}HPO\textsubscript{4}, 10 HEPES-PSS, 5.5 glucose, 137 NaCl, 0.56 MgCl\textsubscript{2}, 4.7 KCl, 1 Na\textsubscript{2}HPO\textsubscript{4}, 10 HEPES, pH 7.4, 1.2 CaCl\textsubscript{2} and then illuminated offline. For the simultaneous measurement of cytosolic and mitochondrial [Ca\textsuperscript{2+}], fura-2- and rhod-FF-loaded cells were excited alternately with light at 340 (10 ms), 380 (10 ms), and 550 nm (50 ms). Fura-2- and rhod-FF-emitted fluorescence was collected sequentially through a fura-2-rhodamine dual emission dichroic beamsplitter (Chroma Technology Corp., Rockingham, VT). Sequential images were acquired at a rate of 1 Hz, and changes in mitochondrial [Ca\textsuperscript{2+}] were represented by ∆F/F, where ∆F = F - F\textsubscript{0} and F\textsubscript{0} is the average starting fluorescence recorded over the initial 10 frames of the image sequence. In some experiments mitochondrial Ca\textsuperscript{2+} uptake was confirmed by dual loading cells with MitoTracker Green FM and rhodFF. These cells were excited with light at 488 ± 15 (10 ms) and 550 ± 25 nm (50 ms), and the emitted fluorescence from both dyes was collected through a fluorescein isothiocyanate/TRITC dual emission di-
ionic, pH, and osmotic environment in the nucleus compared with the cytosol. In an attempt to minimize potential dye artifacts and mimic a more physiological stimulus, intact parotid acinar cells were loaded with the ratiometric dye, fura-2, and stimulated with 300 nM CCh to track the spatiotemporal \([\text{Ca}^{2+}]_i\) signals in a similar manner to Fig. 1A. Shows a bright field image and a series of pseudo-colored fluorescence images of a single patch-clamped parotid acinar cell containing 70 μM OGB2 used to track the spatio-temporal \([\text{Ca}^{2+}]_i\) signal evoked by flash photolysis of 3 μM caged InsP₃. Aii, kinetic profile of InsP₃-evoked Ca²⁺ release in the apical (blue trace), basal (green trace), and the nuclear region (red trace). Inset represents an expanded time scale. The perinuclear \([\text{Ca}^{2+}]_i\) increase was much smaller and exhibited much slower kinetics than the apical and basal \([\text{Ca}^{2+}]_i\) increase (representative trace of 9 of 23 cells stimulated with 3 μM caged InsP₃). Bi, bright field and pseudo-colored fluorescence images (panels A–H) of control fura-2-loaded parotid acinar cells stimulated with 300 nM CCh to track the spatio-temporal \([\text{Ca}^{2+}]_i\) signals (color-coded as in Ai). The location of the nucleus was confirmed by staining cells with 1 μM SYTO-16. Bii, corresponding kinetic profile of CCh-evoked \([\text{Ca}^{2+}]_i\) signals are shown in the apical, basal, and the nuclear region of images in Bii (n = 6). Panels A–H in Bii represent the images in Bi. Ci and Cii, identical experiment to Bi and Bii except cells were pre-incubated with 10 μM Ru360 for 30 min prior to stimulation with CCh (n = 6). Similar results were obtained with 30 μM RuRed (n = 4) (see mean data in Table I).

Fig. 1. CCh- and InsP₃-evoked \([\text{Ca}^{2+}]_i\) signals were delayed in the nucleus of parotid acinar cells. Ai, bright field image and a series of pseudo-colored fluorescence images of a single patch-clamped parotid acinar cell containing 70 μM OGB2 used to track the spatio-temporal \([\text{Ca}^{2+}]_i\) signal evoked by flash photolysis of 3 μM caged InsP₃. Aii, kinetic profile of InsP₃-evoked Ca²⁺ release in the apical (blue trace), basal (green trace), and the nuclear region (red trace). Inset represents an expanded time scale. The perinuclear \([\text{Ca}^{2+}]_i\) increase was much smaller and exhibited much slower kinetics than the apical and basal \([\text{Ca}^{2+}]_i\) increase (representative trace of 9 of 23 cells stimulated with 3 μM caged InsP₃). Bi, bright field and pseudo-colored fluorescence images (panels A–H) of control fura-2-loaded parotid acinar cells stimulated with 300 nM CCh to track the spatio-temporal \([\text{Ca}^{2+}]_i\) signals (color-coded as in Ai). The location of the nucleus was confirmed by staining cells with 1 μM SYTO-16. Bii, corresponding kinetic profile of CCh-evoked \([\text{Ca}^{2+}]_i\) signals are shown in the apical, basal, and the nuclear region of images in Bii (n = 6). Panels A–H in Bii represent the images in Bi. Ci and Cii, identical experiment to Bi and Bii except cells were pre-incubated with 10 μM Ru360 for 30 min prior to stimulation with CCh (n = 6). Similar results were obtained with 30 μM RuRed (n = 4) (see mean data in Table I).
performed on a confocal microscope, care was taken to ensure that the focal plane was as close to the center of the nucleus as possible to avoid contamination from out-of-focus light coming from dye above and below the nucleus. Cells were discarded from analysis if the nucleus was out of focus.

Fig. 1B shows an initial [Ca\(^{2+}\)] signal evoked by 300 nm CCh in the apical, basal, and nucleus of a typical control untreated cell. These data show that the CCh-evoked [Ca\(^{2+}\)] signal is significantly delayed in the nucleus compared with the corresponding non-nuclear basal region, thereby supporting preliminary data using flash photolysis of caged InsP\(_3\) (Fig. 1A). The time to half-maximum response was 0.19 ± 0.07 s slower in the basal region compared with the apical region, whereas the nuclear response was 0.47 ± 0.08 s slower than the apical region (Fig. 1B and Table I). This nuclear [Ca\(^{2+}\)] delay was quantified by subtracting the time between the half-maximum increase in the apical and non-nuclear basal region (A-B) from the time between the half-maximum increase in the apical and nuclear basal region (A-N) in every cell (Table I). On average, the nuclear Ca\(^{2+}\) delay was found to be 0.28 ± 0.1 s. The observed differences in the kinetics of CCh-evoked [Ca\(^{2+}\)] signals in the nucleus versus the non-nuclear basal region could be due to differential fura-2 compartmentalization into intracellular organelles. One might predict that this would be more pronounced in the non-nuclear basal region compared with the nucleus due to the presence of endoplasmic reticulum. The average fluorescence signal from this region would therefore be affected by the “saturated” fura-2 signal emitted from the endoplasmic reticulum. To test this, a series of pilot experiments were performed whereby fura-2-loaded cells were permeabilized by perfusing with a “cytosol-like” solution containing 0.4 IU/ml streptolysin-O (BD Biosciences), identical to that described previously (13). Permeabilization caused a complete loss of cytosolic dye, as monitored by the fura-2 isosbestic 360-nm fluorescence signal (data not shown). Any residual fluorescence would most likely be due to dye trapped within the intracellular organelles. Under these conditions it was found that streptolysin-O treatment decreased fluorescence from 1166 ± 110 gray levels above background to 70 ± 12 gray levels above background (6 experiments, in which >4 cells were imaged). This was not significantly different from autofluorescence of non-loaded cells treated identically (fluorescence decreased from 63 ± 7 to 49 ± 8 gray levels above background; 6 experiments, in which >4 cells were imaged), confirming that fura-2 compartmentalization into intracellular organelles is unlikely to contribute to the nuclear [Ca\(^{2+}\)] delay.

The Delayed CCh-evoked Nuclear [Ca\(^{2+}\)] Signals Are Abolished by Inhibition of Mitochondrial Ca\(^{2+}\) Uptake—To test if this nuclear [Ca\(^{2+}\)] delay is due to mitochondrial Ca\(^{2+}\) uptake slowing the rate at which [Ca\(^{2+}\)] increases in the nucleus, cells were pre-incubated with mitochondrial Ca\(^{2+}\) uptake inhibitors, RuRed and Ru360. Although RuRed is known to have nonspecific effects (18, 19), a derivative Ru360 is a more specific and potent inhibitor of mitochondrial Ca\(^{2+}\) uptake (14, 15). At the concentrations and pre-incubation times tested, both RuRed and Ru360 have been shown previously (20) to inhibit significantly mitochondrial Ca\(^{2+}\) uptake in intact cells.

In the present study pre-incubation with either 30 μM RuRed or 10 μM Ru360 for 30 min prior to stimulation with CCh altered the kinetics of the CCh-evoked [Ca\(^{2+}\)] increase (Fig. 1C and Table I). Most important, the nuclear [Ca\(^{2+}\)] delay was significantly reduced from 0.28 ± 0.1 s (n = 6) to 0.02 ± 0.01 s (n = 4) by RuRed and to 0.03 ± 0.03 s (n = 6) by Ru360 (Fig. 1C and Table I). Further analysis showed that both RuRed and Ru360 significantly reduced the time to peak response in all regions of the cell and abolished any spatial differences in the time to peak values (Table I). Moreover, in control cells the magnitude of the CCh-evoked [Ca\(^{2+}\)] response in the basal region (0.37 ± 0.07 ratio units) and nucleus (0.39 ± 0.08 ratio units) was significantly lower than the corresponding apical region (0.49 ± 0.07 ratio units), although there was no significant difference between all three regions of RuRed- or Ru360-treated cells (Table I). These data indicate that both the magnitude (in the basal and nuclear regions) and rate of Ca\(^{2+}\) release (in all cellular regions) are markedly increased by inhibition of mitochondrial Ca\(^{2+}\) uptake. These data are consistent with previous studies (21) suggesting that mitochondrial Ca\(^{2+}\) uptake suppresses the local feedback activation of Ca\(^{2+}\) release by buffering local [Ca\(^{2+}\)]. One might also predict, from these data, that mitochondrial Ca\(^{2+}\) uptake shapes [Ca\(^{2+}\)] signals in all regions of the cell consistent with a homogeneous distribution of mitochondria. However, upon closer examination, the effect of mitochondrial Ca\(^{2+}\) uptake inhibitors was most striking in the nucleus. This is therefore consistent with perinuclear mitochondria providing a buffer barrier around the nucleus that impedes the propagation or diffusion of [Ca\(^{2+}\)], signals to the nucleus. In addition, the effects of RuRed and Ru360 suggest that the nuclear [Ca\(^{2+}\)] delay is largely independent of any nuclear dye artifacts. Perhaps more importantly, because the affinity of most dyes is reportedly higher in the nucleus (17), the apparent magnitude of the nuclear [Ca\(^{2+}\)] signals may be significantly overestimated.

Parotid Acinar Cell Mitochondria Exhibit a Predominantly Perinuclear Distribution—To investigate whether this anomalous nuclear [Ca\(^{2+}\)], change is due to a specific distribution of mitochondria, we used conventional epifluorescence imaging

| Table I | Summary of mean data from experiments shown in Fig. 1 |
|-----------------|-----------------------------------------------------|
| Control (n = 6) | Ruhenium red (n = 4) | Ru360 (n = 6) |
| Apical to peak (s) | 2.68 ± 0.18 | 1.81 ± 0.23 | 2.08 ± 0.26 |
| Basal to peak (s) | 3.11 ± 0.23 | 1.86 ± 0.22 | 2.07 ± 0.27 |
| Nucleus to peak (s) | 3.50 ± 0.29 | 1.92 ± 0.28 | 2.16 ± 0.32 |
| Apical peak Δ ratio | 0.49 ± 0.07 | 0.42 ± 0.05 | 0.50 ± 0.03 |
| Basal peak Δ ratio | 0.38 ± 0.07 | 0.40 ± 0.05 | 0.47 ± 0.03 |
| Nucleus peak Δ ratio | 0.29 ± 0.08 | 0.40 ± 0.06 | 0.49 ± 0.04 |
| Time between 1/2 max in apical and 1/2 max in basal (A-B) (s) | 0.19 ± 0.07 | 0.15 ± 0.07 | 0.10 ± 0.02 |
| Time between 1/2 max in apical and 1/2 max in nucleus (A-N) (s) | 0.47 ± 0.08 | 0.17 ± 0.07 | 0.12 ± 0.04 |
| Nuclear delay (A-N)-(A-B) (s) | 0.28 ± 0.01 | 0.02 ± 0.01 | 0.03 ± 0.03 |

*p < 0.05, significantly different from control parameter (unpaired Student’s t test).
| *p < 0.0001, significantly different from apical (Wilcoxon test for pairs).
| **p < 0.0001, significantly different from A-B (paired Student’s t test).
| ***p < 0.0001, significantly different from zero (one sample t test).
(Fig. 2A) and confocal imaging in the Z dimension, to obtain three-dimensional reconstructed images (Fig. 2B) of parotid acinar cell clusters co-stained with mitochondrial and nuclear staining dyes. Fig. 2A shows a conventional epifluorescence image of cells loaded with MitoTracker Red (CMXRos), which accumulates in the mitochondrial matrix, and SYTO-16, which labels nuclei by binding to DNA. The mitochondria appear to be distributed as “ring-like” structures around the nucleus. However, due to the much higher signal from SYTO-16 compared with MitoTracker Red, it is difficult to simultaneously image both mitochondria and nuclei in the same cells and accord any three-dimensional association or colocalization of these two organelles. Therefore, we used volume-rendered three-dimensional confocal imaging of cells simultaneously loaded with MitoTracker Green FM and SYTO-61. By using this technique, the most striking observation was that there was a lack of any obvious perigranular mitochondria in parotid acinar cells compared with that observed in pancreatic acinar cells. In addition, although mitochondria appear in other regions of the cell, there was a clear population of mitochondria that had a perinuclear distribution. These mitochondria appear to form a basket-like structure or cavity within which the nucleus sits (Fig. 2B). Each cavity appears to be lined with mitochondria consisting of “finger-like” projections that wrap around the nucleus (see Z stack images, Fig. 2B). Such a distribution of mitochondria may not necessarily behave as an efficient buffer barrier but may be ideally situated to take up Ca\(^{2+}\) during a rapid global [Ca\(^{2+}\)] signal to drive metabolism and thus generate ATP close to the nucleus.

CCh Stimulates Mitochondrial Ca\(^{2+}\) Uptake with Slower Kinetics than the CCh-evoked [Ca\(^{2+}\)] Signal—To confirm that inhibition of the nuclear [Ca\(^{2+}\)] delay by pre-incubation with Ru360 or RuRed is due to inhibition of mitochondrial Ca\(^{2+}\) uptake, cells were loaded with both fura-2 and rhod-FF (or rhod-2) to monitor simultaneously cytosolic and mitochondrial [Ca\(^{2+}\)]. This produced a highly punctate distribution of dye reminiscent of the mitochondrial accumulation of MitoTracker dyes. However, due to the high affinity of rhod-2 for Ca\(^{2+}\) (\(K_d \approx 195\ \text{nm}\) (22)), any residual cytosolic rhod-2 may detect cytosolic [Ca\(^{2+}\)] changes, possibly masking mitochondrial changes. We therefore chose rhod-FF as this has a much lower affinity for Ca\(^{2+}\) (\(K_d \approx 19 \mu\text{M}\) (23)) and is unlikely to detect cytosolic [Ca\(^{2+}\)] changes in the face of small amounts of residual cytosolic dye.

Control cells loaded with both fura-2 and rhod-FF showed that CCh evoked a substantial increase in rhod-FF fluorescence (~7.98 ± 0.98% ΔF/F\(_{0}\), Fig. 3, A and C, mean data), which occurred with slower kinetics than the rapid cytosolic [Ca\(^{2+}\)] increase (Fig. 3A; \(n = 6, 24\) cells). This suggests that the CCh-evoked increase in [Ca\(^{2+}\)] promotes mitochondrial Ca\(^{2+}\) uptake, as reported in other non-excitable cell types (22, 24, 25). Although the changes in rhod-FF fluorescence are uncalibrated signals, the low affinity of rhod-FF for Ca\(^{2+}\) suggests that these changes likely represent changes in mitochondrial [Ca\(^{2+}\)] (several \(\mu\text{M}\)) that are much larger than those observed in the cytosol (~1 \(\mu\text{M}\)). This is not surprising because several studies (using both fluorescent dyes and targeted aequorin) have demonstrated that mitochondrial [Ca\(^{2+}\)] can reach such levels (25, 26). It was also noted that the rate of decrease in rhod-FF fluorescence following the removal of CCh was much slower than the rate of increase (see Fig. 3A). This could be due to the slow activity of the mitochondrial Na\(^+\)/Ca\(^{2+}\)-exchanger, the major pathway for mitochondrial Ca\(^{2+}\) efflux, as has previously been reported in other cells (27). Following stimulation...
**Fig. 3.** Simultaneous measurement of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ reveals that CCh increases $[Ca^{2+}]_m$ that is sensitive to mitochondrial $Ca^{2+}$ uptake inhibitors. Cells were loaded with 2 μM fura-2 for 30 min at room temperature followed by 5 μM rhod-FF for 10 min at 4 °C, which produced optimum loading conditions for the simultaneous measurement of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ (n = 6). Ai shows a bright field image and rhod-FF fluorescent images (A–C) that correspond to the superimposed fura-2 ratio and rhod-FF traces from the same cells in Ai that were acquired quasi-simultaneously. Treatment of cells with 0.5 μM FCCP to depolarize the mitochondria caused the punctate rhod-FF fluorescence to decrease (Aii) and become more diffuse (Ai, panel C), confirming that the dye had accumulated in the mitochondria. B, identical experiment to A, except cells had been pre-incubated with 10 mM Ru360 (representative experiment, n = 6). CCh evoked mitochondrial $Ca^{2+}$ uptake was inhibited, and the effect of FCCP on $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ was dampened. Similar results were obtained with 30 μM RuRed (n = 6). C, mean mitochondrial $Ca^{2+}$ uptake from control cells (A), cells pre-incubated with 10 mM Ru360 (B), or 30 μM RuRed (*, p < 0.05, Mann-Whitney unpaired test).
with CCh, treatment of cells with 0.5 μM FCCP to depolarize the mitochondria caused the punctate rhod-FF fluorescence to decrease and become more diffuse (Fig. 3Ai, panel C) with a corresponding increase in [Ca\(^{2+}\)] (Fig. 3Aii). This suggests that the dye accumulates in mitochondria and that the mitochondria take up Ca\(^{2+}\) during stimulation with CCh (27).

Because stimulation of parotid acinar cells with CCh causes profound cell shrinkage, the slower rate of increase in rhod-FF fluorescence could be due to cell volume changes or mitochondrial volume changes that would be exacerbated using a single wavelength dye such as rhod-FF. To test for this, cells were simultaneously loaded with rhod-FF and MitoTracker Green FM. Both these dyes accumulate in mitochondria, but rhod-FF is Ca\(^{2+}\)-insensitive, whereas MitoTracker Green FM is Ca\(^{2+}\)-sensitive. Changes in the ratio of rhod-FF versus MitoTracker Green FM fluorescence (550/488 ratio) therefore represent mitochondrial Ca\(^{2+}\) uptake and that the subsequent addition of FCCP completely inhibits the rhod-FF fluorescence signal (data not shown), confirming that this was due to mitochondrial Ca\(^{2+}\) changes rather than changes in cell volume.

Pre-treatment of Cells with Ru360 or RuRed Prevent Mitochondrial Ca\(^{2+}\) Uptake—The CCh-evoked increase in rhod-FF fluorescence (representative trace, Fig. 3A, and mean control %ΔF/F, 7.98 ± 0.98, Fig. 3C) was largely inhibited when cells were pre-incubated with 10 μM Ru360 (Fig. 3, Bi and C; %ΔF/F, 1.07 ± 0.47) or 30 μM RuRed (Fig. 2, Cii–Civ). In addition, the FCCP-evoked decrease in rhod-FF fluorescence and the corresponding increase in [Ca\(^{2+}\)], was also largely abrogated (Fig. 3Bii). These data therefore provide evidence that the increase in rhod-FF fluorescence is due to mitochondrial Ca\(^{2+}\) uptake and that the subsequent addition of FCCP promotes mitochondrial Ca\(^{2+}\) efflux. Collectively these data provide convincing evidence that the CCh-evoked increase in [Ca\(^{2+}\)] promotes perinuclear mitochondrial Ca\(^{2+}\) uptake which in turn slows the increase in nuclear [Ca\(^{2+}\)].

CCh Increases NADH Autofluorescence—In addition to shaping nuclear Ca\(^{2+}\) signals, perinuclear mitochondrial Ca\(^{2+}\) uptake is likely to drive mitochondrial metabolism and the generation of ATP by activation of mitochondrial dehydrogenases (28, 29). To test this, we measured NADH autofluorescence, a technique employed extensively to monitor mitochondrial metabolism (30, 31). Fluorescent NADH is generated during the Krebs cycle from NAD\(^+\) (non-fluorescent) where it feeds into the electron transport chain to drive ATP synthesis. Moreover, mitochondrial Ca\(^{2+}\) activates three dehydrogenases of the Krebs cycle (28, 29); thus NADH autofluorescence represents a measure of Ca\(^{2+}\)-dependent mitochondrial metabolism and presumably ATP generation.

NADH autofluorescence was monitored by exciting parotid acinar cells with light at 350 nm and measuring the emitted light at 450 nm. In Fig. 4A, cells were excited at 350 nm (for 1 s exposure) before and during treatment with 300 nM CCh or 0.5 μM FCCP. Continuous NADH autofluorescence was not monitored using these exposure times due to profound photobleaching and possible photo-toxicity. However, this strategy clearly illustrates that NADH autofluorescence has a similar spatial distribution to perinuclear mitochondria. Reducing the exposure time to 100 ms and acquiring every second, NADH autofluorescence was continuously monitored during treatment with CCh or FCCP (Fig. 4, B and C). The kinetic profile of the relative change in autofluorescence (%ΔF/F, see Fig. 4C) and the corresponding fluorescent images (Fig. 4B) clearly demonstrate that CCh increases and FCCP decreases autofluorescence with similar kinetics to mitochondrial [Ca\(^{2+}\)] shown in Fig. 3Aii. Finally, the CCh-evoked increase in NADH autofluorescence was significantly inhibited by pre-incubation with either 10 μM Ru360 (11.28 ± 0.80 to 2.50 ± 0.76, Fig. 4, C and E) or 30 μM RuRed (11.28 ± 0.80 to 2.73 ± 0.53, Fig. 4E) and by transient treatment with 0.5 μM FCCP (11.28 ± 0.80 to 1.00 ± 0.24, Fig. 4, D and E). These data collectively suggest that perinuclear mitochondrial Ca\(^{2+}\) uptake directly drives mitochondrial metabolism and thus likely generates elevated levels of ATP close to the nucleus.

DISCUSSION

The primary function of parotid acinar cells is to secrete copious amounts of salivary fluid. This is achieved by the almost simultaneous activation of Ca\(^{2+}\)-dependent Cl\(^{-}\) channels on the apical membrane and Ca\(^{2+}\)-dependent K\(^{+}\) channels on the basolateral membrane (5). The latter maintain the membrane potential by hyperpolarizing potentials and the driving force for Cl\(^{-}\) efflux thereby ensuring efficient ion and water movement (12). Parotid acinar cells exhibit rapid, global [Ca\(^{2+}\)] signals that are important for the almost simultaneous activation of these spatially separated ion fluxes (5). Apically confined [Ca\(^{2+}\)], signals, which are commonly observed in pancreatic acinar cells and are due, in part, to the localization of active perigranular mitochondria (8–10), were never observed in parotid acinar cells (5). In the face of these observations the following important questions are raised. What is the mitochondrial distribution in parotid acinar cells? What function does mitochondrial Ca\(^{2+}\) uptake serve? Data from the present study revealed an absence of perigranular mitochondria in parotid acinar cells. This likely facilitates the rapid, global [Ca\(^{2+}\)] signals that are important for effective fluid secretion and helps to reconcile some of the spatial and temporal differences in [Ca\(^{2+}\)], signaling observed between parotid and pancreatic acinar cells (5). Instead, it was found that a large proportion of mitochondria exhibited a perinuclear distribution in parotid acinar cells, and that this resulted in both CCh- and InsP\(_{4}\)-evoked [Ca\(^{2+}\)], signals being significantly delayed in the nucleus of these cells.

Differential regulation of nuclear [Ca\(^{2+}\)] signaling or “dampened” nuclear [Ca\(^{2+}\)] signals have been observed in a variety of cells under different experimental conditions (32–35). Several reports have suggested that these observations are due to modulation of the nuclear pore complex (36, 37), differential nuclear Ca\(^{2+}\) buffering (35, 38), or even nuclear dye artifacts (17). Although we cannot completely rule out these effects, an important observation from the present study is that inhibition of mitochondrial Ca\(^{2+}\) uptake completely abolishes the nuclear [Ca\(^{2+}\)] delay.

It has been demonstrated that pancreatic acinar cells contain a minor population of mitochondria that are also localized to the perinuclear region and that have been suggested to give rise to an observed differential nuclear [Ca\(^{2+}\)] signaling (10, 32). In particular, it was shown that perinuclear mitochondria take up Ca\(^{2+}\) specifically when Ca\(^{2+}\) is uncaged locally in the nucleus (10). These elegant experiments suggested that perinuclear mitochondria could play a role in preventing [Ca\(^{2+}\)] signals from invading the nucleus or confine [Ca\(^{2+}\)] signals to the nucleus that originates there (39). However, these studies did not specifically demonstrate functional perinuclear mitochondrial Ca\(^{2+}\) uptake during agonist-evoked [Ca\(^{2+}\)] signaling, but rather emphasized a greater functional role of perigranular mitochondria in shaping the [Ca\(^{2+}\)] changes in pancreatic acinar cells (9, 10).

Differential shaping of nuclear [Ca\(^{2+}\)] signaling by perinuclear mitochondrial Ca\(^{2+}\) uptake has the potential to generate diverse transcriptional responses and thus profoundly affect
cell function and cell fate. This results from the presence of many Ca\(^{2+}\)-dependent effectors in the nucleus, such as the transcriptional repressor DREAM (40), Ca\(^{2+}\)/CaM-dependent kinases (41), as well as CREB-dependent transcription (42). It is, however, important to note that the current data show that CCh-evoked [Ca\(^{2+}\)]\(_i\) signals are on average only ~0.3 s slower in the nucleus than in the corresponding non-nuclear basal region. In addition, there was no significant difference in the magnitude of the observed nuclear and basal [Ca\(^{2+}\)]\(_i\) response. Given this, it seems unlikely that any currently known Ca\(^{2+}\)-dependent effectors could sufficiently decode such subtly different [Ca\(^{2+}\)]\(_i\) signals.

Alternatively, the observed nuclear [Ca\(^{2+}\)] delay may simply be a consequence of the pronounced perinuclear mitochondrial Ca\(^{2+}\) uptake and, rather than specifically shaping nuclear [Ca\(^{2+}\)] signals, perinuclear mitochondria may serve to generate ATP close to the nucleus for the plethora of ATP-dependent nuclear effectors. The present study clearly demonstrated that CCh substantially increases NADH autofluorescence and that this was sensitive to inhibitors of mitochondrial Ca\(^{2+}\) uptake (RuRed and Ru360) or mitochondrial uncouplers (FCCP). Due to the slow kinetics of mitochondrial Ca\(^{2+}\) uptake and Ca\(^{2+}\) efflux (27), mitochondria have the capacity to decode oscillations in [Ca\(^{2+}\)]\(_i\), into efficient metabolism and ATP production (24, 30, 43). For example, NADH autofluorescence has been shown to oscillate during CCK-evoked [Ca\(^{2+}\)]\(_i\) oscillations in pancreatic acinar cells (30). Interestingly, as the frequency of [Ca\(^{2+}\)]\(_i\) oscillations increases the mitochondrial NADH responses fuse into an elevated plateau. Similar results had been observed previously in hepatocytes (24). In the present study, CCh at concentrations that produce [Ca\(^{2+}\)]\(_i\) oscillations evoked a substantial sustained increase in NADH, and oscillations in the NADH response were never observed. This mechanism is clearly designed to match increased energy demand to increased ATP supply, which appears to be highly efficient in parotid acinar cells particularly in the perinuclear region.

There are several important ATP-dependent effects in the nucleus, including regulation of kinases (41), chromatin remodeling (44), DNA replication (45), and cell cycle control (46). In addition, ATP is required for a variety of ion transporters and

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**Fig. 4.** CCh increases NADH autofluorescence indicative of enhanced mitochondrial metabolism and localized ATP generation. NADH autofluorescence was monitored in unloaded parotid acinar cells by exciting at 350 nm light and measuring the emitted light at 480 nm. 

A illustrates that NADH autofluorescence exhibits a similar perinuclear distribution to mitochondria (1-s exposure) before and after stimulation with CCh and FCCP. 

B, continuous monitoring of NADH autofluorescence (100-ms exposure every second) showing bright field and NADH fluorescent images (Bi) and corresponding kinetic profile of the relative (%ΔF/F\(_0\)) change in autofluorescence (Bii, n = 9). This shows that CCh increases and FCCP decreases fluorescence suggesting that NADH autofluorescence was due to changes in mitochondrial metabolism. C–E, representative traces (C and D) and mean data (E) showing that the CCh-evoked increase in NADH autofluorescence was inhibited by pre-incubation of cells with 10 μM Ru360 (C, n = 7 and mean data in E) or 30 μM ruthenium red (RuRed) (mean data in E, n = 6) or by short term treatment with 0.5 μM FCCP (D, n = 11). E, summary of mean data (*, p < 0.05, Mann-Whitney unpaired test).
channels on the nuclear envelope, such as Na\(^+\)/K\(^-\)-ATPases (47), P2X\(_Y\) receptors (48), and the nuclear envelope Ca\(^{2+}\)-ATPase (49). However, one of the most important functions of perinuclear mitochondria is likely the modulation of nuclear translocation of macromolecules, such as CaM, kinases/phosphatases, transcription factors, as well as RNA, by the nuclear pore complex (NPC). There is now increasing evidence that such nuclear translocation is ATP- and/or Ca\(^{2+}\)-dependent (50–54). Specifically, ATP on the cytoplasmic face and Ca\(^{2+}\) on the nuleoplasmic face both promote opening of the NPC and thus nuclear translocation (52, 54). There is, however, some evidence that high [Ca\(^{2+}\)](>1 \mu M) (55, 56) and depletion of the nuclear envelope Ca\(^{2+}\) store inhibits the NPC and nuclear translocation (54, 57, 58). Thus, local ATP generated close to the nuclear envelope could facilitate nuclear translocation either directly or by maintaining nuclear envelope Ca\(^{2+}\) store refilling (39, 57). In addition, more recently it was shown that nuclear translocation of the protein histone H1 in intact cardiomyocytes is critically dependent on functional mitochondria and enzymatic phosphotransfer by creatine kinase and/or adenylyl kinase (59). Perinuclear mitochondrial Ca\(^{2+}\) uptake, which drives metabolism and ATP generation close to the nucleus, therefore likely plays a pivotal role in nuclear translocation.

In summary, parotid acinar cells do not exhibit the striking perigranular mitochondrial distribution observed in pancreatic acinar cells (8–10). In fact a large proportion of mitochondria are localized to the perinuclear region of these cells. The absence of a definitive perigranular mitochondrial belt in parotid acinar cells likely facilitates the rapid, global [Ca\(^{2+}\)] signals that are important for the activation of effective fluid secretion in these cells. These agonist-evoked rapid global [Ca\(^{2+}\)] signals stimulate Ca\(^{2+}\) uptake into perinuclear mitochondria that may shape nuclear [Ca\(^{2+}\)] signals, but perhaps more importantly profoundly increase mitochondrial metabolism, thereby generating ATP close to the nucleus. These data collectively suggest that perinuclear mitochondria likely play an essential role in regulating a variety of nuclear functions while facilitating effective fluid secretion in parotid acinar cells.

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