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Research Article

Mitochondria in Calcium Signaling in the Exocrine Pancreas

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Abstract

Background: Pancreatic acinar cells secrete digestive enzymes in response to hormonal and neural stimuli that trigger complex intracellular calcium ($Ca2^+$) signals. These signals, often originating in the apical pole and exhibiting oscillatory patterns at physiological stimulus strengths, are crucial for driving exocytosis. Mitochondria, dynamic organelles essential for cellular energy metabolism, are also known to accumulate $Ca2^+$ and are strategically positioned near $Ca2^+$ release sites in many cell types, including pancreatic acinar cells. However, the precise contribution of mitochondria to shaping physiological $Ca2^+$ signals and maintaining cellular homeostasis in the exocrine pancreas requires detailed investigation.
Objective: This study aimed to elucidate the role of mitochondria in modulating agonist-induced cytosolic $Ca2^+$ signals and regulating cellular bioenergetics in isolated rat pancreatic acinar cells.
Methods: Pancreatic acinar cells or small acinar clusters were isolated from rat pancreas. Cytosolic $Ca2^+$ ($[Ca2^+]$cyt) was measured using ratiometric imaging of Fura-2 or confocal imaging of Fluo-4. Mitochondrial $Ca2^+$ ($[Ca2^+]$mito) and Membrane Potential were monitored using Rhod-2 and TMRM, respectively, with confocal microscopy. Cells were stimulated with physiological Concentrations of Cholecystokinin (CCK) or acetylcholine analogue carbachol (CCh). Mitochondrial function was manipulated using the mitochondrial $Ca2^+$ uniporter (MCU) inhibitor Ruthenium 360 (Ru360) and mitochondrial uncouplers/inhibitors (FCCP, oligomycin). Cellular ATP levels were measured using a luciferase-based assay.
Results: Stimulation with CCK or CCh induced repetitive cytosolic $Ca2^+$ oscillations localized primarily to the apical, granule-rich pole of the acinar cell. These cytosolic Ca2+ rises were closely followed by transient increases in mitochondrial $Ca2^+$, indicating rapid $Ca2^+$ uptake by perigranular mitochondria. Inhibition of mitochondrial Ca2+ uptake using Ru360, or dissipation of using FCCP (which prevents uptake), significantly altered the pattern of cytosolic $Ca2^+$ signals. Specifically, the decay phase of individual Ca2+ transients was prolonged, peak amplitudes were sometimes increased, and the oscillatory pattern often transitioned towards a more sustained elevation, particularly at moderate agonist concentrations. Physiological mitochondrial Ca2+ uptake was associated with a transient hyperpolarization or stabilization and maintained cellular ATP levels during stimulation. Conversely, conditions mimicking pathological $Ca2^+$ overload led to sustained mitochondrial Ca2+ elevation, depolarization, and a decline in ATP levels.
Conclusion: Mitochondria play a critical dual role in shaping $Ca2^+$ signals and maintaining bioenergetic homeostasis in pancreatic acinar cells. By rapidly taking up $Ca2^+$ released from apical ER stores, they act as dynamic buffers that influence the amplitude, duration, and oscillatory pattern of physiological cytosolic $Ca2^+$ signals. This $Ca2^+$ uptake simultaneously stimulates mitochondrial ATP production, providing the energy required for $Ca2^+$ extrusion and secretory processes. However, this system is vulnerable, as excessive $Ca2^+$ influx can overwhelm mitochondrial capacity, leading to dysfunction potentially relevant to pancreatic pathology.

Introduction

The exocrine pancreas plays a vital role in digestion by synthesizing, storing, and secreting large quantities of digestive enzymes and bicarbonate-rich fluid into the duodenum. The primary functional unit responsible for enzyme secretion is the

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pancreatic acinar cell. These highly polarized epithelial cells respond to hormonal stimuli, mainly Cholecystokinin (CCK), and neurotransmitters, primarily Acetylcholine (ACh), released postprandially [1]. Receptor activation triggers intracellular signaling cascades, with changes in cytosolic free calcium concentration ([Ca^{2+}]cyt) playing a central role in initiating the fusion of enzyme-containing zymogen granules with the apical plasma membrane (exocytosis) [2,3].Calcium signaling in pancreatic acinar cells is characterized by its complex spatio-temporal organization. Stimulation with physiological concentrations of agonists typically evokes repetitive oscillations in [Ca^{2+}]cyt, which often initiate in and remain largely confined to the apical pole, the region containing the zymogen granules and adjacent to the acinar lumen [4,5]. These localized, oscillatory signals are thought to be optimal for driving efficient secretion while minimizing potentially toxic effects associated with sustained global elevations in [Ca2+]cyt [6]. The primary source of Ca^{2+} for these signals is the Endoplasmic Reticulum (ER), mobilized via activation of Phospholipase C (PLC) and subsequent generation of Inositol 1,4,5-Trisphosphate (IP3), which binds to and opens IP3 receptors (IP3Rs) predominantly located on the apical ER [7]. Store depletion can then trigger Store-Operated Ca^{2+} Entry (SOCE) across the basolateral membrane to replenish ER stores and sustain signaling [8].

Mitochondria are essential organelles primarily known for generating ATP through oxidative phosphorylation. However, they are also increasingly recognized as crucial players in intracellular Ca^{2+} homeostasis and signaling across various cell types [9,10]. Mitochondria possess the ability to rapidly accumulate Ca^{2+} from the cytosol, driven by the steep electrochemical gradient across the inner mitochondrial membrane (mitochondrial membrane potential, $\Delta\psi m$, typically -150 to -180 mV) [11]. Ca^{2+} enters the mitochondrial matrix primarily via the Mitochondrial Calcium Uniporter (MCU), a highly selective channel whose molecular components were identified around 2010-2011 [12,13]. Calcium efflux from mitochondria occurs mainly via the Na^+/Ca^{2+} exchanger (NCLX) or the H^+/Ca^{2+} exchanger.In many excitable and non-excitable cells, mitochondria are strategically positioned near ER Ca^{2+} release sites (e.g., IP3Rs or ryanodine receptors), forming close structural and functional associations [14]. This proximity allows mitochondria to efficiently sense and shape local Ca^{2+} dynamics. By taking up released Ca^{2+}, mitochondria can act as temporary buffers, modulating the amplitude and duration of cytosolic Ca^{2+} transients and potentially contributing to the generation or termination of Ca^{2+} oscillations [15]. Furthermore, mitochondrial Ca2+ uptake serves a key bioenergetic role: Ca2+ influx into the matrix stimulates several dehydrogenases of the Krebs cycle and potentially oxidative phosphorylation itself, boosting ATP production to meet the increased energy demands associated with cellular activity, such as ion pumping and exocytosis [16].

Pancreatic acinar cells possess abundant mitochondria, which are notably concentrated in the perigranular region surrounding the zymogen granule store at the apical pole, precisely where Ca2+ signals originate and exocytosis occurs [17,18]. This strategic localization strongly suggests a significant role for mitochondria in regulating local Ca2+ dynamics and providing ATP for secretion in these cells. Indeed, early studies demonstrated mitochondrial Ca^{2+} uptake following agonist stimulation and links between mitochondrial function and Ca2+ signaling or secretion were proposed [19]. However, with the advent of improved fluorescent indicators, specific inhibitors (like those targeting the MCU), and advanced imaging techniques, a more detailed dissection of the precise role of mitochondria in shaping physiological, oscillatory Ca2+ signals and supporting cellular bioenergetics during stimulation became feasible.Acinar cell Ca2+ signaling is also highly relevant to pancreatic pathology. Supramaximal or pathological stimulation leads to sustained, global elevations in [Ca2+]cyt, mitochondrial Ca^{2+} overload, mitochondrial depolarization, ATP depletion, and ultimately acinar cell injury and the initiation of pancreatitis [20]. Understanding how mitochondria handle Ca^{2+} under physiological conditions is therefore crucial for understanding the mechanisms that protect against, or predispose to, pathological outcomes.

Material and Methods

Isolation of Pancreatic Acinar Cells

Pancreatic acini or single acinar cells were isolated from male Wistar rats (150-200 g) using collagenase digestion, as previously described with minor modifications [21,22]. Briefly, rats were euthanized following institutional guidelines (approved by Animal Ethics Committee). The pancreas was perfused in situ via the common bile duct with a Ca^{2+}free buffer, then removed and minced. Tissue fragments were digested with purified collagenase (e.g., Type IV, Worthington or Sigma) in a buffer containing soybean trypsin inhibitor and BSA, with gentle agitation at 37°C. Digestion was stopped by washing with Ca^{2+}containing buffer. Dispersed acini/cells were filtered, washed, and resuspended in a HEPES-buffered physiological salt solution (PSS) containing (in mM): NaCl 137, KCl 4.7, KH2PO4 1.2, MgSO4 1.1, CaCl2 1.28, Glucose 10, HEPES 10, supplemented with essential amino acids and 0.1% BSA, pH 7.4. Cells were used within 4-6 hours of isolation.

Measurement of Cytosolic $Ca2^+$ ([$Ca2^+$] cyt)

Fura-2 Ratiometric Imaging: Isolated acini were loaded with the ratiometric Ca^{2+} indicator Fura-2 AM (3-5 μM, Molecular Probes/Invitrogen) for 30-45 minutes at room temperature in PSS. After loading, acini were washed and allowed to de-esterify for at least 15 minutes. Acini were placed in a perfusion chamber on the stage of an inverted microscope equipped with a xenon arc lamp, filter wheels, and a fluorescence imaging system (e.g., MetaFluor/MetaMorph, Molecular Devices). Cells were excited alternately at 340 nm and 380 nm, and emission was collected >510 nm. The ratio of fluorescence intensities (F340/F380) was calculated pixel-by-pixel or averaged over selected regions of interest (ROIs), typically targeting the apical pole of individual cells within an acinus. Ratios were converted to approximate [Ca^{2+}]cyt values using calibration parameters if required, or presented as ratio changes.

Fluo-4 Confocal Imaging:For higher spatio-temporal resolution, acinar cells were loaded with the non-ratiometric indicator Fluo-4 AM (2-4 μM, Molecular Probes) for 20-30 minutes. Cells were washed and imaged using a laser scanning confocal microscope (e.g., Leica SP5, Zeiss LSM 710) equipped with an argon laser (488 nm excitation) and appropriate detectors (emission 500-550 nm). Images were acquired rapidly (e.g., 1-5 frames/second) from a focal plane capturing the apical region. Changes in Fluo-4 fluorescence intensity (F/F0, where F0 is baseline fluorescence) were used as an indicator of [Ca^{2+}]cyt.

Measurement of Mitochondrial $Ca2^+$ ([$Ca2^+$] mito)

Acinar cells were loaded with the mitochondrial Ca^{2+} indicator Rhod-2 AM (2-5 μM, Molecular Probes) for 30-45 minutes at 37°C (loading is temperature-dependent and requires membrane potential). Co-loading with a mitochondrial marker like MitoTracker Green FM (100 nM, Molecular Probes) was some-

times performed to confirm mitochondrial localization of the Rhod-2 signal. Cells were washed and imaged using confocal microscopy (e.g., 543 nm or 561 nm excitation, emission >580 nm). Changes in Rhod-2 fluorescence intensity (F/F0) within individual mitochondrial structures (identified by morphology or co-staining) were measured in response to stimuli.

Measurement of Mitochondrial Membrane Potential

Acinar cells were loaded with the potentiometric dye Tetramethylrhodamine Methyl Ester (TMRM, 20-50 nM, Molecular Probes) for 20-30 minutes at 37°C. TMRM accumulates in mitochondria driven by $\Delta\psi m$, and its fluorescence intensity is proportional to the potential (in non-quenching mode). Cells were imaged using confocal microscopy (e.g., 543/561 nm excitation, emission >580 nm). A decrease in TMRM fluorescence indicates mitochondrial depolarization, while an increase suggests hyperpolarization. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 1-2 μM) or FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone, 1-2 μM), protonophores that collapse $\Delta\psi m$, were used as controls to establish baseline fluorescence.

Cell Stimulation and Treatments

ACells loaded with indicators were continuously perfused with PSS at 37°C. Agonists - Cholecystokinin octapeptide (CCK-8, sulfated, 10-100 pM, Bachem or Sigma) or Carbachol (CCh, 0.3-1 μM, Sigma) - were added to the perfusion solution for specified durations. For inhibition studies, cells were pre-incubated with:

- Ruthenium 360 (Ru360, MCU inhibitor, 5-10 μM, Calbiochem/EMD Millipore) for 15-30 minutes. Alternatively, Ruthenium Red (broader inhibitor, 10-20 μM) could be used, acknowledging its off-target effects.
- FCCP (1 μM) or Oligomycin (inhibitor of ATP synthase, 1-5 μg/mL, Sigma) to manipulate mitochondrial energetics.
- Thapsigargin (SERCA pump inhibitor, 1 μM, Calbiochem) to deplete ER Ca^{2+} stores.

Measurement of Cellular ATP Levels

Total cellular ATP content was measured using a luciferase-based assay kit (e.g., ATP Bioluminescence Assay Kit HS II, Roche, or CellTiter-Glo, Promega). Acinar cell suspensions (~10^5 cells per condition) were incubated under basal conditions or stimulated with agonists +/- inhibitors for relevant time periods (e.g., 10-30 minutes). Cells were lysed according to the kit protocol, and luminescence, proportional to ATP concentration, was measured using a luminometer. ATP levels were normalized to protein content (measured by BCA assay, Pierce) or cell number and expressed relative to basal levels.

Statistical Analysis

Data from imaging experiments typically represent responses from multiple cells (n=10-30) within multiple acini from several independent preparations (N=3-5 pancreata). Data are presented as mean ± standard error of the mean (SEM). Peak amplitudes, oscillation frequencies, decay times, and integrated responses were quantified where appropriate. Comparisons between control and treated groups were made using Student's t-test or ANOVA with suitable post-hoc tests. P-values < 0.05 were considered statistically significant.

Results

Physiological Agonist Stimulation Induces Apical Cytosolic Ca2⁺ Oscillations

Stimulation of isolated rat pancreatic acini with physiological concentrations of CCK (e.g., 30-50 pM) or CCh (e.g., 0.5 μM) induced repetitive oscillations in cytosolic Ca^{2+}, as measured by Fura-2 or Fluo-4 imaging. Confocal imaging with Fluo-4 confirmed that these oscillations typically initiated in and were of largest amplitude within the apical, granule-rich pole of the acinar cells, consistent with previous reports. At these concentrations, sustained global elevations in $[Ca^{2+}]$cyt were generally avoided.

Mitochondria Rapidly Accumulate Ca2⁺ Following Cytosolic Rises

Simultaneous or sequential imaging of cytosolic Ca^{2+} (Fluo-4) and mitochondrial Ca^{2+} (Rhod-2) revealed a close temporal coupling between the two compartments. Each apical cytosolic Ca^{2+} transient was followed, with a very short delay (within seconds), by a rise in mitochondrial Ca^{2+} specifically within the mitochondria located in the apical perigranular region. Basal mitochondria showed little or no change in $[Ca^{2+}]$mito. This demonstrates efficient Ca^{2+} uptake by strategically positioned apical mitochondria during physiological signaling events (Figure 1).

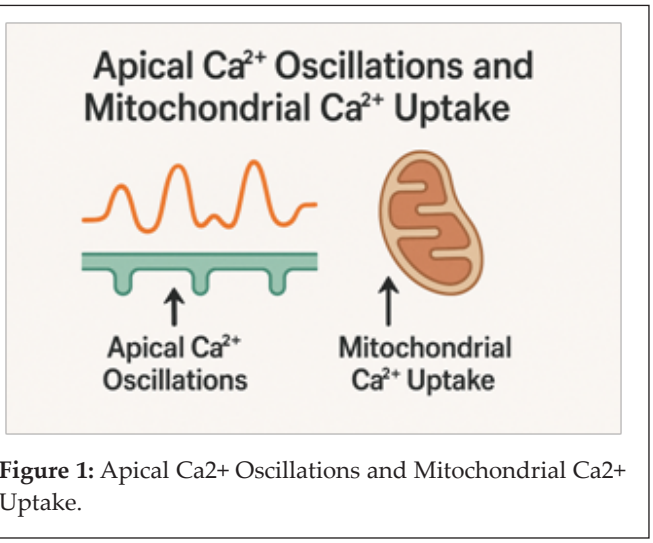

Figure 1: Apical Ca2+ Oscillations and Mitochondrial Ca2+ Uptake.

Inhibition of Mitochondrial Ca2⁺ Uptake Alters Cytosolic Ca2+ Signaling

To assess the role of mitochondrial Ca^{2+} uptake in shaping cytosolic signals, we inhibited the MCU using Ru360 or collapsed the driving force ($\Delta\psi m$) using FCCP. Pre-treatment with Ru360 (10 μM) significantly attenuated the CCK-induced mitochondrial Ca^{2+} transients (confirming uptake inhibition, data not shown) and markedly altered the cytosolic Ca^{2+} response. While oscillations could still occur, the decay phase of individual cytosolic Ca^{2+} transients was significantly prolonged. In many cells, especially at moderate agonist concentrations, the oscillatory pattern was lost, transitioning into a more sustained elevation of apical $[Ca^{2+}]$cyt. Similar effects prolonged decay and tendency towards sustained elevation were observed upon treatment with FCCP (1 μM). These results strongly suggest that rapid mitochondrial Ca^{2+} uptake normally contributes to the sharp termination of cytosolic Ca2+ peaks and helps maintain the oscillatory pattern by buffering Ca^{2+} near the release sites(Figure 2).

Mitochondrial Ca2⁺ Uptake Supports Bioenergetics During Stimulation

We monitored mitochondrial membrane potential ($\Delta\psi m$) using TMRM during physiological stimulation. Agonist-induced Ca^{2+} oscillations were often associated with either a stabilization or a slight transient hyperpolarization of $\Delta\psi m$ in apical mitochondria consistent with Ca^{2+} stimulating respiration. To assess the impact on cellular energy levels, total ATP content was measured. Stimulation with CCK or CCh for 10-20 minutes generally

maintained or slightly increased cellular ATP levels compared to unstimulated controls. However, if mitochondrial Ca^{2+} uptake was prevented (e.g., by Ru360 pre-treatment) or if mitochondrial ATP synthesis was directly inhibited (e.g., by oligomycin), agonist stimulation led to a significant decrease in cellular ATP levels. This indicates that physiological mitochondrial Ca^{2+} uptake is coupled to enhanced ATP production, which is necessary to meet the energy demands of stimulated secretion (e.g., $Ca2^{+}$ pumping by PMCA/SERCA, exocytosis).

Pathological $Ca2^{+}$ Signals Lead to Mitochondrial Dysfunction

In contrast to physiological oscillations, stimulation with high, supramaximal concentrations of CCK (e.g., 10 nM) induced a large, sustained rise in global $[Ca^{2+}]$cyt. This was associated with a massive and sustained elevation of $[Ca^{2+}]$mito and a profound, irreversible depolarization of $\Delta\psi$m (collapse of TMRM fluorescence), Under these conditions, cellular ATP levels significantly decreased even without inhibitors. This confirms that while mitochondria efficiently handle physiological Ca^{2+} signals, pathological Ca^{2+} overload overwhelms their capacity, leading to mitochondrial dysfunction and ATP depletion, key events in the initiation of pancreatitis (Figure 3).

Discussion

The crucial and multifaceted role of mitochondria in regulating intracellular Ca^{2+} signaling and cellular bioenergetics in pancreatic acinar cells. Our findings demonstrate that mitochondria, strategically positioned near apical Ca^{2+} release sites, actively participate in shaping the physiological Ca^{2+} signals required for enzyme secretion. The rapid uptake of Ca^{2+} by perigranular mitochondria following each cytosolic Ca^{2+} transient provides strong evidence for their role as dynamic Ca^{2+} buffers. By sequestering Ca^{2+} released from the ER, mitochondria limit the spatial spread and temporal duration of the cytosolic signal. The observed prolongation of Ca^{2+} transient decay times and the shift from oscillatory to sustained responses upon inhibition of mitochondrial uptake (using Ru360 or FCCP) directly support this buffering function. This shaping of the Ca^{2+} signal is likely critical for maintaining the specificity and efficiency of downstream processes like exocytosis, while preventing the detrimental effects of prolonged global Ca^{2+} elevation. The ability of mitochondria to buffer Ca^{2+} might be particularly important in the restricted apical pole where Ca^{2+} release is concentrated. This function complements Ca^{2+} extrusion by plasma membrane Ca^{2+} ATPases (PMCAs) and re-uptake into the ER by SERCA pumps in terminating Ca^{2+} signals.mBeyond simply buffering, mitochondrial Ca^{2+} uptake serves a vital bioenergetic purpose. Our results show that physiological Ca^{2+} signals are associated with maintained or enhanced mitochondrial function (stabilized/hyperpolarized $\Delta\psi$m) and preserved cellular ATP levels. Inhibiting mitochondrial Ca^{2+} uptake or ATP synthesis during stimulation led to ATP depletion, underscoring the importance of mitochondrial activation in meeting the increased energy demands of the secreting cell. Ca^{2+} entry into the mitochondrial matrix stimulates key dehydrogenases and potentially components of the respiratory chain, boosting NADH production and oxidative phosphorylation [23]. The ATP generated is essential for powering ion pumps (SERCA, PMCA) that restore Ca^{2+} homeostasis after each transient, as well as for fueling the process of zymogen granule exocytosis itself [24]. Thus, mitochondria create a positive feedback loop where the Ca^{2+} signal they help shape also stimulates the energy supply needed to sustain that signal and the resulting cellular work.

However, this study also illustrates the vulnerability of this system. Pathological, sustained Ca^{2+} elevations induced by supramaximal agonist stimulation overwhelmed the buffering and bioenergetic capacity of mitochondria, leading to massive Ca^{2+} accumulation, mitochondrial depolarization (a sign of permeability transition pore opening or metabolic collapse), and ATP depletion. This mitochondrial dysfunction is considered a key early event in the pathogenesis of acute pancreatitis. Understanding the mechanisms that allow mitochondria to efficiently handle physiological Ca^{2+} signals but succumb to pathological overload is therefore critical. Factors like the expression levels of MCU and NCLX, the buffering capacity of the mitochondrial matrix, and the status of the antioxidant defense system likely play important roles.In summary, mitochondria in pancreatic acinar cells are not passive bystanders but dynamic regulators integrated into the Ca^{2+} signaling and metabolic machinery. They shape physiological signals, support cellular energetics, and represent a critical control point whose dysfunction contributes to pathology.

Conclusion

Mitochondria play an indispensable dual role in pancreatic acinar cell function. Strategically located near apical Ca^{2+} release sites, they act as rapid buffers, taking up released Ca^{2+} to shape the amplitude, kinetics, and oscillatory pattern of physiological cytosolic Ca^{2+} signals essential for regulated enzyme secretion. Concurrently, this mitochondrial Ca^{2+} uptake stimulates oxidative phosphorylation, ensuring adequate ATP supply to meet the energy demands of ion transport and exocytosis during stimulation. This intricate coupling of Ca^{2+} signaling and mitochondrial bioenergetics is vital for normal pancreatic function, but its capacity can be overwhelmed by pathological Ca^{2+} overload, leading to mitochondrial dysfunction and contributing to the initiation of pancreatitis. Understanding the mechanisms governing mitochondrial Ca^{2+} handling in acinar cells is therefore crucial for both physiology and pathology.

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