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Role of mitochondria in Ca^{2+} oscillations and shape of Ca^{2+} signals in pancreatic acinar cells

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Abstract

We studied the role of mitochondria in Ca^{2+} signals in fura-2 loaded exocrine pancreatic acinar cells. Mitochondrial depolarization in response to carbonylcyanide-*p*-tryfluoromethoxyphenyl hydrazone or rotenone (assessed by confocal microscopy using rhodamine-123) induced a partial but statistically significant reduction in the decay of Ca^{2+} signals under different experimental conditions. Spreading of Ca^{2+} waves evoked by the pancreatic secretagogue cholecystokinin cholecystokinin octapeptide was accelerated by mitochondrial inhibitors, whereas the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillations in response to physiological levels of this hormone were suppressed by rotenone and carbonylcyanide-*p*-tryfluoromethoxyphenyl hydrazone. Oligomycin, an inhibitor of mitochondrial ATP synthase, did no affect either propagation of calcium waves nor $[Ca^{2+}]_i$ oscillations. Individual mitochondria of rhod-2 loaded acinar cells showed heterogeneous matrix Ca^{2+} concentration increases in response to oscillatory and maximal levels of cholecystokinin octapeptide. On the other hand, using Ba^{2+} for unequivocal study of capacitative calcium entry we found that mitochondrial inhibitors did not affect this process. Our results show that although the role of mitochondria as a Ca^{2+} clearing system in exocrine cells is quantitatively secondary, they play an essential role in the spatial propagation of Ca^{2+} waves and in the development of $[Ca^{2+}]_i$ oscillations.

Keywords: Ca^{2+} oscillations; Mitochondria; Capacitative Ca^{2+} influx; Pancreas

1. Introduction

Changes in cytosolic-free calcium concentration are an ubiquitous messenger system involved in the regulation of a plethora of cellular processes, from contraction or secretion to differentiation or apoptosis. This factor is exquisitely controlled by two types of transport systems. On one hand, different types of channels allow diffusion of Ca^{2+} ions into the cytosol from the intracellular calcium stores (inositol 1,4,5-trisphosphate (IP3) and ryanodine receptors) or from the extracellular medium (Ca^{2+} influx channels). On the other hand, Ca^{2+} is actively removed from the cytosol towards the internal pools (by sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA)) or to the extracellular space (via plasma membrane Ca^{2+} ATPase (PMCA) and Na^+/Ca^{2+} exchange).

The fact that mitochondria accumulate Ca^{2+}, described more than 20 years ago in liver [1], and since then in other tissues ([2], for a review see [3]), prompted the concept that mitochondria might shape Ca^{2+} signals in mammalian cells. In the last few years, new techniques have allowed both determination of mitochondrial matrix Ca^{2+} concentration and a detailed evaluation of perturbations of Ca^{2+} signals in response to experimental impairment of mitochondrial function. These techniques have confirmed *in situ* the previously assumed role of mitochondria as a low affinity Ca^{2+} buffer, showing that these organelles take up Ca^{2+} during cytosolic Ca^{2+} signals ([4–7], for reviews see [3,8]). An immediate consequence of mitochondrial Ca^{2+} import is the control of mitochondrial metabolic function [7,9].

The other consequence of this activity is that mitochondria contribute to shape $[Ca^{2+}]_i$ signals. First, mitochondria contribute to the decay phase of $[Ca^{2+}]_i$ signals, an aspect studied in numerous tissues [10–14]. Second, this uptake activity has an impact on spatial propagation of signals, although there are conflicting reports ranging from

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Abbreviations: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_m$, mitochondrial Ca^{2+} concentration; CCE, capacitative calcium entry; CCK, cholecystokinin octapeptide; IP3, inositol 1,4,5-trisphosphate; FCCP, carbonylcyanide-*p*-tryfluoromethoxyphenyl hydrazone; PMCA, plasma membrane Ca^{2+} ATPase; Ψ_m, mitochondrial membrane potential; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase.

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support [15–17] to restriction [13,18,19] of spreading of $[Ca^{2+}]_i$ signal. However, information is very scarce regarding the relationship between mitochondria and $[Ca^{2+}]_i$ oscillations.

Another putative consequence of Ca^{2+} buffering by mitochondria is a permissive role in extracellular Ca^{2+} influx, given that by restricting the Ca^{2+} increases beneath the plasma membrane mitochondria would relieve the negative feedback of Ca^{2+} on the influx channels, as has been proposed in T cells [20]. This is also supported by the presence of mitochondria close to plasma membrane [21], in line with the effects of mitochondrial uncouplers on voltage-operated Ca^{2+} entry [22].

The aim of our study is to assess in intact pancreatic acinar cells the role of mitochondria in several aspects of Ca^{2+} signals, from removal of cytosolic Ca^{2+} to maintenance of Ca^{2+} oscillations and spreading of Ca^{2+} waves. Our data indicate that in exocrine cells these organelles are critical in the spreading of Ca^{2+} waves and the development of $[Ca^{2+}]_i$ oscillations, and take up calcium during cytosolic $[Ca^{2+}]_i$ signals, although they do not participate in capacitative calcium entry and have only a secondary role in global Ca^{2+} clearance.

2. Materials and methods

2.1. Preparation of acinar cells

A suspension of mouse pancreatic single cells and small acini was prepared by collagenase treatment following a previously described method [23]. Throughout the preparation procedure as well as during the loading and perfusion we employed a physiological solution containing (mM): NaCl 140, KCl 4.7, $CaCl_2$ 2, $MgCl_2$ 1.1, glucose 10, *N*-2-hydroxyethylpiperazine-*N*′-2-sulphonic acid (HEPES) 10, pH 7.4. Trypsin inhibitor (soybean) 0.01% was only present during the isolation and loading procedures. Ca^{2+}-free solutions had a similar composition but Ca^{2+} was omitted and 1 mM EGTA was added. All the procedures were performed at room temperature.

2.2. Cell loading and $[Ca^{2+}]_i$ determination

After isolation, the cells were suspended in physiological solution and loaded with the fluorescent ratiometric calcium indicator fura-2 by incubation with fura-2 AM (1–2 μM, for 30 min). Once loaded, the cells were washed an used within 2–4 hr.

For experiments, a small volume of cell suspension was placed on a thin glass coverslip attached to a perspex perfusion chamber. After a 2 min period to allow spontaneous attachment of the cells to the coverslip, the perfusion with physiological solution (approximately 1 mL/min) was started. No coating treatment was necessary to immobilize the cells. The chamber was placed on the stage of an inverted fluorescence-equipped microscope (Nikon Diaphot). Cells were excited at 340 and 380 nm by a computer-controled filterwheel (Lambda 10-2, Sutter Instruments) at 0.3–1 cycles/s and the emitted images were captured by a cooled digital CCD camera (C-4880-81, Hamamatsu Photonics) and recorded using dedicated software (Argus-HisCa, Hamamatsu Photonics). After calculating the ratio 340/380 pixel by pixel, the intracellular-free calcium concentration ($[Ca^{2+}]_i$) was determined using standard methods [24]. The calibration parameters R_{max}, R_{min}, K_d and S_f/S_b were determined *in vitro* using the free salt of fura-2 and Ca^{2+} standards. In some experiments the rate of acquisition was increased to 8–10 ratio/s.

2.3. Mitochondrial potential determination

Cells were loaded for 5 min with 5 μM dihydrorhodamine-123, which upon oxidation produces rhodamine-123. This cationic dye is retained in active mitochondria by the negative potential of the inner membrane, so that depolarization releases the dye into the cytosol [3]. Cells were studied with a laser scanning confocal microscope (BioRad 1024) using the 488 nm line of an Argon laser as excitation source and a 515 nm (±30 nm) interference filter to collect the emitted fluorescence. Autofluorescence was not detectable at the laser power and photomultiplicator gain used in this study.

2.4. Determination of mitochondrial calcium concentration

Cells were incubated during 15 min at 4° with 8 μM rhod-2 AM, which upon hydrolysis allows accumulation of the cationic Ca^{2+} dye rhod-2 in negatively charged mitochondria [3]. Cells were studied with a laser scanning confocal microscope (BioRad 1024) using the 543 nm line of a He:Ne laser as excitation source and a 570 nm long pass filter to collect the emitted fluorescence. In some experiments, rhod-2 loaded cells were subsequently loaded with fluo-3 by incubation with 4 μM fluo-3 AM (30 min, room temperature), for simultaneous registration of cytosolic calcium concentration, using the 488 nm line of an Argon laser and a 522 band pass filter. All the data are given as fractional changes of emitted fluorescence (F/F_0).

2.5. Statistics

To analyze $[Ca^{2+}]_i$ decays, values were normalized to the first point of the decay (Ca^{2+}/Ca^{2+}_{max}) after subtracting the resting $[Ca^{2+}]_i$ value for each individual cell. To analyze rates of decay, the time derivative of $[Ca^{2+}]_i$ was calculated (nM/s) and data were averaged and represented for 50 nM $[Ca^{2+}]_i$ intervals. The $t_{1/2}$ of decays was defined as the time necessary (in seconds) for the normalized Ca^{2+} level to complete 50% of the recovery. All statistical comparisons were performed using Student's *t*-test or χ^2 test.

2.6. Materials

Chemicals were purchased from Sigma except collagenase CLSPA, which was obtained from Worthington Biochemical Corporation. Fura-2, fluo-3, rhod-2, calcium standards and dihydrorhodamine from Molecular Probes Europe.

3. Results and discussion

3.1. Role of mitochondria in removal of cytosolic Ca^{2+}

To disrupt mitochondrial function we perfused cells with 1 μM carbonylcyanide-*p*-tryfluoromethoxyphenyl hydrazone (FCCP), a protonophore which collapses the mitochondrial inner membrane potential, or with 10 μM rotenone, an inhibitor of the complex I of the electron transport chain [3]. At these concentrations, FCCP and rotenone induced mitochondrial depolarization, as revealed by redistribution of rhodamine-123, a fluorescent marker for mitochondrial potential. As can be observed in Fig. 1, the typical punctate distribution of this cationic dye (due to potenial-driven mitochondrial accumulation) disappeared after treatment with FCCP or rotenone. These treatments

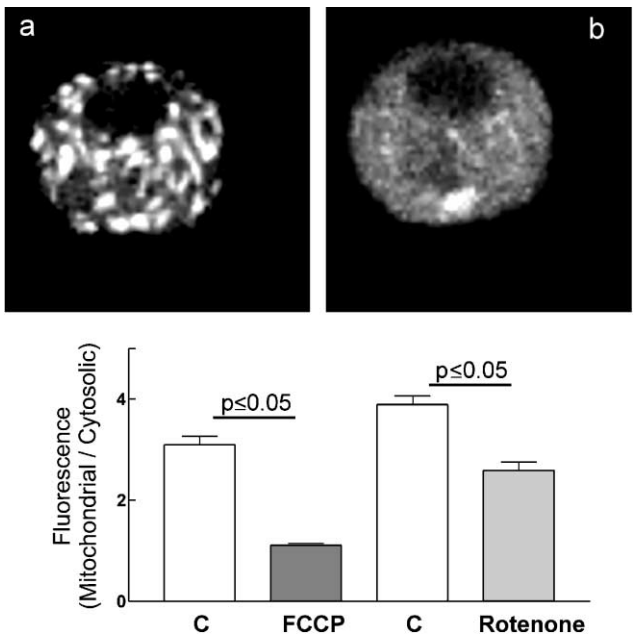

Fig. 1. FCCP and rotenone depolarize mitochondria in pancreatic acinar cells. Top: confocal images showing fluorescence of rhodamine-123 accumulated in mitochondria of acinar cells before (a) and after (b) a 5 min treatment with FCCP (1 μM). As shown, the treatment induces a clear decrease in mitochondrial fluorescence with an accompanying increase in the background cytosolic fluorescence. Bottom: histogram showing the change in the mitochondrial fluorescence relative to the cytosolic fluorescence before (c, open bars) and after (filled bars) treatment with mitochondrial inhibitors (10 μM for rotenone, 1 μM for FCCP). Bars are mean ± SEM (n = FCCP 58 mitochondria of 4 cells, rotenone 110 mitochondria 10 cells).

led to a slight and steady state $[Ca^{2+}]_i$ increase (FCCP: 153.2 ± 18.7 nM, 23 experiments; rotenone: 89.7 ± 9.6 nM, 24 experiments; values above resting levels). This increase was independent of extracellular Ca^{2+}, since the use of Ca^{2+}-free solution neither prevented this increase nor modified it once established (not shown). This finding is in keeping with a recent report [25].

In several cell types, such as neurons [10,11], chromaffin cells [14] or astrocytes [13], mitochondria have been reported to behave as an important Ca^{2+} clearing mechanism, contributing to accelerate the decay phase of Ca^{2+} signals. To investigate if this is also the case for non-excitable exocrine cells we investigated the clearance of cytosolic Ca^{2+} under different conditions. First, we studied the recovery phase of the response to 10 nM CCK in Ca^{2+}-free solution, a process mainly driven by extrusion to the external medium (Ca^{2+} reincorporated into SERCA-operated pools re-circulates to cytosol due to continuous stimulation) [26]. Fig. 2A shows that mitochondrial inhibition induced a slight decrease in the final $[Ca^{2+}]_i$ recovery, although the effect was statistically significant. Final normalized $[Ca^{2+}]_i$ values were 0.067 ± 0.022 for FCCP treated cells and 0.048 ± 0.017 for rotenone, in both cases statistically significant vs. control values (0.023 ± 0.0036; $P < 0.05$). This suggests that during the response to agonists mitochondria are only a secondary Ca^{2+} clearing system. Since in pancreatic acinar cells Ca^{2+} reuptake into the stores and Ca^{2+} extrusion are powerful Ca^{2+} removing mechanisms [26,27], it could be possible that contribution of mitochondria is simply masked by these transport systems. Thus, in other series of experiments we blocked any possible contribution of SERCA-operated Ca^{2+} pools during the recovery of the CCK response using the specific inhibitor thapsigargin. As shown in Fig. 2B, mitochondrial inhibitors induced a partial inhibition in Ca^{2+} removal: FCCP slowed the rate of the decay ($t_{1/2}$ 145.3 ± 3.4 s: 42 cells vs. 80.3 ± 3.2 s: 70 cells for control cells, $P < 0.05$), while rotenone decreased the final recovery (final normalized $[Ca^{2+}]_i$ 0.018 ± 0.014: 44 cells vs. −0.04 ± 0.004 for control cells, $P < 0.05$). Next, we used the protocol shown in Fig. 2C, aimed at blocking Ca^{2+} extrusion. Sudden interruption of cholinergic stimulation with atropine and 2 mM La^{3+} (to block PMCA) induces a fast recovery of $[Ca^{2+}]_i$, which is due to Ca^{2+} reuptake into the stores [26]. In the presence of inhibitors the $t_{1/2}$ of the recovery was increased (FCCP 68.3 ± 6.6 s: 37 cells, rotenone 21.7 ± 1.2 s: 48 cells, $P < 0.05$ vs. control cells 16.6 ± 0.8 s: 106 cells), and in FCCP-treated cells the final recovery (0.14 ± 0.017) was clearly decreased with respect to control values (0.05 ± 0.008, $P < 0.05$), although in rotenone-treated cells the value was similar to control cells.

Taken together, the results of Fig. 2 show that in exocrine acinar cells mitochondria are only an auxiliary Ca^{2+} removing system, given that even after blocking either Ca^{2+} transport into the pools (Fig. 2B) or Ca^{2+} extrusion

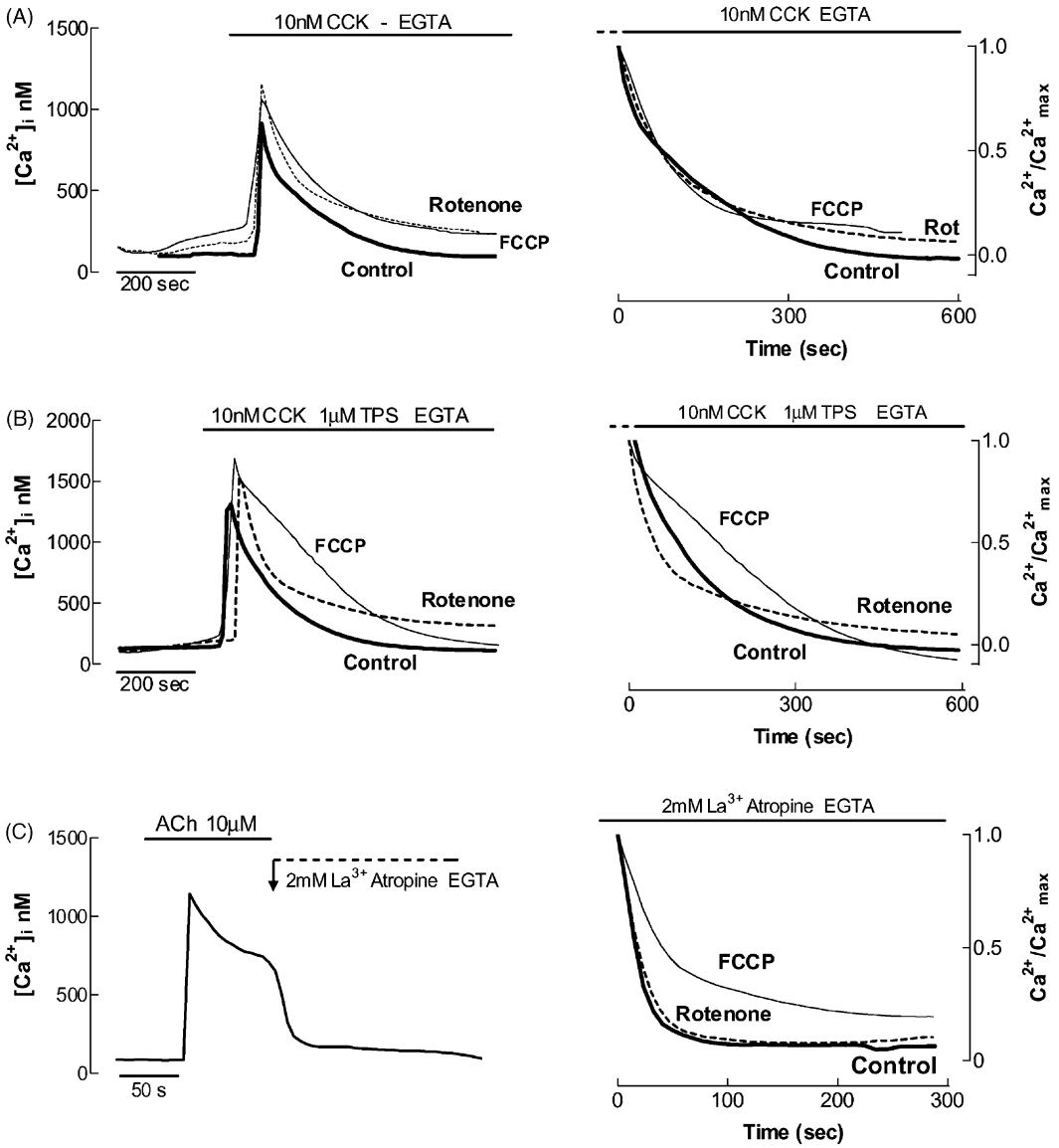

Fig. 2. Effect of FCCP and rotenone on removal of cytosolic Ca^{2+} in fura-2 loaded pancreatic acinar cells. (A) Cells were stimulated with CCK in absence (thick line) or presence (starting 5 min before CCK) of 10 μM rotenone (dashed lines) or 1 μM FCCP (thin lines). Left panel shows mean values for 33 to 91 cells from five or more independent experiments, and right panel are the mean decay phase of the $[Ca^{2+}]_i$ increase normalized to the peak values (Ca^{2+}/Ca^{2+}_{max}). Errors are omitted for the sake of clarity. (B) $[Ca^{2+}]_i$ transients in response to CCK plus 1 μM thapsigargin (TPS) in absence (thick line) or presence of 10 μM rotenone (dashed lines) or 1 μM FCCP (thin lines). Left panel shows mean $[Ca^{2+}]_i$ values (42–70 cells from at least six independent experiments), and right panel shows the decay phase of the $[Ca^{2+}]_i$ increase normalized to the peak values. (C) Left panel: protocol to induce accelerated $[Ca^{2+}]_i$ decay by reuptake into the stores. Cholinergic stimulation (10 μM ACh) was suddenly interrupted with a nominally Ca^{2+}-free solution containing 100 μM atropine and 2 mM La^{3+} (to block Ca^{2+} influx). Right panel: decrease of $[Ca^{2+}]_i$ normalized to the first point of the decrease during the reuptake-induced recovery, both in absence and presence of mitochondrial inhibitors. Data are mean values from 37 to 106 cells from at least six independent experiments.

(Fig. 2C) FCCP and rotenone only had a small (though significant) effect. However, mitochondrial uptake is likely to be relevant during the first phase of the decay (Fig. 2B and C; see also $t_{1/2}$ increases in previous paragraph), while the second component relies mainly on SERCA, in agreement with the high activity of the thapsigargin-sensitive stores [26] and the highly developed endoplasmic reticulum [28] in this cell type. This is in line with previous models proposed for neuronal handling of Ca^{2+} recovery [29,30]. In addition, an important corollary of these results is that mitochondrial inhibitors do not perturb the ability of the main Ca^{2+} transport systems of acinar cells to effectively reduce $[Ca^{2+}]_i$ during responses to agonists. Therefore, the effects of mitochondrial inhibition in $[Ca^{2+}]_i$ signals are not due to energetic deprivation or to loss of Ca^{2+} homeostasis.

3.2. Mitochondria and spatiotemporal aspects of Ca^{2+} signals

In spite of the previous conclusion, mitochondria could modulate the physiologically relevant aspects of Ca^{2+}

signals, i.e. spatial arrangement of the signals and $[Ca^{2+}]_i$ oscillations. For example, in presinaptic terminals mitochondria placed in close apposition to neurotransmitter vesicles accumulate Ca^{2+} and thus modulate exocytosis [31], and it is known that some mitochondria, located at specific sites, support spreading of cytosolic $[Ca^{2+}]_i$ signals in glial cells [16,17]. It is well documented that in pancreatic acinar cells agonist-evoked Ca^{2+} signals start as calcium release from intracellular stores located at the secretory (or apical) pole of the cell, and then spreads to the basolateral pole as a Ca^{2+} wave [32]. The probability of an apical signal spreading as a global response depends on the level of stimulation and the Ca^{2+} buffering mechanisms, including any transport systems. It is known that buffering systems determine the pattern of oscillations and the probability of propagation of the subcellular $[Ca^{2+}]_i$ signals evoked by post-prandial levels of CCK in the secretory pole of the cell [33,34], which is densely packed with zymogen granules containing the digestive enzymes of the gland. In fact, recent studies demonstrate that in pancreatic acinar cells mitochondria take up calcium during $[Ca^{2+}]_i$ signals [35,36], confirming previous reports [2], so that mitochondrial uptake could modulate the spreading of agonist-evoked Ca^{2+} waves.

Therefore, we assayed the effect of mitochondrial inhibition on the spatiotemporal development of the Ca^{2+} response to CCK. Fig. 3A shows propagation of a typical response to CCK from the secretory to the basolateral pole of the cell, recorded using high speed digital imaging. As shown in the histogram of Fig. 3B, pretreatment with either FCCP or rotenone increased the calcium wave speed (47.4 ± 7.6 μm/s, $n = 13$, and 58.8 ± 8.1 μm/s, $n = 7$, 27.6 ± 3.0 μm/s, $n = 19$ for rotenone, FCCP and control group, respectively). The mean space between basal and apical areas was 18.5 ± 0.09 μm (45 cells). The difference with respect to the control group was statistically significant for both treatments ($P < 0.05$). The effect was not reproducible with oligomycin, an ATP synthase inhibitor.

This result demonstrates that mitochondria are a limiting factor for propagation of Ca^{2+} waves. In fact, using the same model, Tinel *et al.* have recently described that the $[Ca^{2+}]_i$ signals evoked by low levels of IP3 at the secretory pole are converted into global signals by administration of a mitochondrial uncoupler [19], and the authors interpreted this finding as a consequence of Ca^{2+} buffering by mitochondria surrounding the luminal pole. Another recent paper, published during finalization of our work, also describes spreading of local Ca^{2+} signals by mitochondrial inhibitors [37]. This is an important aspect of the spatiotemporal arrangement of calcium signals, specially in polarized and relatively small cells, such as pancreatic acini, because two reasons make it necessary to avoid global spreading of calcium signals across cytoplasm. First, because it is frequently desirable to target calcium signals to specific organelles, such as the exocytotic machinery in the case of pancreatic acinar cells. Second,

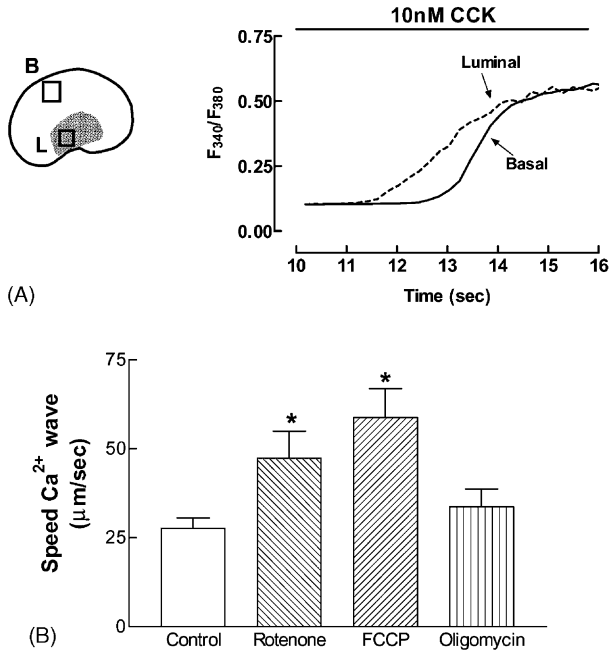

Fig. 3. Rotenone and FCCP increase speed of Ca^{2+} waves in fura-2 loaded pancreatic acinar cells. (A) Schematic representation showing propagation the CCK-evoked Ca^{2+} wave from the luminal pole (labeled as L in the sketch) to the basal edge of the cell. The traces represent $[Ca^{2+}]_i$ increases (F340/F380) in square areas of the luminal and basal poles of the cell sketched on the left. (B) Speed of propagation of the wave in control cells, and in rotenone, FCCP and oligomycin pretreated cells (5 min treatment). Speed was calculated as the time necessary for the initial part of the response to travel from the luminal to the basal pole, divided by the space in μm. Bars depict mean ± SEM, $n = 19$, 13, 7 and 6 cells for control, rotenone, FCCP and oligomycin, respectively. $^*P < 0.05$.

because widespread $[Ca^{2+}]_i$ increases are potentially cytotoxic, leading to cell death. In fact, we have shown that these cells have a high calcium binding capacity [38], and it has been shown that polarized extrusion of Ca^{2+} contributes to restrict Ca^{2+} signals from the secretory pole [27]. Thus, the present data support the view that mitochondria could operate as a buffer to restrict spreading of Ca^{2+} signal from the initiation site [19], although quantitatively their Ca^{2+} clearing activity is much less important than that of SERCA-driven pools or extrusion to extracellular medium.

However, in other tissues mitochondria enhance rather than restrict Ca^{2+} signals [15–17], so that mitochondrial uncouplers inhibit propagation of $[Ca^{2+}]_i$ signals. The rationale for these tissue-specific differences is probably associated with differences in the type and spatial arrangement of IP3 and ryanodine receptors. In pancreatic acinar cells the most abundant type is IP3R-3 [39], which is stimulated by Ca^{2+} but does not show the biphasic, bell-shaped $[Ca^{2+}]_i$ sensitivity displayed by the IP3R-1 type [40] so that it is not expected that $[Ca^{2+}]_i$ will have a negative feedback effect on IP3-sensitive stores.

Regarding oscillations, pancreatic acinar cells provide an ideal model for their study since this tissue develops oscillations in response to post-prandial concentrations of

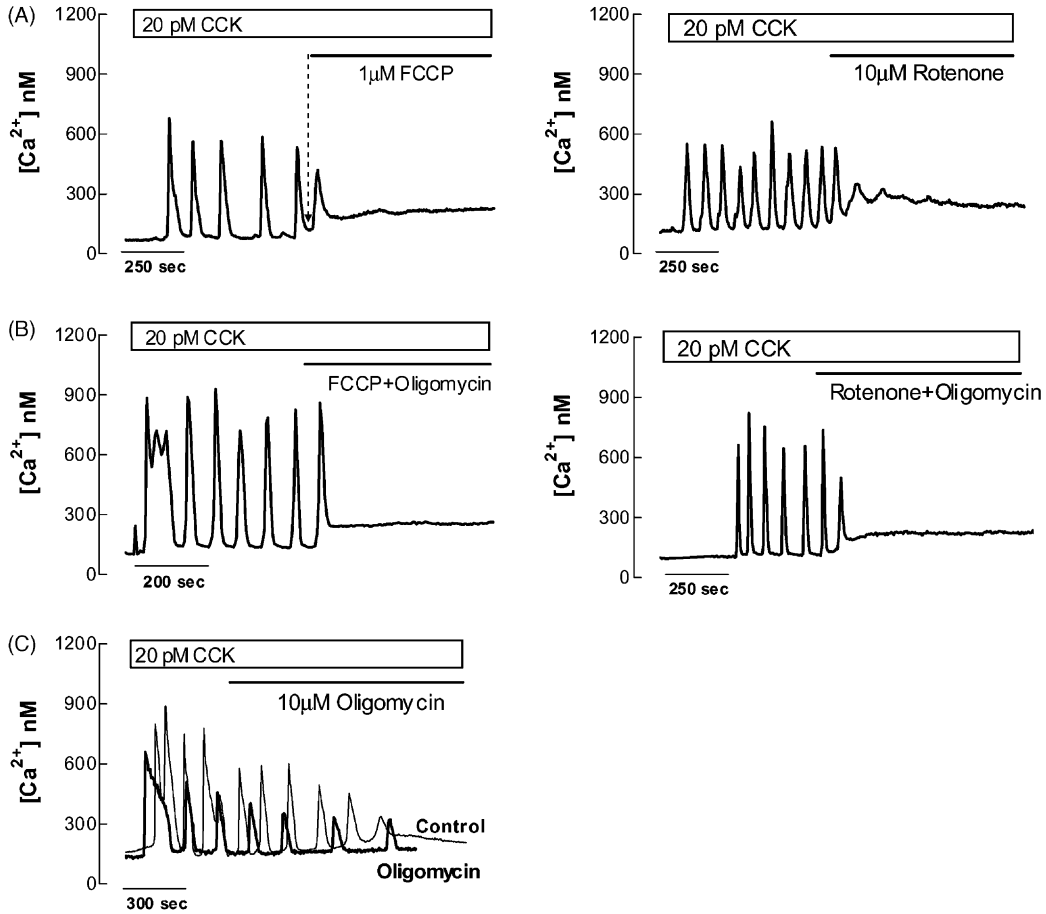

Fig. 4. Maintenance of Ca^{2+} oscillations requires mitochondrial function. Fura-2 loaded acinar cells were stimulated with 20 pM CCK. (A) After establishment of the oscillations, FCCP or rotenone were applied as indicated. Traces are representative of 44 and 31 cells for FCCP and rotenone, respectively. (B) Cells were treated as in A but adding oligomycin to FCCP or rotenone. Data are typical of 36 and 29 cells for FCCP and rotenone. (C) Superimposed traces from control (untreated, thin line) and oligomycin-treated cells (thick line). Representative of 50 (control) and 29 cells (oligomycin).

CCK, its main physiological stimulus . Thus, we investigated the effect of mitochondrial inhibition on oscillations evoked by 20 pM CCK. Fig. 4A shows that after application of FCCP or rotenone the typical pattern of $[Ca^{2+}]_i$ transients evoked by CCK was dramatically interrupted, so that the cyclic $[Ca^{2+}]_i$ transients disappeared in 1–2 min. This pattern was present in 42 of 44 cells and 28 of 31 cells for FCCP- and rotenone-treated cells, respectively. Both percentages are significantly higher than the spontaneous disappearance of oscillations observed in control cells (3 out of 50 cells, $P < 0.005$, χ^2 test). To assess the role of ATP depletion in this effect we also performed the same protocol adding oligomycin to rotenone or FCCP, in order to avoid ATP hydrolysis by the operation of mitochondrial ATP synthase in reverse mode [3]. As shown in Fig. 4B, this combination still induced a similar inhibition, blocking the oscillations in 26 of 36 cells and 18 of 29 cells for FCCP and rotenone, respectively ($P < 0.005$ respect control, χ^2 test). Moreover, oligomycin alone only had a slight and non-significant effect (Fig. 4C).

To our knowledge, this is the first detailed description of an acute dependence of Ca^{2+} oscillations on mitochondrial function in non-excitable cells. The only direct precedent is a report in excitable gonadotrope cells [41], showing that $[Ca^{2+}]_i$ oscillations are disrupted by mitochondrial inhibitors. In addition, a paper published during finalization of this work, briefly reports inhibition of $[Ca^{2+}]_i$ oscillations in acinar cells upon mitochondrial inhibition [37], although the authors focused in the involvement of ryanodine receptors in Ca^{2+} waves.

If the main mechanism for participation of mitochondria in oscillations is calcium buffering, one would expect an enhancement of the signals or a strong release of calcium during inhibition of the oscillations, but this was not the case. In addition, unpublished data show that Ru360, a specific inhibitor of the mitochondrial Ca^{2+} uniporter, did not reproduce the effect of the inhibitors (Camello-Almaraz *et al.*, unpublished results). Alternative explanations should consider the existence of mitochondrial-based factors supporting IP3-driven oscillations. It is tempting the possibility that mitochondria in close apposition to strategic domains of the endoplasmic reticulum generate reactive oxygen species in small but sufficient amounts to sensitize the IP3R, which is known to increase its sensi-

tivity upon oxidation in several systems, including acinar cells [42]. In fact, we have recently found that CCK-evoked oscillations can be inhibited by pretreatment with the antioxidant *N*-acetyl-cysteine [43]. This hypothesis is currently under examination in our laboratory.

In any case, it is likely that mitochondrial uptake is somehow related to this effect. It has been previously shown that pharmacological levels of agonists increase mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) in pancreatic acinar cells [35], and a very recent paper reports $[Ca^{2+}]_m$ oscillations in response to oscillatory levels of ACh [36]. We wanted to know if the physiological levels of CCK used in our study also resulted in elevation of mitochondrial matrix Ca^{2+} concentration. Fig. 5A shows that, upon application of 20 pM CCK rhod-2 loaded cells displayed a steady increase in many (but not all) mitochondria, although this response was quite heterogeneous. As can be observed, within one single cell it is possible to observe strong differences between individual mitochondria. The responses were variable not only in terms of size, but also in the temporal profile: while some mitochondria quickly reached a steady state level, other developed a slow increase which in some cases terminated with a decrease.

Previous reports have shown $[Ca^{2+}]_m$ oscillations during Ca^{2+} mobilization [7,36]. In acinar cells Park *et al.* described that $[Ca^{2+}]_m$ oscillations were more prominent

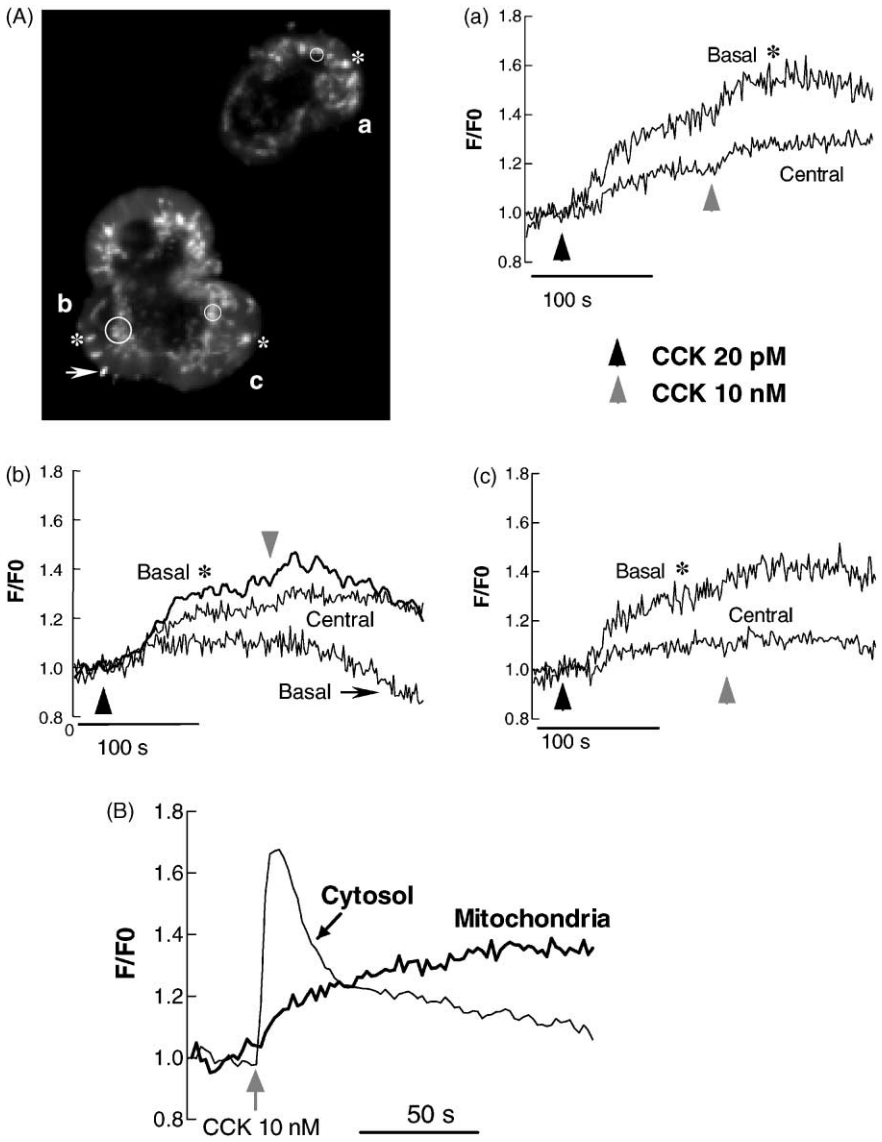

Fig. 5. Changes in $[Ca^{2+}]_m$ upon CCK stimulation of pancreatic acinar cells. (A) The photograph is a confocal image of the rhod-2 fluorescence accumulated in mitochondria of two small acini. As shown, the indicator accumulated irregularly in the basal and central zones of the cell, partially surrounding the secretory pole (dark central areas). Panels a, b and c depict changes in $[Ca^{2+}]_m$ (as rhod-2 fluorescence) corresponding to mitochondria situated in central areas (marked with circles) and basal areas (marked with asterisks or arrow) of the cells labeled as a, b and c, respectively. Application of 20 pM and 10 nM CCK is denoted by black and gray arrowheads. Representative of five experiments. (B) Simultaneous record of $[Ca^{2+}]_m$ and $[Ca^{2+}]_i$ in response to CCK in a cell labeled with rhod-2 (thick line, $[Ca^{2+}]_m$) and fluo-3 (thin line, $[Ca^{2+}]_i$). Representative of three independent experiments.

in the central area surrounding the secretory pole of the cells [36]. However, we found no evidence of CCK-evoked $[Ca^{2+}]_m$ oscillations in six independent experiments. In addition, we could not observe any correlation between the specific pattern of $[Ca^{2+}]_m$ response and the situation of the mitochondria within the cell. This is not due to failure of our experimental conditions to detect $[Ca^{2+}]_m$ increases in response to cytosolic $[Ca^{2+}]_i$ signals, as demonstrated by the clear correlation between $[Ca^{2+}]_m$ and cytosolic $[Ca^{2+}]_i$ during maximal stimulation with CCK, as revealed by simultaneous determination in fluo-3 and rhod-2 double labelled cells (Fig. 5B). In addition, in our studies we observed a clearly punctuate and discrete localization of the rhod-2 fluorescence on top of a faint and diffuse cytosolic background (see image 5A), discarding possible misinterpretation of cytosolic rhod-2 signals. A possible explanation for this discrepancy is the existence of differences between ACh- and CCK-stimulated signalling pathways (it is known that the normal pattern of $[Ca^{2+}]_i$ oscillations evoked by these two agonists is clearly different, e.g. [33]). In any case, there are other reports of sustained mitochondrial Ca^{2+} signals in presence of cytosolic oscillations (e.g. [44]).

Our data are in keeping with detailed studies showing clear heterogeneity of $[Ca^{2+}]_m$ signals in oligodendrocytes [17] and smooth muscle [45], and is probably associated with differences in the mitochondrial membrane potential (Ψ_m), which drives Ca^{2+} import into the mitochondrial matrix. Inter-mitochondrial differences in Ψ_m can be established during $[Ca^{2+}]_m$ increases for example by Ca^{2+}-induced stimulation of oxidative phosphorylation (which would reinforce or sustain Ψ_m) or by dissipation of Ψ_m (either directly or by activation of ATP synthase), as discussed previously [17]. This could explain the heterogeneous pattern of $[Ca^{2+}]_m$ responses: mitochondria reacting to stimulation with an enhancement of Ψ_m would show a high or sustained $[Ca^{2+}]_m$ increase, while those suffering Ψ_m dissipation would exhibit small or transient $[Ca^{2+}]_m$ changes.

It is noteworthy that there was a poor correlation between the level of stimulation (20 pM or 10 nM CCK) and the $[Ca^{2+}]_m$ response: when the cell was subsequently stimulated with a high level of CCK a further increase could be observed in some mitochondria, while other exhibited only a slight increase, no response or even a decrease in $[Ca^{2+}]_m$. The same conclusion can be drawn from comparison of the responses to initial stimulation with 20 pM (Fig. 5A) and 10 nM (Fig. 5B). However, although this can be considered another aspect of the heterogeneity described above, it must be kept in mind that it is possible that mitochondrial Ca^{2+} indicators do not reflect the real accumulation, because the high levels of phosphate in the matrix compete with the dyes (avoiding further $[Ca^{2+}]_m$ increase in spite of continuous Ca^{2+} transport) [46] and because rhod-2 could approach saturation.

3.3. Mitochondria and capacitative Ca^{2+} entry

It has been postulated that capacitative calcium entry (CCE) is also modulated by mitochondria. This hypothesis stemmed from the observation that some mitochondria located close to the plasma membrane accumulate calcium during calcium influx in endothelial cells [21], and from the fact that refilling of Ca^{2+} pools in T lymphocytes is impaired upon mitochondrial inhibition [20]. The authors proposed that during entry of extracellular Ca^{2+} mitochondrial buffering of Ca^{2+} ions close to plasma membrane enhance CCE. A similar suggestion has been made for voltage-operated Ca^{2+} influx in smooth muscle cells [22]. We assessed the validity of this hypothesis in pancreatic acinar cells using the protocol shown in Fig. 6. After initial application of thapsigargin (1 μM) in Ca^{2+}-free solution to deplete internal Ca^{2+} stores, perfusion with a 5 mM Ca^{2+}-containing medium resulted in a sustained $[Ca^{2+}]_i$ plateau which is commonly used as index of capacitative calcium influx [47,48]. Fig. 6A shows that application of rotenone before readmission of external calcium enhanced CCE. Moreover, when either rotenone or FCCP were applied once CCE was fully established (top of the $[Ca^{2+}]_i$ plateau), cells displayed a further $[Ca^{2+}]_i$ increase (26 out of 39). This experimental approach strongly suggested that mitochondria inhibit rather than facilitate calcium entry, contrary to previous observations in other non-excitable cells.

However, in the present experimental context the described procedure could be flawed by the fact that the plateau $[Ca^{2+}]_i$ used as influx estimation is really the result of two opposite processes: on one hand, Ca^{2+} entry through plasma membrane, on the other hand, Ca^{2+} extrusion and mitochondrial uptake (SERCA-driven removal is blocked by thapsigargin). Since results of Fig. 2 show that mitochondrial inhibition partially reduces removal of cytosolic Ca^{2+}, our approach could be misleading because a reduction in mitochondrial Ca^{2+} uptake could increase the plateau without modifying CCE. Fig. 6B shows the relationship between $[Ca^{2+}]_i$ and rate of $[Ca^{2+}]_i$ decay for data of Fig. 2B ($[Ca^{2+}]_i$ decay in presence of thapsigargin). As can be observed, when $[Ca^{2+}]_i$ is similar to the Ca^{2+} values of the CCE plateau ($292 + 15.8$, $n = 80$), below 600 nM, the rate of decay was diminished in FCCP- and rotenone-treated cells. So, mitochondrial inhibitors will reduce rate of cytosolic removal therefore increasing the plateau level.

To circumvent this limitation we followed the same protocol but using 30 mM Ba^{2+} instead of Ca^{2+} to initiate influx. This cation binds to fura-2, is permeable through store-operated Ca^{2+} channels [49] and is not pumped by Ca^{2+} ATPases, so that monitoring its intracellular levels during CCE gives an univocal estimate of influx through Ca^{2+} channels (e.g. [48]). Fig. 6C shows that mitochondrial inhibitors did not affect influx of extracellular Ba^{2+}. Neither the rate of influx or its steady state changed in presence of the inhibitors. Thus, the apparent increase of

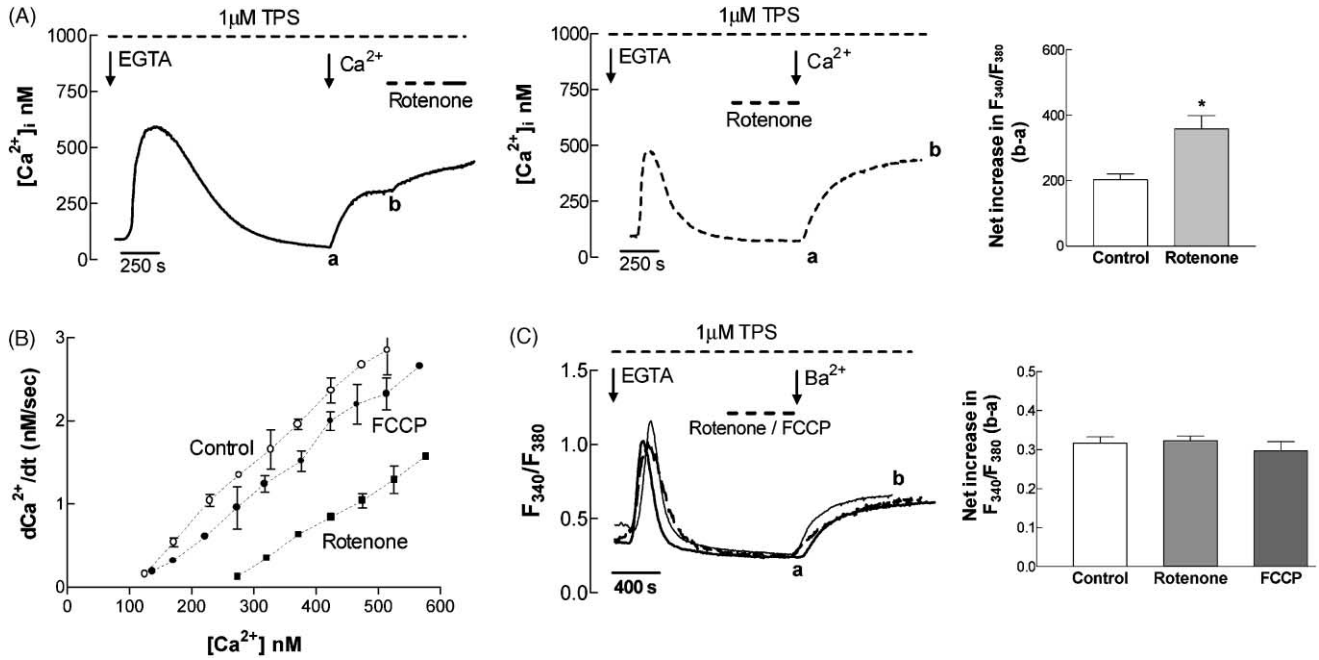

Fig. 6. Capacitative calcium entry (CCE) is not modulated by mitochondria in pancreatic acinar cells. (A) After depletion of internal Ca^{2+} pools with thapsigargin (TPS) readmission of extracellular Ca^{2+} (2 mM) induced a $[Ca^{2+}]_i$ plateau due to CCE. Application of rotenone (10 μM) on top (left graph) or before (right graph) the plateau enhanced the apparent CCE. The histogram shows mean ± SEM of CCE in control and rotenone-treated cells, using b–a as index. (B) Rate of absolute $[Ca^{2+}]_i$ decay (nM/s, mean ± SEM) during the control CCK-evoked Ca^{2+} transients in presence of thapsigargin shown in Fig. 2B. Data were grouped in 50 nM $[Ca^{2+}]_i$ intervals. (C) Determination of CCE using Ba^{2+} as a surrogate for Ca^{2+} influx. Application of Ba^{2+} (30 mM) induced an increase in F340/F380 which reflects unidirectional influx. This response was not affected by rotenone nor FCCP. The bars of the histogram represent mean ± SEM from 49 to 75 cells. $^{*}P < 0.05$.

Ca^{2+} influx evoked by rotenone (Fig. 6A) must be due to loss of cytosolic Ca^{2+} removal by mitochondria (consistent with Fig. 2). This result indicate that, at least in pancreatic acinar cells, CCE is not modulated by mitochondria. Furthermore, the present data indicate that effects of mitochondrial inhibition on calcium entry must be interpreted with caution, because the associated decrease of buffering, even if moderate, could affect the apparent Ca^{2+} influx, leading to erroneous interpretation.

In conclusion, we present here new data showing that during agonist-evoked $[Ca^{2+}]_i$ signals in exocrine cells mitochondria play an important role in propagation of Ca^{2+} waves and in the development of $[Ca^{2+}]_i$ oscillations, in spite of the fact that they are only an ancillary system for global Ca^{2+} clearance and do not modulate capacitative calcium influx. We also show that during physiological $[Ca^{2+}]_i$ signals mitochondria show sustained and heterogeneous $[Ca^{2+}]_m$ increases, though the biological meaning of this finding remains to be established and deserves further investigation.

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