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MODULATION OF CCK-8-EVOKED INTRACELLULAR Ca^{2+} WAVES BY HYDROGEN PEROXIDE IN MOUSE PANCREATIC ACINAR CELLS

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In the present study we have employed single cell imaging analysis to monitor the propagation of cholecystokinin-evoked Ca^{2+} waves in mouse pancreatic acinar cells. Stimulation of cells with 1 nM CCK-8 led to an initial Ca^{2+} release at the luminal cell pole and subsequent spreading of the Ca^{2+} signal towards the basolateral membrane in the form of a Ca^{2+} wave. Inhibition of sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity by 1 μM thapsigargin, preincubation in the presence of 100 μM H_2O_2 or inhibition of PKC with either 5 μM Ro31-8220 or 3 μM GF-109203-X all led to a faster propagation of CCK-8-induced Ca^{2+} signals. The propagation of CCK-8-evoked Ca^{2+} signals was slowed down by activation of PKC with 1 μM PMA, and preincubation of cells in the presence of H_2O_2 counteracted the effect of PKC inhibition. The protonophore FCCP (100 nM) and the inhibitor of the mitochondrial Ca^{2+}-uniporter Ru360 (10 μM) led to an increase in the propagation rate of CCK-8-evoked Ca^{2+} waves. Finally, depolymerisation of actin cytoskeleton with cytochalasin D (10 μM) led to a faster propagation of CCK-8-evoked Ca^{2+} signals. Stabilization of actin cytoskeleton with jasplakinolide (10 μM) did not induce significant changes on CCK-8-evoked Ca^{2+} waves. Preincubation of cells in the presence of H_2O_2 counteracted the effect of cytochalasin D on CCK-8-evoked Ca^{2+} wave propagation. Our results suggest that spreading of cytosolic Ca^{2+} waves evoked by CCK-8 can be modulated by low levels of oxidants acting on multiple Ca^{2+}-handling mechanisms.

Key words: *CCK-8, hydrogen peroxide, calcium wave, fluorescence, imaging analysis*

INTRODUCTION

Ca^{2+} is a unique ion that is used in a wide variety of biological processes such as gene transcription, ion channel function, contraction, secretion and

proliferation. To control multiple functions specifically, the distribution of Ca^{2+} is highly regulated in the dimensions of space, time and concentration (1). Stimulation of pancreatic acinar cells by secretagogues leads to activation of phospholipase C (PLC) and, as a consequence of its activity, produces an increase in the concentration of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) in the cytosol. IP_3 in turn releases Ca^{2+} from stores leading to an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). DAG will activate protein kinase C (PKC) (2). Following depletion of intracellular stores a "capacitative" Ca^{2+} entry pathway from the extracellular medium is activated (3).

Because of the spatial distribution of organelles in the pancreatic acinar cell, related to a functional polarization, polarized Ca^{2+} signals can be observed that are linked to a unidirectional and polarized enzyme and fluid secretion. The Ca^{2+} signal has been shown to propagate from the luminal toward the basolateral side of the cell in form of a Ca^{2+} wave (4 - 6). Underlying the propagation of Ca^{2+} signals throughout the cytosol, a mechanism of Ca^{2+}-induced Ca^{2+} release (CICR) from stores in series has been proposed (7, 8). It has been shown that the speed of propagation of Ca^{2+} signals throughout the cytosol depends not only on the concentration of the agonist used, but also on the type of agonist (8, 9). The activation of different intracellular metabolic pathways, i.e., protein kinase C (PKC) (8), phospholipase A_2/arachidonic acid (PLA_2/AA) (9), or even intracellular pH changes (6) can modulate the propagation of Ca^{2+} waves.

In addition, participation of intracellular Ca^{2+} stores on the propagation of Ca^{2+} waves has been documented in studies based on inhibition of the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) (8, 9). Therefore, spreading of cytosolic Ca^{2+} signals does not only depend on secondary release of Ca^{2+} from intracellular stores but also on Ca^{2+} reuptake into intracellular pools. Another cytosolic organelle involved in Ca^{2+} signalling is mitochondria. In pancreatic acinar cells, mitochondria can be observed surrounding the zymogens granule area, beneath the basolateral plasma membrane and around the nucleus; moreover, single mitochondria can also be found between the zymogens granules in the luminal cell pole (10). In this cellular type it has been proposed that mitochondria block spreading of Ca^{2+} signal from the initiation points in the apical secretory cell pole toward the basolateral pole (11); therefore, these organelles are important modulators of Ca^{2+} signalling. Recently, the Golgi system has been proposed to be potentially implicated in shaping Ca^{2+} signals and serve either as a Ca^{2+} sink or an amplifier of the Ca^{2+} responses (12).

Differential participation of Ca^{2+} stores in Ca^{2+} signalling in response to agonists has been confirmed in a work by Yamakasi *et al.* (13), who have shown that different agonists generate their specific Ca^{2+} signals by signalling through Ca^{2+} mobilization from different intracellular stores, a process termed "organelle selection". Furthermore, the maintenance of cellular architecture and the polarized localization of cytoplasmic organelles are crucial for a precise operation

of the Ca^{2+} signalling machinery and for regulated exocytosis in the pancreatic acinar cells (14).

Reactive oxygen species (ROS) are a molecular group that can be produced in the course of different physiological processes and react with a large variety of oxidizable cellular components. Oxidation-reduction reactions involving ROS have gained attention as important chemical processes with implications in cellular signal transduction (15). ROS, including H_2O_2, are able to increase $[Ca^{2+}]_i$ by terms of its release from endoplasmic reticulum (ER) and mitochondria, thereby impairing normal Ca^{2+} homeostasis (16, 17). In addition, ROS inhibit amylase secretion in response to secretagogues (18) and, in excess, are able to induce profound changes in mitochondrial activity inhibiting those produced by physiological agonists (19). By terms of its effects on $[Ca^{2+}]_i$, excessive ROS production has been considered pathological in different tissues and cells, including the pancreas (20). An increase in $[Ca^{2+}]_i$ due to disturbance of Ca^{2+} homeostasis by ROS can cause morphological and functional alterations of the cell, and therefore, it has been clearly established to contribute to disease and cell death (21). On the other hand, it has been suggested that elevated, but sublethal, levels of ROS can function as signalling molecules participating as intermediates to influence intracellular metabolic pathways and, therefore, modulate the normal physiological activity of the cell. In pancreatic acinar cells oxidants facilitate oscillations in $[Ca^{2+}]_i$ by enhancing release of Ca^{2+} from internal stores (22), and postprandial levels of pancreatic agonists increase mitochondrial ROS production, which accompany and support $[Ca^{2+}]_i$ oscillations (23).

The mechanisms of action of ROS at the cellular level are not completely understood. It is therefore important to gain more information in order to better understanding their role in the physiology of the cell. Due to the importance of Ca^{2+} signals polarization in the function of pancreatic acinar cells and the implications of ROS in Ca^{2+} homeostasis, we sough to investigate the propagation of Ca^{2+} waves in the presence of hydrogen peroxide (H_2O_2) in this cellular model.

MATERIALS AND METHODS

Animals and chemicals

Adult male Swiss mice were used for this study. Mice were humanely handled and sacrificed in accordance to national ethics committee guidelines for the use of animals in research (RD 1201/2005). Fura-2/AM and JC-1 were obtained from Molecular Probes Inc. (Europe); Biosindolylmaleimide IX methanesulfonate salt (Ro-31-8220), GF-109203-X, cell permeable oxygen-bridged dinuclear ruthenium amine complex (Ru360), carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP), cytochalasin D and jasplakinolide from Calbiochem (Bionova Científica S.L., Spain) and phorbol 12-myristate 13-acetate (PMA) from Tocris (Biogen Científica S.L., Spain). Oligomycin and all other used materials were obtained from Sigma Chemicals Co. (Spain).

Preparation of isolated pancreatic acinar cells

A suspension of mouse pancreatic single cells and small acini (2 - 4 cell clusters) was prepared by collagenase treatment following a previously described method (24). Throughout the preparation procedure as well as during the loading and perfusion we employed a physiological solution containing (in mM): NaCl 140, KC1 4.7, $CaCl_2$ 1, $MgCl_2$ 1.1, glucose 10, N-2-hydroxyethylpiperazine-N'-2-sulphonic acid 10, pH 7.4. Trypsin inhibitor (soybean) 0.01% and 0.2% bovine serum albumin were added to the medium during the isolation and loading procedures. Ca^{2+}-free solution had a similar composition but Ca^{2+} was omitted and 1 mM EGTA was added. Cell viability was higher than 95% as assayed by trypan blue exclusion test, at least for the range of time during which our determinations were carried out. All the procedures were performed at room temperature.

Dye loading

For the determination of $[Ca^{2+}]_i$ freshly isolated mouse pancreatic acinar cells were loaded with the fluorescent ratiometric Ca^{2+} indicator fura-2 by incubation of cells with fura-2 acetoxymethyl ester (2 μM) at room temperature (23-25 °C) for 30 min following previously established methods (22). Changes in fluorescence emitted by this fluorophore reflect changes in $[Ca^{2+}]_i$ (25).

Changes in mitochondrial inner membrane potential (ψ_m) were determined after loading of cells with 5,5′-6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 10 μg/ml) at 37 °C for 15 min (10). Once loaded, the cells were washed twice and kept at 4 °C until use. All experiments were performed within the next 2 - 4 h.

Fluorescence measurements-video imaging

For monitoring Ca^{2+}-dependent fluorescence signals, small aliquots of fura-2-loaded cells were placed onto a coverslip attached to the bottom of a perfusion chamber on the stage of an epifluorescence-inverted microscope (Nikon Diaphot T200). After a 2 min period to allow spontaneous attachment of the cells to the coverslip, the cells were continuously superfused with the physiological solution (approximately 3 ml/min). No coating treatment was necessary to immobilize the cells.

Cells were alternatively excited with light from a xenon arc lamp passed through a high-speed monochromator (Polychrome IV, Photonics) at 340/380 nm. Fluorescence emission at 505 nm was detected using a cooled digital CCD camera (Hisca CCD C-6790, Hamamatsu) and recorded using dedicated software (Aquacosmos 2.5, Hamamatsu). Fluorescence images of 48 × 48 pixels with a resolution of 0.99 μm/pixel were recorded at 5 - 6 ratios/s (12 images/s). Small square areas were selected in the luminal and the basolateral pole within each individual cell. From the distance between both areas selected at each side of the cell and the difference in time between the increases in the fluorescence in these regions, we could determine the speed (μm/s) of the Ca^{2+} wave. For monitorisation of ψ_m small square areas were selected corresponding to fluorescent spots localized within the cells. JC-1 has been employed as a potential-sensitive probe. It accumulates in energized mitochondria and subsequently forms J-aggregates from monomers. A decrease in the JC-1 aggregate fluorescence at 590 nm as well as an increase in the monomer fluorescence at 535 nm reflects depolarization ψ_m.

It has been shown that H_2O_2 induces a non-specific decrease in JC-1 fluorescence at 595 nm, which is unrelated to changes in ψ_m. Thus, when the effect of an H_2O_2-induced oxidative stress is studied on ψ_m, only the monomer fluorescence monitored at 535 nm provides reliable information on changes in ψ_m (26). Therefore, when the effect of H_2O_2 of ψ_m was studied, we considered only fluorescence emission corresponding to J-monomer. In the experiments presented in this work only

fluorescence at this wavelength was taken into consideration, avoiding possible non-specific fluorescence responses of this probe due to unexpected behaviour of J-aggregates in the presence of H_2O_2. Throughout the experiments, cells were excited at 488 nm and emitted fluorescence was detected at 510 nm. An increase in fluorescence emission at this wavelength is related to depolarization of ψ_m.

Results obtained in fura-2-loaded cells are expressed as the ratio of fluorescence emitted at both excitation wavelengths. Results obtained in JC-1-loaded cells are expressed as the absolute values of fluorescence emission at the excitation wavelength employed. Data were normalized previously to the basal (resting) fluorescence values. All stimuli were dissolved in the physiological solution and applied directly to the cells in the perfusion chamber. Experiments were performed at room temperature (23 - 25°C).

Analysis of data

Data show the mean propagation rate of Ca^{2+} waves in μm/s ± SEM. Statistical analysis was performed by Student's *t* test and only *P* values less than 0.05 were considered as significant.

RESULTS

Cholecystokinin-evoked Ca^{2+} signals

As it has been previously shown, stimulation of pancreatic acinar cells with 1 nM of CCK-8 resulted in an initial increase in $[Ca^{2+}]_i$ at the luminal cell pole and subsequent spreading of the Ca^{2+} signal towards the basolateral cell membrane in the form of a Ca^{2+} wave (*Fig. 1*). No statistically significant differences were observed in the speed of propagation of Ca^{2+} signals when the cells were stimulated either in the presence or in the absence of Ca^{2+} in the extracellular medium, suggesting that there is no contribution of extracellular Ca^{2+} to the propagation of the Ca^{2+} wave (data not shown).

Effect of H_2O_2 and Tps on CCK-evoked Ca^{2+} waves

Propagation of Ca^{2+} signals in pancreatic cells can be modulated by intracellular metabolites and the activity of Ca^{2+} pumps (8, 9). Following an increase in $[Ca^{2+}]_i$ after hormonal stimulation, Ca^{2+} is extruded via the plasma membrane Ca^{2+}-ATPase and sequestered by a SERCA located in the membrane of intracellular stores (27, 28).

Since we have previously observed that H_2O_2 inhibits SERCA in pancreatic acinar cells (16), we evaluated if H_2O_2 has any effect on CCK-8-evoked Ca^{2+} waves. When pancreatic acinar cells were stimulated with 1 nM CCK-8 in the presence of 100 μM H_2O_2 (1.5 min preincubation) a statistically significant faster propagation rate of the Ca^{2+} signal compared to CCK-8 was observed (23.75 ± 0.96 μm/s, n = 12 expt/25 cells *vs*. 17.13 ± 0.55 μm/s, n = 18 expt/41 cells, respectively; $P < 0.001$). The propagation rate of Ca^{2+} signals following stimulation of cells with CCK-8 after preincubation in the presence of lower doses of H_2O_2, 1 and 10 μM, did not differ statistically from that observed in the

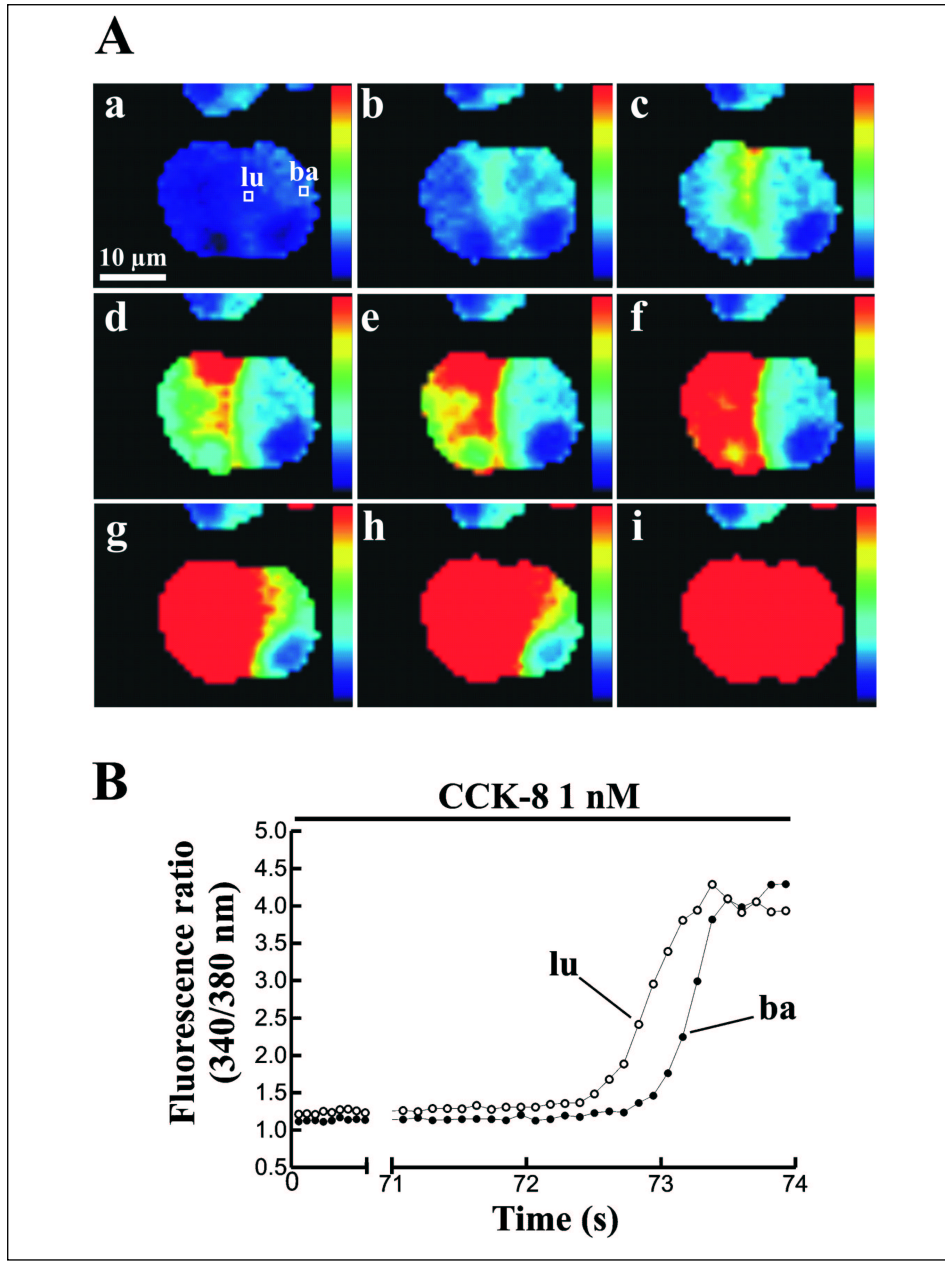

Fig. 1. Time-course of Ca^{2+} wave propagation in mouse pancreatic acinar cells. (**A**) Image series showing the propagation of Ca^{2+} waves. Stimulation of the cells with 1 nM CCK-8 led to an initial Ca^{2+} release at the luminal cell pole (lu) and subsequent spreading of the Ca^{2+} signal towards the basolateral (ba) membrane within the same cell in the form of a Ca^{2+} wave (a-i; images shown at 0.12 s interval). (**B**) Time course of changes in $[Ca^{2+}]_i$ at luminal (lu) and basolateral (ba) cell poles of a pancreatic acinar cell (shown in A) stimulated with 1 nM CCK-8.

presence of CCK-8 alone (17.41 ± 0.83 μm/s, n = 9 expt/18 cells and 17.17 ± 0.94 μm/s, n = 9 expt/18 cells, for 1 and 10 μM H_2O_2 respectively; *Fig. 2*). Higher concentrations of H_2O_2 led to a general increase in $[Ca^{2+}]_i$ all through the cytosol and abolished the response induced by CCK-8 (data not shown).Therefore, throughout our experiments, we used the concentration of 100 μM H_2O_2 for our analysis.

SERCA can be inhibited by Tps (29). As a control, when pancreatic acinar cells were stimulated with 1 nM CCK-8 in the presence of 1 μM Tps (1.5 min preincubation), spreading of CCK-8-induced Ca^{2+} waves was significantly faster as compared to the experiments in which cells were stimulated with CCK-8 alone (23.92 ± 1.01 μm/s, n = 12 expt/24 cells *vs*. 17.13 ± 0.55 μm/s, n = 18 expt/41 cells, respectively; $P < 0.001$; *Fig. 2*). The combination of Tps plus H_2O_2 had no additive effect on Ca^{2+} wave propagation compared to the effect of each treatment alone (data not shown).

The calculated ratios of fluorescence at the peak response following stimulation of pancreatic acinar cells with CCK-8 alone or in the presence of Tps or H_2O_2 (1.79 ± 0.03, 1.71 ± 0.03 and 1.72 ± 0.12, respectively) showed no significant differences, suggesting that speed of propagation of Ca^{2+} signals does not depend on the amount of Ca^{2+} released from the agonist-sensitive pools. Therefore, we can conclude that the differences in the propagation rate of Ca^{2+} waves must be due to modulation of secondary Ca^{2+} release from stores that promote spreading of the Ca^{2+} signal throughout the cytosol. Facilitated Ca^{2+} release from these secondary Ca^{2+} pools leads to a faster spreading of CCK-8-induced Ca^{2+} signals, whereas inhibition of secondary Ca^{2+} release leads to a

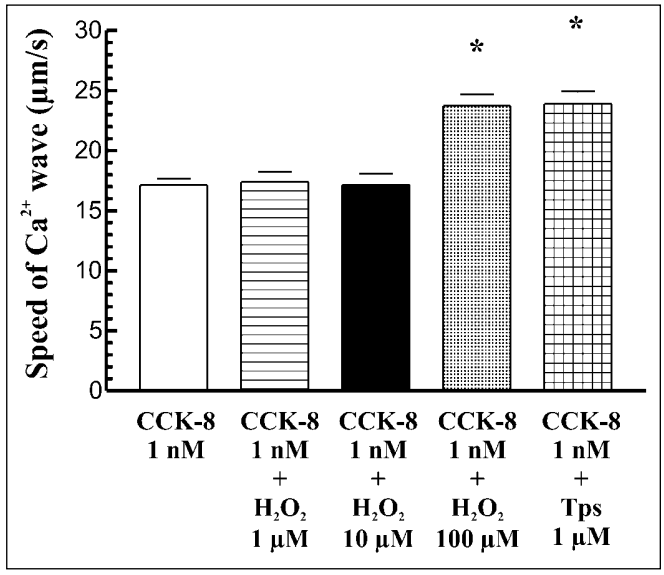

Fig. 2. CCK-8-evoked Ca^{2+} wave propagation in the presence of Tps and H_2O_2. Histograms depicting the speed of propagation of Ca^{2+} signals in response to 1 nM CCK-8 and after preincubation of pancreatic acinar cells in the presence of 1 μM Tps or 100 μM H_2O_2 (* Differences statistically significant compared to CCK-8-evoked responses; $P < 0.001$).

slower propagation rate of Ca^{2+} waves. Our results suggest that inhibition of SERCA activity leads to a faster propagation rate of CCK-8-evoked Ca^{2+} signals, and that, although the low concentrations of 1 and 10 μM H_2O_2 did not induce any statistically significant effect, a higher concentration induces a similar effect as Tps on CCK-8-evoked Ca^{2+} waves.

Effect of PKC on the propagation rate of Ca^{2+} waves

In order to investigate the participation of PKC on the propagation of Ca^{2+} signals, we performed a series of experiments in the presence of activators and inhibitors of the kinase.

When the pancreatic acinar cells were stimulated with 1 nM CCK-8 after a 5 min preincubation in the presence of 1 μM of the phorbol ester phorbol 12-myristate 13-acetate (PMA), to preactivate PKC, the spreading of Ca^{2+} waves was significantly slower as compared to the controls (13.49 ± 1.41 μm/s, n = 10 expt/22 cells *vs*. 17.13 ± 0.55 μm/s, n = 18 expt/41 cells, respectively; $P < 0.01$; *Fig. 3*).

On the other hand, the inhibition of PKC by preincubation of pancreatic acinar cells for 5 min in the presence of the PKC-inhibitor, GF-109203-X (5 μM) (30) induced a faster propagation of Ca^{2+} signals in response to 1 nM CCK-8, compared to the response in the presence of CCK-8 alone (29.18 ± 2.40 μm/s, n = 4 expt/8cells *vs*. 17.13 ± 0.55 μm/s, n = 18 expt/41 cells, respectively; $P < 0.001$; *Fig. 3*). Similar results were obtained using another inhibitor of PKC.

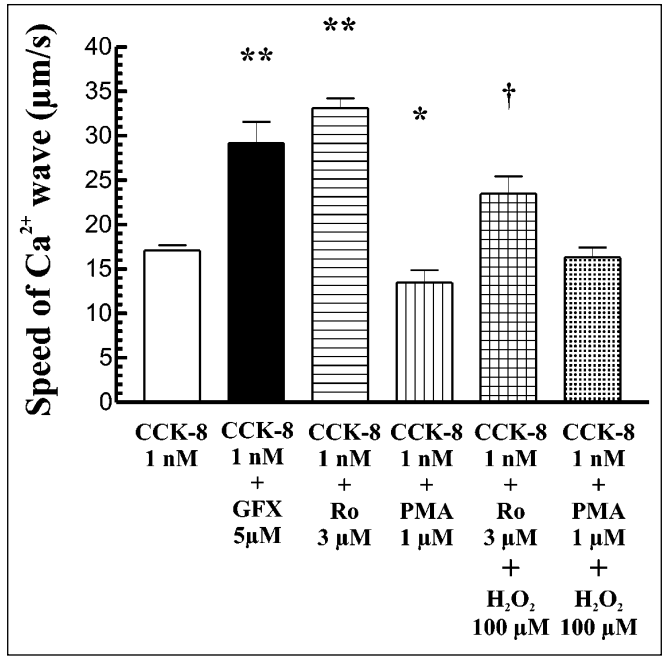

Fig. 3. Effect of PKC activation or inhibition on the propagation of Ca^{2+} waves. Speed of propagation of CCK-8-evoked Ca^{2+} waves after preincubation of pancreatic acinar cells in the presence of GFX (GF-109203-X) or Ro (Ro31-8220) and PMA to respectively inhibit or activate PKC, and a combination of PKC activators or inhibitors plus H_2O_2 (* = $P < 0.01$, ** = $P < 0.001$ compared to CCK-8-evoked responses; † = $P < 0.001$ compared to CCK-8-evoked responses in the presence of Ro31-8220).

Preincubation of pancreatic acinar cells during 5 min with the inhibitor of PKC, Ro-31-8220 (3 μM) (9) led as well to a faster propagation rate of Ca^{2+} waves in response to 1 nM CCK-8 (33.14 ± 1.09 μm/s, n = 16 expt/33 cells *vs*. 17.13 ± 0.55 μm/s, n = 18 expt/41 cells, $P< 0.001$; *Fig. 3*).

In another set of experiments, preincubation of pancreatic acinar cells in the presence of 100 μM H_2O_2, in addition to PMA (1 μM), did not induce any statistically significant differences in the speed of propagation of CCK-8-evoked Ca^{2+} waves compared to those evoked by the hormone in the presence of PMA alone (16.33 ± 1.09 μm/s, n = 18 expt/40 cells *vs*. 13.49 ± 1.41 μm/s, n = 10 expt/22 cells; *Fig. 3*), but it was significantly slower compared to the response of cells in the presence of H_2O_2 alone (16.33 ± 1.09 μm/s, n = 18 expt/40 cells *vs*. 23.75 ± 0.96 μm/s, n = 12 expt/25 cells; $P < 0.001$). This could be explained by an additive effect of PMA and H_2O_2 on PKC activation to slow down the speed of propagation of Ca^{2+} waves.

On the other hand, stimulation with CCK-8 (1 nM) after preincubation of cells in the presence of H_2O_2 (100 μM) plus the PKC inhibitor Ro-31-8220 (3 μM), led to the generation of Ca^{2+} signals that propagated throughout the cytosol at a lower speed compared to the response observed after stimulation of cells with CCK-8 in the presence of Ro-31-8220 alone (23.48 ± 1.98 μm/s, n = 6 expt/11 cells *vs*. 33.14 ± 1.09 μm/s, n = 16 expt/33 cells, $P< 0.001$; *Fig. 3*).

When we compared the response of cells in the presence of H_2O_2 alone (23.75 ± 0.96 μm/s, n = 12 expt/25 cells) with that obtained in the presence of a combination of the oxidant with Ro-31-8220 (23.48 ± 1.98 μm/s, n = 6 expt/11 cells), the differences were not statistically significant, whereas the latter is statistically significant compared to the response evoked by CCK-8 alone, i.e., the waves propagated faster (23.48 ± 1.98 μm/s, n = 6 expt/11 cells *vs*. 17.13 ± 0.55 μm/s, n = 18 expt/41 cells, $P< 0.001$).

These results show that PKC activation slows down the rate of propagation of Ca^{2+} waves evoked by CCK-8. The findings further suggest that PKC is activated to some extent by H_2O_2 and regulates the propagation of Ca^{2+} waves evoked by CCK-8, since preincubation with the oxidant counteracted the stimulatory effect on the speed of Ca^{2+} waves observed in the presence of PKC inhibitors.

Effect of mitochondrial Ca^{2+} uptake inhibition on CCK-evoked Ca^{2+} waves

In order to evaluate the role of mitochondria in the spatiotemporal progress of the Ca^{2+} response to CCK-8, we assayed the effect of mitochondrial inhibitors on the propagation of Ca^{2+} signals. The protonophore carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP) impairs the proton gradient across the mitochondrial inner membrane and the intramitochondrial negative potential (ψ_m), and leads to an inhibition of Ca^{2+} uptake into the organelle (10). When pancreatic acinar cells were stimulated with 1 nM CCK-8 following incubation of cells during 5 min in the presence of 100 nM of FCCP, we observed a faster

propagation rate of Ca^{2+} signals in comparison to the experiments in which the cells were stimulated with CCK-8 alone (20.53 ± 0.70 μm/s; n = 21 expt/41 cells *vs*. 17.13 ± 0.55 μm/s; n = 18 expt/41 cells, respectively; $P < 0.001$; *Fig. 4A*).

To discard an effect of ATP depletion on FCCP-evoked changes in the speed of Ca^{2+} wave propagation we performed a series of experiments in the presence of oligomycin, to avoid depletion of cellular ATP through reversal of ATP-synthase working mode. In the presence of 100 nM FCCP plus 10 μM oligomycin a similar result was observed, indicating that the responses obtained in the presence of FCCP are not due to ATP depletion (data not shown).

Similar results were obtained when pancreatic acinar cells were stimulated with CCK-8 after a preincubation for 30 min in the presence of 10 μM of the cell permeable oxygen-bridged dinuclear ruthenium amine complex (Ru360), an inhibitor of mitochondrial Ca^{2+} uptake (31, 32) (28.95 ± 0.98 μm/s; n = 17 expt/34 cells *vs*. 17.13 ± 0.55 μm/s; n = 18 expt/41 cells; $P < 0.001$; *Fig. 4A*).

Incubation of cells in the presence of the H_2O_2 (100 μM), in addition to either FCCP (100 nM) or Ru360 (10 μM), did not show any additive effects on the propagation rate of Ca^{2+} signals compared to the response observed after stimulation of cells with CCK-8 in the presence of either inhibitor alone (20.24 ± 1.57 μm/s; n = 8 expt/16 cells *vs*. 20.53 ± 0.70 μm/s; n = 21 expt/41 cells for FCCP and 26.43 ± 1.63 μm/s; n = 5 expt/10 cells *vs*. 28.95 ± 0.98 μm/s; n = 17 expt/34 cells for Ru360; *Fig. 4A*). Furthermore, this effect did not differ statistically from the response evoked by CCK-8 in the presence of H_2O_2 alone.

Given that inhibition of Ca^{2+} uptake into mitochondria by disruption of ψ_m would render a higher amount of Ca^{2+} free in the cytosol that, in turn, would stimulate CICR, the stimulatory effects of H_2O_2 on CCK-8 evoked Ca^{2+} waves could be also a partial consequence of its action on mitochondrial activity. To test whether the oxidant has an effect on ψ_m we carried out another set of experiments in which pancreatic acinar cells were loaded with the potential-sensitive dye 5,5′-6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) and then stimulated with H_2O_2 (100 μM). The oxidant led to a progressive depolarization of ψ_m that reached a constant value after 8-10 minutes in the presence of H_2O_2 (n = 5 exp/10 cell/56 mitochondrial area; *Fig. 4B*). These results demonstrate that mitochondria represent a limiting factor for the propagation of Ca^{2+} waves, as a consequence of the Ca^{2+} buffering by these organelles, and that depolarization of ψ_m by H_2O_2 could impair Ca^{2+} uptake into the organelle that would lead to a faster propagation rate of Ca^{2+} signals in response to the hormone.

Effect of cytoskeleton disruption on CCK-evoked Ca^{2+} waves

Participation of actin cytoskeleton in the propagation of Ca^{2+} waves was assayed in experiments in which pancreatic acinar cells were incubated in the presence of disruptors of actin cytoskeleton dynamics. Stimulation of pancreatic acinar cells with 1 nM CCK-8 after preincubation for 45 min in the presence of

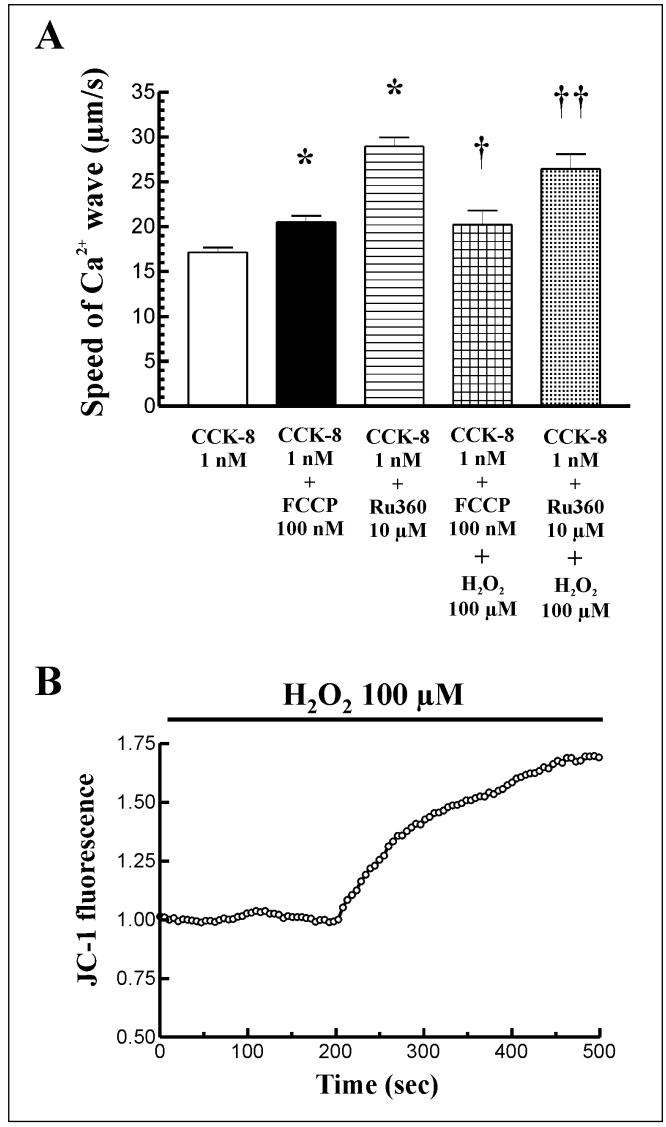

Fig. 4. Effect of mitochondrial inhibition on the propagation of CCK-8-evoked Ca^{2+} waves. (A) Speed of propagation of CCK-8-evoked Ca^{2+} waves after preincubation of pancreatic acinar cells in the presence of the mitochondrial uncoupler FCCP and Ru360, an inhibitor of Ca^{2+} uptake into mitochondria, as well as a combination of the uncoupler or the inhibitor plus H_2O_2 (* Differences statistically significant compared to CCK-8-evoked responses; $P < 0.001$; † = $P < 0.05$, †† = $P < 0.001$ compared to CCK-8-evoked responses). (B) Effect of H_2O_2 on ψ_m.

cytochalasin D (10 µM), which disrupts actin cytoskeleton architecture (33), led to a statistically significant acceleration in the propagation rate of Ca^{2+} signals in comparison to control experiments with CCK-8 alone (22.25 ± 0.84 µm/s, n = 30 expt/58 cells *vs*. 17.13 ± 0.55 µm/s, n = 18 expt/41 cells, respectively; $P < 0.001$; *Fig. 5*). However, when cells were stimulated with the hormone after preincubation with jasplakinolide (10 µM) for 30 min, a cell-permeant peptide isolated from *Jaspis johnstoni*, which has been shown to induce polymerization and stabilization of actin filaments (34), no statistically significant differences

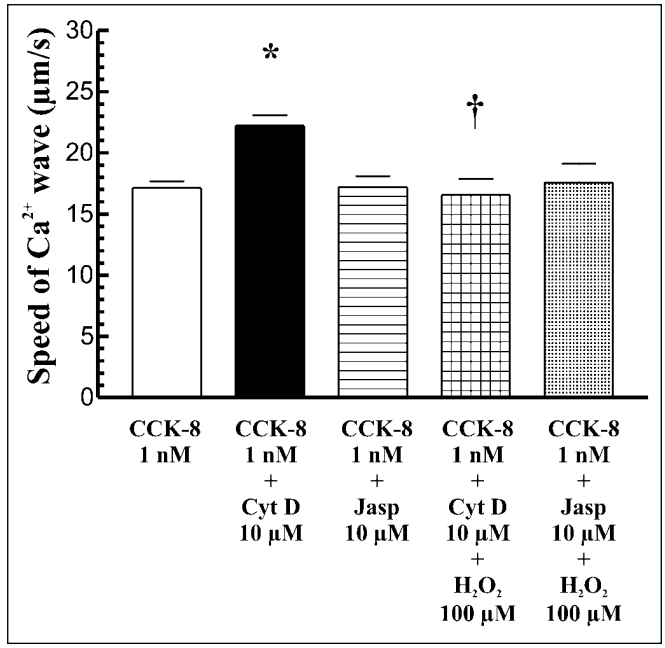

Fig. 5. Effect of actin cytoskeleton on the propagation of CCK-8-evoked Ca^{2+} waves. Pancreatic acinar cells were stimulated with CCK-8 after preincubation in the presence of cytochalasin D (Cyt D) to disrupt actin cytoskeleton architecture or jasplakinolide (Jasp) which stabilizes actin cytoskeleton, and a combination of them plus H_2O_2 (* Differences statistically significant compared to CCK-8-evoked responses; $P < 0.001$; † Differences statistically significant compared to CCK-8-evoked responses in the presence of cytochalasin D; $P < 0.01$).

were observed compared to control experiments with CCK-8 alone (17.21 ± 0.90 μm/s, n = 24 expt/47 cells *vs*. 17.13 ± 0.55 μm/s, n = 18 expt/41 cells, respectively; *Fig. 5*).

Incubation of cells in the presence of H_2O_2 (100 μM), in addition to cytochalasin D (10 μM), reduced the speed of propagation of Ca^{2+} signals compared to the response obtained after stimulation of cells with the hormone in the presence of cytochalasin D alone (16.57 ± 1.33 μm/s, n = 9 expt/17 cells *vs*. 22.25 ± 0.84 μm/s, n = 30 expt/58 cells; $P < 0.01$), and the effect was not statistically different from the propagation rate evoked by CCK-8 alone (16.57 ± 1.33 μm/s, n = 9 expt/17 cells *vs*. 17.13 ± 0.55 μm/s, n = 18 expt/41 cells; *Fig. 5*). On the other hand, incubation of pancreatic acinar cells with the oxidant in addition to jasplakinolide (10 μM), did not induce statistically significant differences in the propagation rate of CCK-8-evoked Ca^{2+} waves compared to that evoked by CCK-8 either in the presence of jasplakinolide (17.59 ± 1.52 μm/s, n=8 expt/16 cells *vs*. 17.21 ± 0.90 μm/s, n = 24 expt/47 cells) or alone (17.59 ± 1.52 μm/s, n=8 expt/16 cells *vs*. 17.13 ± 0.55 μm/s, n = 18 expt/41 cells; *Fig. 5*).

Actin cytoskeleton plays a critical role in Ca^{2+} homeostasis and, therefore, any action to depolymerise or stabilize actin will affect the processes depending on cytoskeleton dynamics. The results show that, actin cytoskeleton integrity plays a function in the regulation of Ca^{2+} signals induced by CCK-8 in pancreatic acinar cells.

DISCUSSION

Between all the mammalian cells models employed for investigation of agonist-evoked Ca^{2+} signalling, one of the most studied non-excitable cells is the acinar cell of the exocrine pancreas. Pancreatic acinar cells present a highly polarized distribution of subcellular components, which is related to a functional polarization. Areas of specialization can clearly be observed within the cytoplasm, varying between the zymogens granules area located at the apical (luminal) side of the cell and the nucleus, located at the basal pole of the acinar cell (35). In the pancreas, it has been described that CCK-8 can activate high- as well as low-affinity CCK-receptors (36 - 38). This may result in a differential involvement of second messenger systems generating different biological responses (9, 39).

In pancreatic acinar cells ROS are produced intracellularly as part of normal metabolic reactions (23, 40). ROS are highly reactive oxidants and their excessive or uncontrolled production can have detrimental effects on cellular physiology and function, leading to apoptosis and a variety of diseases. On the other hand, it has been suggested that low levels of ROS can modulate the normal physiological activity of the cell, serving as signalling molecules, as is the case for pancreatic acinar cells $[Ca^{2+}]_i$ oscillations in response to physiological stimulation.

Our data confirm previous investigations which show that SERCA activity, PKC activation, and mitochondrial activity can modulate the speed of propagation of CCK-8-evoked Ca^{2+} waves and propose a novel effect of actin polymerization on the spatial organization of the Ca^{2+} signals. We also show that H_2O_2 induces changes in the propagation rate of CCK-8-evoked Ca^{2+} signals, and propose that the generation of oxidants under normal or pathological activity of the cell modulate Ca^{2+} waves acting on multiple Ca^{2+}-handling mechanisms.

Ca^{2+} waves may represent an important early step for the coordination of cellular functions. Spreading of the Ca^{2+} signal is facilitated by regenerative mechanisms of Ca^{2+} mobilization derived from interactions between adjacent Ca^{2+} release points, the so-called CICR. Therefore, the synchronization of Ca^{2+} release events is crucial for the propagation of Ca^{2+} waves (41). It has been previously shown that there are hormone-dependent differences evoked in the time course for spreading of Ca^{2+} signals, and that the propagation of Ca^{2+} waves is regulated by SERCA activity and activation of PKC (8, 9). Our results are in agreement with these previous findings.

Here we show that CCK-8-evoked Ca^{2+} waves are accelerated after inhibition of SERCA by Tps. We also show that CCK-8-evoked Ca^{2+} waves are accelerated in the presence of H_2O_2. The effect of the oxidant could be explained on the basis of an inhibition of SERCA by H_2O_2 in a Tps-like manner, as it has been proposed (16, 40, 42). The inhibition of Ca^{2+} uptake into ER by Tps or H_2O_2 would lead to an elevated $[Ca^{2+}]_i$ and therefore to an acceleration of the speed of propagation of Ca^{2+} waves by CICR.

Our results further show that PKC activation slows down the rate of propagation of Ca^{2+} waves evoked by CCK-8, consistent with observations mentioned above.

Incubation of pancreatic acinar cells with H_2O_2 reduced the speed of CCK-8-evoked Ca^{2+} signals in cells treated with Ro-31-8220, reaching a value close to that observed after stimulation of cells with CCK-8 in the presence of H_2O_2 alone, which suggests that PKC could be activated to some extent by H_2O_2 and hence regulate the propagation of Ca^{2+} waves evoked by the hormone. Our results support previous observations by others, which show that PKC is activated by phosphorylation in H_2O_2-treated cells and is involved in H_2O_2-evoked apoptosis in different cell types (43, 44). It seems paradoxical that the oxidant accelerates the speed of CCK-8-evoked Ca^{2+} waves propagation acting in a Tps-like manner (as we have shown above), whereas acting to activate PKC it slows down the propagation rate of Ca^{2+} signals. A possible explanation for this observation could be that the oxidant acts to modulate both sites, in the sense that there is a final response arising from both the effect of SERCA inhibition and PKC activation, leading to a propagation rate of Ca^{2+} signals that result from a push-pull equilibrium.

It has been also shown that mitochondria play an important role in the regulation of Ca^{2+} signalling due to its ability to accumulate Ca^{2+}. In this sense, inhibition of mitochondrial Ca^{2+} import correlated to the conversion of oscillations in $[Ca^{2+}]_i$ to sustained responses, and permitted apical localized increases in $[Ca^{2+}]_i$ to propagate throughout the cell (11, 45). Therefore, mitochondrial activity can influence the propagation of Ca^{2+} signals throughout the cytosol. If mitochondria do not act as rapid Ca^{2+} buffering organelles, this would lead to an elevated $[Ca^{2+}]_i$ and therefore to an acceleration of the speed of propagation of Ca^{2+} waves by CICR. Our findings in which uncoupling of mitochondria by FCCP and the inhibition of mitochondrial Ca^{2+} uptake by Ru360 led to faster propagation rates of CCK-8-evoked Ca^{2+} signals are in agreement with this and support previous reports (46).

Our results further show that H_2O_2 induces depolarization of mitochondrial ψ_m, confirming previous observations (19). The effect of the oxidant on ψ_m would inhibit Ca^{2+} accumulation into mitochondria, and thereby would lead to a faster rate of Ca^{2+} wave propagation in response to CCK-8 stimulation by CICR. This observation is confirmed by the results in which the effect of H_2O_2 is not additive to that induced by FCCP or Ru360. Therefore, the acceleration in the rate of propagation of CCK-8-evoked Ca^{2+} waves in the presence of the oxidant could be a partial consequence of its action on mitochondrial activity.

The actin microfilaments of the cytoskeleton form a complex network whose stable interactions provide structural basis for the construction and remodelling of a variety of polarized subcellular structures, determining their positioning and dynamic movements (47).

Bozem *et al.* (48) showed that disruption of actin cytoskeleton results in an impairment of Ca^{2+} release in response to hormone stimulation of pancreatic acinar cells, presumably due to insufficient coupling of G-protein to PLC. This situation leads to subsequent decreases in IP_3 production and Ca^{2+} release. Similarly, Sergeeva *et al.* (49) have shown that disruption of actin cytoskeleton

suppresses oscillations in $[Ca^{2+}]_i$ by changing the balance between the Ca^{2+} regulating processes. And recently, Luo *et al.* (14) have shown that normal organelle localization is of critical importance in generating apical-to-basal Ca^{2+} signals. Furthermore, mitochondrial distribution within the cell as well as that of ryanodine-receptors is controlled by cytoskeleton (50, 51).

The results we have observed under stimulation of cells with CCK-8 in the presence of cytochalasin D and jasplakinolide can be explained on the basis of these previous investigations. Our results show that disruption of actin cytoskeleton by cytochalasin D treatment led to an acceleration of CCK-8-evoked Ca^{2+} waves. However, its stabilization by jasplakinolide treatment did not induce significant changes in the rate of propagation of CCK-8-evoked Ca^{2+} signals. Disruption of actin cytoskeleton would lead to abnormal organelle distribution within the cytosol, for example mitochondria, impairing its buffering capacity at the luminal cell pole and, therefore, leading to global Ca^{2+} signals instead of apical localized increases in $[Ca^{2+}]_i$ (11, 45) or to faster propagation rates of Ca^{2+} signals as we have shown. However, we can rule out a modulation of CCK-8-evoked Ca^{2+} signals by an action of H_2O_2 to disrupt actin cytoskeleton since we have previously shown that H_2O_2 increases, without net depolymerisation, actin filament content in pancreatic acinar cells, located both in the cell periphery and widespread in the cytosol (18).

Taken together, our results show that SERCA activity, PKC activation, and mitochondrial activity can regulate the speed of propagation of CCK-8-evoked Ca^{2+} signals, and that this process depends on the integrity of the actin cytoskeleton. H_2O_2, a ROS that induces changes in Ca^{2+} homeostasis in the exocrine pancreas, regulates the propagation rate of CCK-8-evoked Ca^{2+} signals, acting on multiple steps within the signal transduction machinery with an important role as Ca^{2+} handling mechanisms in mouse pancreatic acinar cells. In cellular physiology ROS can be considered as a double sided sword, presenting a wide range of actions on cellular homeostatic mechanisms, in the sense that high concentrations have deleterious effects to the cell, whereas at low concentrations can serve as signalling molecules. Locally generated or global diffusion of ROS therefore modulate the cellular mechanisms governing its physiology.

Symbols and abbreviations

Ca^{2+}, calcium; $[Ca^{2+}]_{i,}$ intracellular free Ca^{2+} concentration; CCK-8, cholecystokinin oktapeptide; CICR, Ca^{2+}-induced Ca^{2+} release; Cyt D, cytochalasin D; ER, endoplasmic reticulum; FCCP, carbonylcyanide-p-trifluoromethoxyphenyl hydrazone; H_2O_2, hydrogen peroxide; IP_3, inositol 1,4,5-trisphosphate; Jasp, jasplakinolide; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; Tps, thapsigargin; ψ_m, mitochondrial inner membrane potential.

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REFERENCES

1. Berridge MJ, Bootman MD, Roderick IIL. Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 2003; 4: 517-529.
2. Exton JH. Phosphoinositide phospholipases and G proteins in hormone action. *Annu Rev Physiol* 1994; 56: 349-369.
3. Putney JW. A model for receptor-regulated calcium entry. Cell Calcium 1986; 7: 1-12.
4. Kasai H, Augustine GJ. Cytosolic Ca^{2+} gradients triggering unidirectional fluid secretion from exocrine pancreas. *Nature* 1990; 348: 735-738.
5. Thorn P, Lawrie AM, Smith PM, Gallacher DV, Petersen OH. Local and global cytosolic Ca^{2+} oscillations in exocrine cells evoked by agonists and inositol trisphosphate. *Cell* 1993; 74: 661-668.
6. González A, Pfeiffer F, Schmid A, Schulz I. Effect of intracellular pH on acetylcholine-induced calcium waves in mouse pancreatic acinar cells. *Am J Physiol* 1998; 275: C810-C817.
7. Nathanson MH, Padfield PJ, O´Sullivan AJ, Burgstahler AD, Jamieson JD. Mechanism of Ca^{2+} wave propagation in pancreatic acinar cells. *J Biol Chem* 1992; 267: 18118-18121.
8. Pfeiffer F, Sternfeld L, Schmid A, Schulz I. Control of Ca^{2+} wave propagation in mouse pancreatic acinar cells. *Am J Physiol* 1998; 274: C663-C672.
9. González A, Schmid A, Sternfeld L, Krause E, Salido GM, Schulz I. CCK -evoked Ca^{2+} waves in isolated mouse pancreatic acinar cells are modulated by activation of cytosolic phospholipase A_2, phospholipase D and protein kinase C. *Biochem Biophys Res Commun* 1999; 261: 726-733.
10. González A, Schulz I, Schmid A. Agonist-evoked mitochondrial Ca^{2+} signals in mouse pancreatic acinar cells. *J Biol Chem* 2000; 275: 38680-38686.
11. Tinel H, Cancela JM, Mogami H, *et al.* Active mitochondria surrounding the pancreatic acing granule region prevent spreading of inositol trisphosphate-evoked local cytosolic Ca^{2+} signals. *EMBO J* 1999; 18: 4999-5008.
12. Dolman NJ, Gerasimenko JV, Gerasimenko OV, Voronina SG, Petersen OH, Tepikin AV. Stable Golgi-mitochondria complexes and formation of Golgi Ca^{2+} gradients in pancreatic acinar cells. *J Biol Chem* 2005; 280: 15794-15799.
13. Yamakasi M, Masgrau R, Morgan AJ, *et al.* Organelle selection determines agonist-specific Ca^{2+} signals in pancreatic acinar and β cells. *J Biol Chem* 2004; 279: 7234-7240.
14. Luo X, Shin DM, Wang X, Konieczny SF, Muallem S. Aberrant localization of intracellular organelles, $Ca2^{+}$ signalling and exocytosis in Mist1 null mice. *J Biol Chem* 2005; 280: 12668-12675.
15. Suzuki YJ, Forman HJ, Sevanian A. Oxidants as stimulators of signal transduction. *Free Rad Biol Med* 1997; 22: 269-285.
16. Pariente JA, Camello C, Camello PJ, Salido GM. Release of calcium from mitochondrial and nonmitochondrial intracellular stores in mouse pancreatic acinar cells by hydrogen peroxide. *J Membr Biol* 2001; 179: 27-35.
17. González A, Schmid A, Salido GM, Camello PJ, Pariente JA. XOD-catalyzed ROS generation mobilizes calcium from intracellular stores in mouse pancreatic acinar cells. *Cell Signal* 2002; 14: 153-159.

18. Rosado JA, González A, Salido GM, Pariente JA. Effects of reactive oxygen species on actin filament polymerisation and amylase secretion in mouse pancreatic acinar cells. *Cell Signal* 2002; 14: 547-556.
19. Granados MP, Salido GM, Pariente JA, González A. Effect of H_2O_2 on CCK-evoked changes in mitochondrial activity in isolated mouse pancreatic acinar cells. *Biol Cell* 2005; 97: 847-856.
20. Weber H, Roesner JP, Nebe B, *et al.* Increased cytosolic Ca^{2+} amplifies oxygen radical-induced alterations of the ultrastructure and the energy metabolism of isolated rat pancreatic acinar cells. *Digestion* 1998; 59: 175-185.
21. Jacobson J, Duchen, MR. Mitochondrial oxidative stress and cell death in astrocytes-requirement for stored Ca^{2+} and sustained opening of the permeability transition pore. *J Cell Sci* 2002; 115: 1175-1188.
22. Camello C, Camello PJ, Pariente JA, Salido GM. Effects of antioxidants on calcium signal induced by cholecystokinin in mouse pancreatic acinar cells. *J Physiol Biochem* 2000; 56: 173-180.
23. Camello-Almaraz MC, Pozo MJ, Murphy MP, Camello PJ. Mitochondrial production of oxidants is necessary for physiological calcium oscillations. *J Cell Physiol* 2006; 206: 487-494.
24. Camello-Almaraz C, Pariente JA, Salido GM, Camello PJ. Differential involvement of vacuolar H^+-ATPase in the refilling of thapsigargin- and agonist-mobilized Ca^{2+} stores. *Biochem Biophys Res Commum* 2000; 271: 311-317.
25. Grynkiewicz G, Poenie M, Tsien RY. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; 260: 3440-3450.
26. Chinopoulos C, Tretter L, Adam-Vizi V. Depolarisation of in situ mitochondria due to hydrogen peroxide-induced oxidative stress in nerve terminals: inhibition of α-ketoglutarate dehydrogenase. *J Neurochem* 1999; 73: 220-228.
27. Lytton J, Westlin M, Burk SE, Shull GE, MacLennan DH. Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J Biol Chem* 1992; 267: 14483-14489.
28. Camello PJ, Gardner J, Petersen OH, Tepikin AV. Calcium dependence of calcium extrusion and calcium uptake in mouse pancreatic acinar cells. *J Physiol* 1996; 490: 585-593.
29. Ozawa T, Thévenod F, Schulz I. Characterization of two different Ca^{2+} uptake and IP_3-sensitive Ca^{2+} release mechanisms in microsomal Ca^{2+} pools of rat pancreatic acinar cells. *J Membr Biol* 1995; 144: 111-120.
30. Gomez-Puertas P, Satrustegui J, Bogonez E. Synaptic vesicles isolated from 32P-prelabeled synaptosomes contain a phosphoprotein of apparent M(r) 65,000 (pp65), a possible substrate for PKC. *J Biol Chem* 1994; 269: 7564-7570.
31. Zhou Z, Bers DM. Time course of action of antagonists of mitochondrial Ca^{2+} uptake in intact ventricular myocytes. *Pflügers Arch* 2002; 445: 132-138.
32. Bruce JIE, Giovannucci DR, Blinder G, Shuttleworth TJ, Yule DI. Modulation of $[Ca^{2+}]_i$ signaling dynamics and metabolism by perinuclear mitochondria in mouse parotid acinar cells. *J Biol Chem* 2004; 279: 12909-12917.
33. Flanagan MD, Lin S. Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. *J Biol Chem* 1980; 255: 835-838.
34. Bubb MR, Senderowicz AM, Sausville EA, Duncan KL, Korn ED. Jasplakinolide, a cytotoxic natural product, induces actin polymerisation and competitively inhibits the binding of phalloidin to F-actin. *J Biol Chem* 1994; 269: 14869-14871.
35. Gorelick FS, Jamieson JD. The pancreatic acinar cell: structure-function relationships. In Physiology of the gastrointestinal tract vol 2 (3rd ed), LR Johnson (ed). New York, Raven Press, 1994, pp. 1353-1376.

36. Sankaran H, Goldfine ID, Bailey A, Licko V, Williams JA. Relationship of cholecystokinin receptor binding to regulation of biological functions in pancreatic acini. *Am J Physiol* 1982; 242: G250-G257.
37. Matozaki T, Martinez J, Williams JA. A new CCK analogue differentiates two functional distinct CCK receptors in rat and mouse pancreatic acini. *Am J Physiol* 1989; 257: G594-G600.
38. Tsunoda Y, Owyang C. High-affinity CCK receptors are coupled to phospholipase A_2 pathways to mediate pancreatic amylase secretion. *Am J Physiol* 1995; 269: G435-G444.
39. Williams JA, Blevins GT Jr. Cholecystokinin and regulation of pancreatic acinar cell function. *Physiol Reviews* 1993; 73: 701-723.
40. Granados MP, Salido GM, González A, Pariente JA. Dose-Dependent effect of hydrogen peroxide on calcium mobilisation in mouse pancreatic acinar cells. *Biochem Cell Biol* 2006; 84: 39-48.
41. Lin X, Várnai P, Csordás G, *et al.* Control of calcium signal propagation to the mitochondria by inositol 1,4,5-trisphosphate-binding proteins. *J Biol Chem* 2005; 280: 12820-12832.
42. Sánchez S, Fernández-Belda F, Soler F. Functional effect of hydrogen peroxide on the sarcoplasmic reticulum membrane: uncoupling and irreversible inhibition of the Ca^{2+}-ATPase protein. *Arch Biochem Biophys* 2004; 431: 245-251.
43. Konishi H, Yamauchi E, Taniguchi H, *et al.* Phosphorylation sites of protein kinase C delta in H_2O_2-treated cells and its activation by tyrosine kinase *in vitro*. *Proc Natl Acad Sci USA* 2001; 98: 6587-6592.
44. Kumar S, Bharti A, Mishra NC, *et al.* Targeting of the c-Abl tyrosine kinase to mitochondria in the necrotic cell death response to oxidative stress. *J Biol Chem* 2001; 276: 17281-17285.
45. Straub SV, Giovannucci DR, Yule ID. Calcium wave propagation in pancreatic acinar cells: functional interaction of inositol 1,4,5-trisphosphate receptors, ryanodine receptors, and mitochondria. *J Gen Physiol* 2000; 116: 547-560.
46. Camello-Almaraz C, Salido GM, Pariente JA, Camello PJ. Role of mitochondria in Ca^{2+} oscillations and shape of Ca^{2+} signals in pancreatic acinar cells. *Biochem Pharmacol* 2002; 63: 283-292.
47. Mironov SL, Ivanniko MV, Johannson M. $[Ca^{2+}]_i$ signalling between mitochondria and endoplasmic reticulum in neurons is regulated by microtubules. *J Biol Chem* 2005; 280: 715-721.
48. Bozem M, Kuhlmann R, Blum R, Feick P, Schulz I. Hormone-stimulated calcium release is inhibited by cytoskeleton-disrupting toxins in AR4-2J cells. *Cell Calcium* 2000; 28: 73-82.
49. Sergeeva M, Ubl JJ, Reiser G. Disruption of actin cytoskeleton in cultured rat astrocytes suppresses ATP- and bradykinin-induced $[Ca^{2+}]_i$ oscillations by reducing the coupling efficiency between Ca^{2+} release, capacitative Ca^{2+} entry and store refilling. *Neurosci* 2000; 97: 765-769.
50. Baumann O. Disruption of actin filaments causes redistribution of ryanodine receptor Ca^{2+} channels in honeybee photoreceptor cells. *Neurosci Lett* 2001; 306: 181-184.
51. Foissner I. Microfilaments and microtubules control shape, motility and subcellular distribution of cortical mitochondria in charcean internodal cells. *Protoplasma* 2004; 224: 145-157.

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