THE GERONTOLOGICAL SOCIETY OF AMERICA®

*Journals of Gerontology: Biological Sciences*
cite as: *J Gerontol A Biol Sci Med Sci*, 2016, Vol. 71, No. 2, 145–152
doi:10.1093/gerona/glv018
Advance Access publication March 24, 2015

OXFORD

Original Article

# Propagation of Intracellular $Ca^{2+}$ Signals in Aged Exocrine Cells

**Francisco E. Martin-Cano,[1] Cristina Camello-Almaraz,[1] Jesús González Macías,[2] Maria J. Pozo,[1] and Pedro J. Camello[1]**

[1]Department of Physiology, Faculty of Nursing and Occupational Therapy, University of Extremadura, Cáceres, Spain.
[2]Hospital Marqués de Valdecilla, University of Cantabria, IDIVAL, Santander, Spain.

Address correspondence to Pedro J. Camello, PhD, DVM, Department of Physiology, Faculty of Nursing and Occupational Therapy and Fac. Veterinary Sciences, University of Extremadura, Campus Universitario, 10003 Cáceres, Spain. Email: pcamello@unex.es

Received August 12, 2014; Accepted February 3, 2015

**Decision Editor:** Rafael de Cabo, PhD

## Abstract

There is little information on the effects of aging in the propagation of calcium signals and its underlying mechanisms. We studied the effects of aging on propagation of $Ca^{2+}$ signals in pancreatic acinar cells. Fura-2 loaded cells isolated from young (3–4 months old) and aged (24 months old) mouse responded to acetylcholine (ACh) and cholecystokinin (CCK) with a polarized $Ca^{2+}$ response initiated at the secretory pole before spreading to the basal one. Aging slowed down the propagation of the response to ACh but enhanced the velocity of the CCK response. This pattern can be explained by the age-induced depolarization of mitochondria, because it can be reproduced in young cells by mitochondrial inhibitors. Aging also increased the role of acidic stores in the CCK signal, as judged by the folimycin-induced suppression of the polarization in aged but not in young cells. The involvement of ryanodine receptors in the ACh response was also enhanced, as indicated by the loss of polarization after the treatment with 8Br–cyclic ADP ribose. Therefore, we conclude that aging modifies differentially the propagation of ACh and CCK-evoked $Ca^{2+}$ signals through mitochondrial depolarization and changes in the role of the acidic $Ca^{2+}$ stores and ryanodine receptors in the initiation of the signals.

**Key Words:** Calcium signaling—Exocrine pancreas—Mouse—Mitochondria

One of the consequences of aging is the alteration of the cytosolic $Ca^{2+}$ increases ($[Ca^{2+}]_i$ signals) induced by cell agonists (1–7). The importance of these changes is obvious given the involvement of this signaling system in all the aspects of the cell physiology. This functional diversity involves a precise and segregated regulation of diverse cell functions, which is accomplished through the development and propagation of subcellular $[Ca^{2+}]_i$ signals (8). In spite of this, there is almost no information on the effects of aging in the spatiotemporal propagation of $[Ca^{2+}]_i$ signals within the cells.

We have previously shown that aging down-regulates the secretory function of pancreatic acinar cells. This was underlaid by the impairment of different aspects of calcium signal, from the response to physiological and supraphysiological stimulation to $Ca^{2+}$ transporting mechanisms (1).

Pancreatic acinar cells are a classic model to study $[Ca^{2+}]_i$ signaling mechanisms and exocytosis. This polarized cell type exhibits local $[Ca^{2+}]_i$ responses restricted to the apical or secretory pole that eventually propagate toward the basolateral pole after exceeding the mitochondrial network surrounding the apical pole [for a review see ref. (9)]. Low-level stimulation (cholinergic input during the cephalic phase of digestion and also postprandial cholecystokinin [CCK] levels during the intestinal phase) induces repetitive $[Ca^{2+}]_i$ transients localized at the apical pole of the cell to stimulate exocytosis and the accompanying ionic transport. Eventually, some of these signals spread all across the cytosol toward the basal pole in order to regulate ion transport, protein secretion, and metabolism to support the secretory function of the cell (9,10). Any change in the mechanisms propagating the intracellular $Ca^{2+}$ signals has important consequences on the cellular physiology. However,

similar to other cell types, the subcellular propagation of the polarized $[Ca^{2+}]_i$ response of this cell type has not been studied in aged individuals.

The triggering and spreading of subcellular $[Ca^{2+}]_i$ responses in nonexcitable cells relies on $Ca^{2+}$ release from stores operated by the second messengers inositol 1,4,5-trisphosphate ($IP_3$), cyclic ADP ribose (cADPr), and nicotinic acid adenine dinucleotide phosphate (NAADP). The apical area of pancreatic acinar cells is very sensitive to these intracellular messengers because it contains receptors for these messengers ($IP_3R$, RyR, and probably NAADP receptors). This allows this area to behaves as a "trigger" zone for $Ca^{2+}$ signals which then spread as $Ca^{2+}$-induced $Ca^{2+}$-release through the same receptors (11,12). Mitochondria have been reported to regulate the propagation of these signals although there are conflicting reports (13–15). As mitochondria in aged acinar cells show a partial depolarization (1), we explored in this study the possibility that aging modifies the propagation of agonist-evoked $[Ca^{2+}]_i$ signals in pancreatic acinar cells.

## Methods

### Animals

Mice (Swiss OF1) were divided into two groups: young adults (3 months old) and aged (22–24 months old). Animals were housed in light (12 hours light–12 hours dark cycle) and temperature (20°C) controlled rooms. The experiments were performed according to European guidelines for animal research and approved by the Animal Ethics Committee of the University of Extremadura.

### Cell Isolation and Handling

After sacrifice (following ethical guidelines of the Ethical Committee of the University of Extremadura), a suspension of single cells and small acini was prepared from pancreas by injection with purified collagenase (from *Clostridium histolyticum*, 200 U/mL, 37°C, 6–12 minutes) followed by vigorous manual agitation and gentle pippetting (16). Isolated cells were suspended in physiological solution (Na-HEPES) containing (mM): 140 NaCl, 4.7 KCl, 2 $CaCl_2$, 1.1 $MgCl_2$, 10 glucose, 10 N-2-hydroxyethylpiperazine-N′-2-sulfonic acid (HEPES), and 0.01% trypsin inhibitor (soybean), pH 7.4.

### $[Ca^{2+}]_i$ Determination

Once loaded with the fluorescent calcium indicator fura-2 AM (1–2 μM, 30 minutes, room temperature), cells were washed, allowed to rest for 20–30 minutes for dye de-esterification, and used within the next 2–4 hours. A small volume of the cell suspension was placed on a perfusion chamber made with a poly-D-Lys-coated coverslip (thickness 0.17 mm), placed on the stage of an inverted fluorescence-equipped microscope (Nikon TE2000, Barcelona, Spain) and viewed with a 100× objective (oil immersion). Extracellular application of agonists and inhibitors was performed by a gravity perfusion system at room temperature. Cells were excited by a computer controlled monochromator (Optoscan, Cairn Research Ltd., Kent, UK) at 340/380 nm, and the emitted fluorescence (510 nm, band pass filter) was recorded with a cooled CCD camera (Orca-II-ER, Hamamatsu Photonics, Cerdanyola, Spain) and dedicated software (Metafluor, Molecular Devices, Downingtown, PA). For each experiment the field of view was cropped to the size of the studied cell. This allowed a recording speed of 5–10 pairs of 340/380 nm images per second.

Analysis and quantification of the images was performed using Metafluor Analyst (Molecular Devices) and Fiji distribution of ImageJ [Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/, 1997–2012; (17)]. Changes in $[Ca^{2+}]_i$ are expressed as changes in the ratio between the fura-2 fluorescence emission at 340 and 380 nm excitation wavelengths ($F_{340}/F_{380}$) after background subtraction. For presentation purposes, images were colored using a pseudocolor palette matching the range of the response of the cell of interest. To calculate the velocity of propagation of the signal (pixels × $s^{-1}$) we estimated the time between initiation of the signal at the apical pole of the cell and initiation at the last spot of the basolateral pole developing the response. The criteria for initiation were the time point when the ratio of fluorescence reached 5% of the amplitude of the response (equal to 2–3 *SD* of the resting values). The size of the pixel in the experimental setup was 0.2577 μm, but we did not translate the speed into μm since it was irrelevant for the conclusions of the study. The rate of rise of $[Ca^{2+}]_i$ was calculated as the first differential of ratio respect to time (dRatio fura-2/dt) and is presented as ratio units × $s^{-1}$. Amplitude of the responses is expressed as the fractional increase of the fura-2 ratio respect to resting ratio (Ro) (Δ Ratio/R0).

### Determination of Mitochondrial Potential

To assess mitochondrial potential we used the potentiometric dye TMRM under quenching conditions (see later), which in this cell type is more sensitive than nonquenching conditions (18): accumulation of the dye by polarized mitochondria quenches fluorescence, so that depolarization increases the fluorescence of mitochondrial areas. Pancreatic acinar cells were loaded with 1,000 nM TMRM during 10 minutes at room temperature, placed on a perfusion chamber made with a poly-D-Lys-coated coverslip (thickness 0.17 mm) in the stage of a inverted laser scanning confocal microscope (Nikon A1, Barcelona, Spain) and viewed with a 60× objective (oil immersion) using 1.2 Airy units iris aperture. Cells were excited at 490 nm and the emitted fluorescent images (>525 ± 15 nm, band-pass filter) was recorded at room temperature at 1 Hz.

### Statistics

Results were expressed as mean ± SEM (standard error of the mean), and we indicate the number of cells (isolated from at least three independent preparations) for each experimental group. One-way or two-way ANOVA test was used for comparison of age groups and treatments as appropriate, followed by planned comparisons tests between selected groups. Student's *t* test was used for comparison between two groups. Differences were considered significant for $p < .05$.

### Reagents and Drugs

Collagenase (type CLSPA) was obtained from Worthington Biochemical Corp. (Lakewood, NJ), fura-2 (acetoxymethyl ester), and TMRM from Molecular Probes (Invitrogen SA, Barcelona, Spain). Melatonin, trypsin inhibitor, acetylcholine (ACh), and CCK octapeptide sulphate and other common reagents were from Sigma–Aldrich Química (Madrid, Spain).

## Results

Application of either ACh (10 μM) or CCK (1 nM) induced a polarized $[Ca^{2+}]_i$ response in mouse pancreatic acinar cells. In both cases,

the signals started in the apical or secretory pole and then spread toward the basolateral area of the cell as a wave or tide (Figure 1A). This initiating or trigger area was clearly visible as a rising $[Ca^{2+}]_i$ spot, and is a well known feature of the $[Ca^{2+}]_i$ signal in this cell type. The velocity of propagation was higher for ACh (68.73 ± 4.91 pixels × $s^{-1}$, $n$ = 32) than for CCK (39.92 ± 2.41 pixels × $s^{-1}$, $n$ = 25) in cells isolated from young mice ($p < .005$). The maximal rate of rise (expressed as ratio units × $s^{-1}$, r.u. × $s^{-1}$) also showed higher values for ACh (1.95 ± 0.17 r.u. × $s^{-1}$) than for CCK (1.39 ± 0.09; $p < .05$). Aging induced a clear differential effect on the two responses, as shown in Figure 1B. Propagation of the ACh response was impaired in aged cells (39.15 ± 5.92 pixels × $s^{-1}$, $n$ = 22, $p < .005$ respect to young cells), similar to rate of the response (1.40 ± 0.11 r.u. × $s^{-1}$, $p < .05$). On the contrary, in aged cells the CCK-induced response showed increases in the velocity of propagation (73.28 ± 7.58 pixels × $s^{-1}$, $n$ = 27, $p < .005$) and in the rate of the rise (2.16 ± 0.18 r.u. × $s^{-1}$, $p < .01$).

A detailed analysis of the rates of rise of the apical and basal poles of the cells showed that the effect of ACh and CCK was age-dependent ($F$ = 3.65, $p < .0003$; Figure 1.B1). Aging induced a significant decrease in the rate of response to ACh in the basal but not

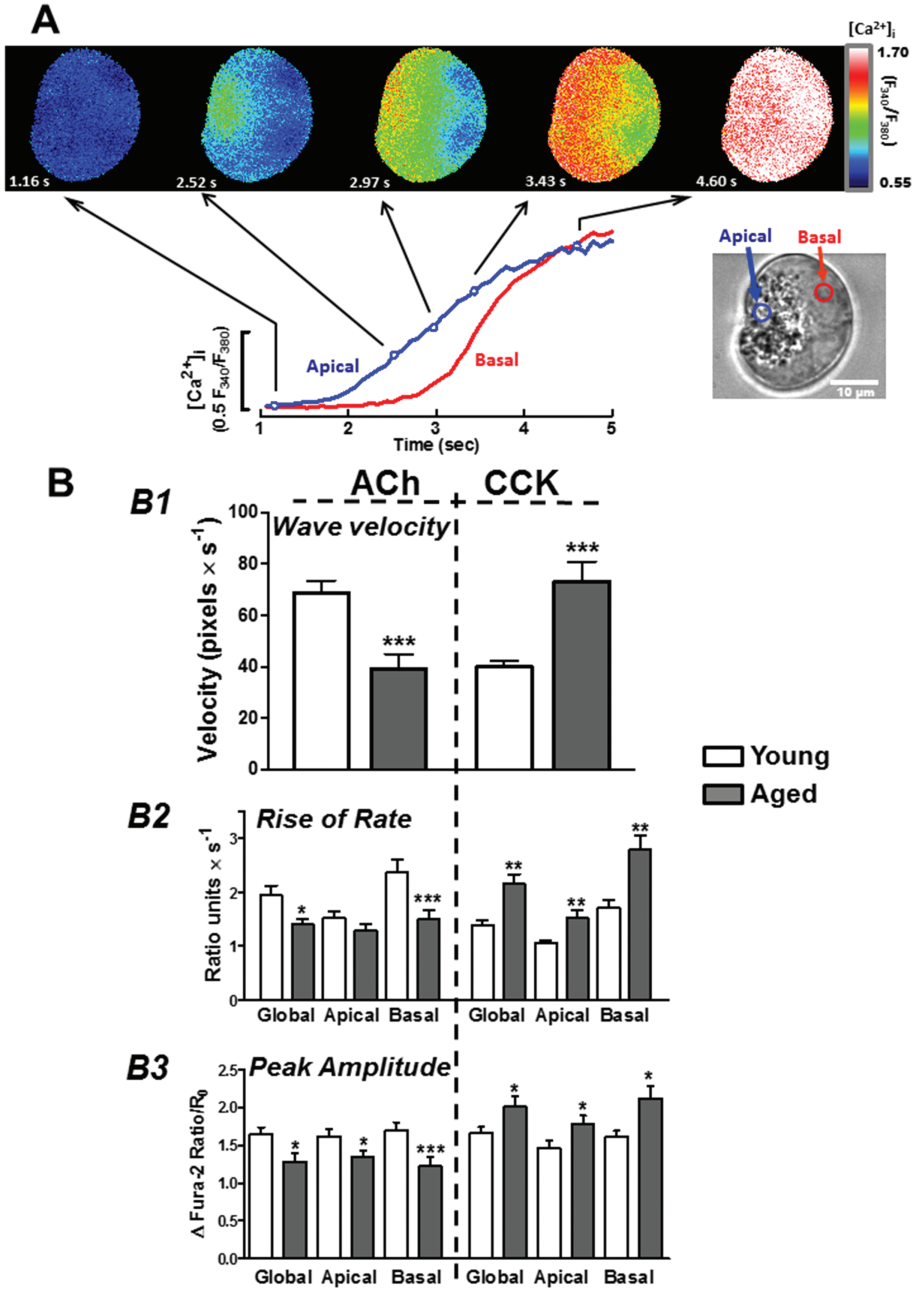


**Figure 1.** Propagation of $Ca^{2+}$ signals in response to ACh (10 µM) and cholecystokinin (CCK; 1 nM) in pancreatic acinar cells isolated from young and aged mice. (**A**) A representative response to CCK (applied at Time 0) at the apical and basal areas of the cell marked in the bright field image of the right. The top sequence shows pseudocolor images of the fura-2 ratio ($F_{340}/F_{380}$) at different time points of the response (emphasized as open circles). The pseudocolor palette is shown on the right. (**B**) Histograms showing the average values of the velocity of propagation (**B1**), and the rise of rate (**B2**) and amplitude (**B3**) of the global and local responses. *$p < .05$, **$p < .01$, ***$p < .005$ respect to young. $n$ = 22–32 cells.

in the apical pole (which showed a non significant decrease), whereas the response to CCK was enhanced in poles (Figure 1.B2). This result suggests that aging induced different alterations in the mechanisms of the response of the two cell areas.

A similar pattern was observed in the amplitude of the $[Ca^{2+}]_i$ peak: as can be observed in Figure 1.B3, aging impaired the ACh response but enhanced the CCK response, similar to the effect on velocity and rate of rise.

A possible explanation for the differential effect of aging on ACh and CCK responses are the mitochondrial alterations in aged cells. If mitochondria delay the propagation of CCK response but reinforce the propagation of the ACh signal, the partial depolarization of mitochondria reported in aged pancreatic acinar cells (1) could contribute to the changes described previously. To assess this possibility, in a series of experiments we studied the effect of a short application (3–5 minutes) of the mitochondrial inhibitors rotenone (0.3 μM) or CCCP (50 nM) on the responses to ACh and CCK in young cells. Figure 2B shows a protocol illustrating that in our experimental conditions rotenone and CCCP induced the expected decrease of mitochondrial potential, as revealed by an increase in the fluorescence of TMRM loaded into mitochondria, a nernstian probe whose fluorescence is quenched at the concentration used in this study [see Methods section (18)]. Figure 2.A1 shows that, as predicted, the propagation of the ACh response was slowed down by simple application of either rotenone or CCCP ($F = 11.41$, $p < .0001$), resembling the effect of aging. On the contrary, in the case of CCK the inhibitors induced a significant ($F = 6.92$, $p < .003$) increase in the speed of propagation, similar to the effect of aging on this parameter.

We also examined the regional effects of mitochondrial inhibitors on the rate of rise and the amplitude of the $Ca^{2+}$ responses. As observed in panels A2 and A3 of Figure 2, mitochondrial inhibition induced changes analogous to those observed in aged cells (Figure 1.B2 and B3); once more, both the rate and the amplitude of the response were inhibited for ACh and increased for CCK, although for the latter the effect was significant only in CCCP treated cells. Taken together, these results indicate that mitochondrial depolarization is a plausible mechanism for age-induced impairment of $Ca^{2+}$ signal propagation.

Acidic $Ca^{2+}$ stores are a source for $Ca^{2+}$ release in pancreatic acinar cells (19,20), where the apical poke is densely packed with secretory and endosomal acidic vesicles which respond to intracellular $Ca^{2+}$ releasing messengers (20,21). Therefore, we tested the effect of folimycin, a specific inhibitor of the V-type $H^+$ pump located on the acidic stores. In young cells, folimycin induced a reduction in the speed of propagation of CCK-evoked response (treated 33.01 ± 3.71 vs untreated 45.66 ± 3.33 pixels × $s^{-1}$, $n = 8$, $p < .05$), indicative of the role of acidic stores in the signal initiation. In the case of aged cells the effect of folimycin was stronger, inducing a clear suppression of the polarized response: 9 out of 12 cells showed a simultaneous response, with absent or negligible polarization (Figure 3A; whereas in control aged cells this pattern was observed only in 4 out of 31 cells). In the three cells showing a polarized response the velocity was clearly inhibited (48.56 ± 7.78 pixels × $s^{-1}$, $p < .05$) when compared to untreated cells (91.25 ± 12.13, $n = 12$). On the contrary, in the case

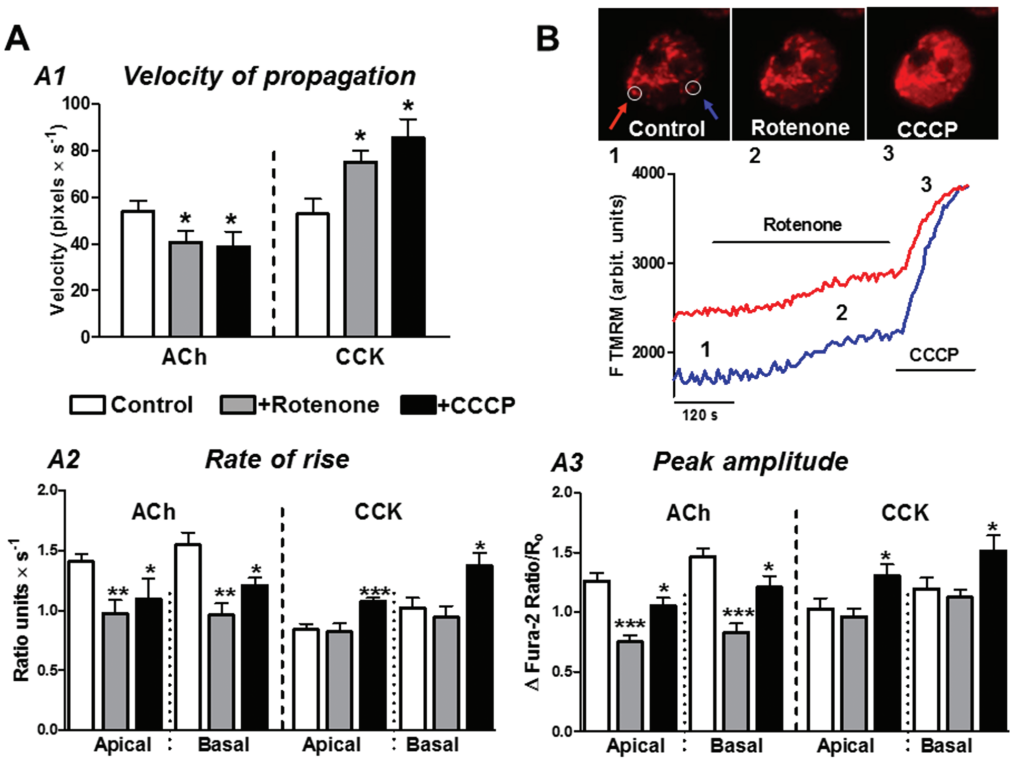


**Figure 2.** Effect of mitochondrial inhibitors on the propagation of the $Ca^{2+}$ signal. (**A**) Effects of rotenone (0.3 μM) or CCCP (50 nM) pretreatment on the propagation velocity (**A1**), and on the local rate of rise (**A2**) and amplitude (**A3**) of the responses to ACh (10 μM) and cholecystokinin (CCK; 1 nM) in young pancreatic acinar cells. One-way ANOVA for ACh or for CCK showed significant effect ($p < .05$ or smaller) of treatment for all the parameters. $p < .05$, $^{**}p < .01$, $^{***}p < .005$ versus control. $n = 5$–16 cells. (**B**) Protocol assessing the typical effect of rotenone and CCCP on the mitochondrial potential. The three top images show the fluorescence of a pancreatic acinar cell loaded at quenching conditions (1 μM) at rest (1) and after subsequent rotenone (2) and CCCP (3) application. The traces show the changes of TMRM fluorescence in the mitocohondrial regions marked by circles and corresponding color arrows in Frame 1: rotenone- and CCCP-evoked depolarization released the dye from mitochondria and therefore de-quenched fluorescence.

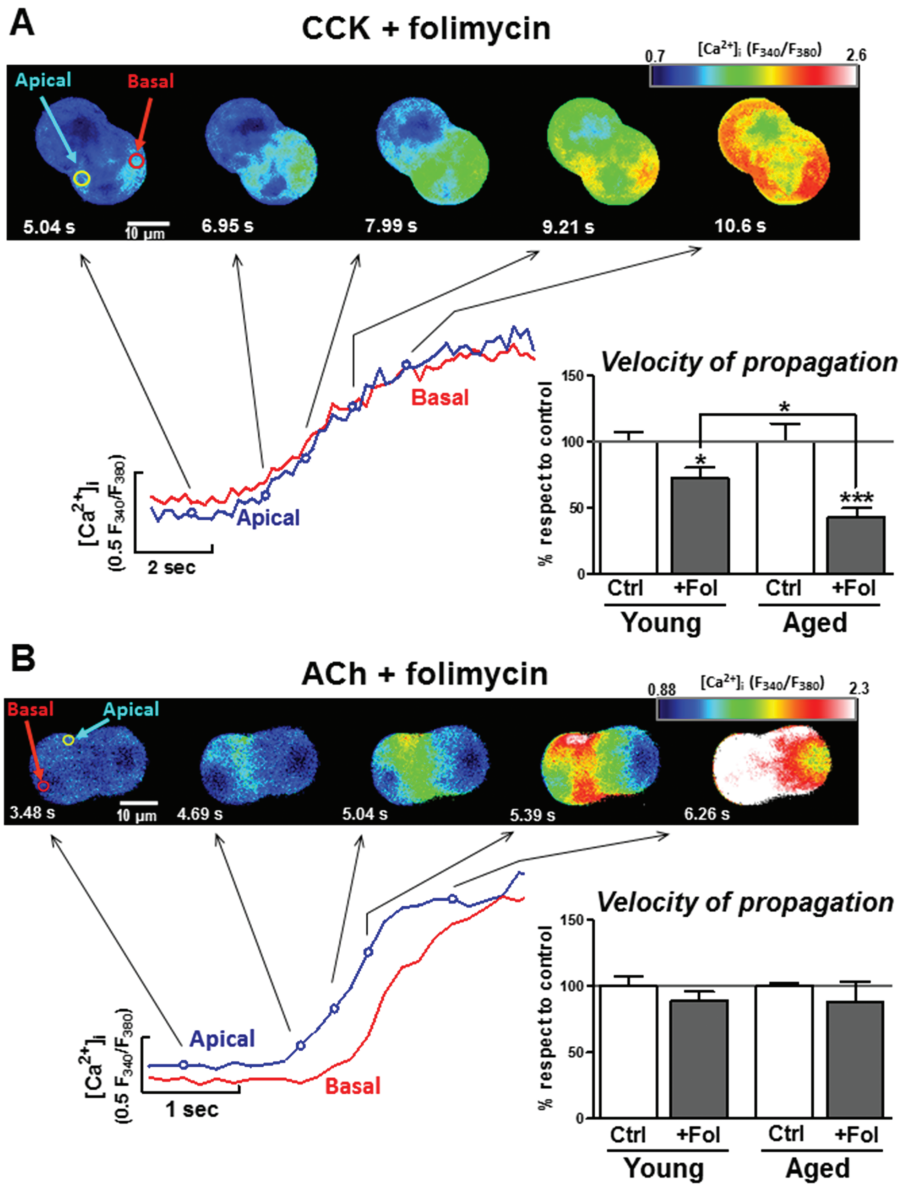


**Figure 3.** Folimycin suppressed in aged cells the polarization of the calcium signal induced by cholecystokinin (CCK) 1 nM (**A**) but not by ACh 10 µM (**B**). The traces are representative of 12 (CCK) and 8 (ACh) aged cells, and represent the subcellular $[Ca^{2+}]_i$ changes in the apical and basal poles of the cell shown in the ratio images at different time points of the record. The two insets represent the velocity of the $[Ca^{2+}]_i$ wave propagation (mean ± SEM) for untreated (Ctrl) and folimycin-treated (Fol) cells normalized to untreated cells for each preparation. One-way ANOVA for CCK group: $F = 10.56$, $p < .005$. $^*p < .05$, $^{**}p < .01$ and $^{***}p < .005$ respect to control or as indicated. $n = 3$–8 for CCK, 6–9 for ACh.

of ACh folimycin did not modify significantly the polarized response neither in young (not shown) nor aged (Figure 3B) cells.

To facilitate the analysis of age effect on the role of acidic stores the responses of folimycin-treated cells were normalized to control cells from the same preparations. Inset of Figure 3A indicates that folimycin inhibited the $Ca^{2+}$ wave velocity for CCK-stimulated cells and that aging increased significantly this folimycin-induced inhibition. By the contrary, folimycin had no effect in ACh-treated cells (Figure 3B, inset). Figure 4 shows the analysis of the local responses; folimycin effect on the rate of rise and amplitude was also greater for CCK (Figure 4A) than for ACh (Figure 4B). Similar to the velocity of propagation, aging enhanced significantly the inhibitory effect of folimycin on the rate of response at the apical pole (Figure 4A). As a whole, the results of folimycin treatment indicate that aging enhances the role of apical acidic stores in the CCK response.

To initiate the polarized response, ACh and CCK use respectively the $Ca^{2+}$ release channels $IP_3R$ and RyR (9). To investigate the role of RyR $Ca^{2+}$ pools they were depleted by pretreating the cells with the RyR agonist 8Br-cADPr. This compound increased $[Ca^{2+}]_i$, both as a slowly rising plateau (in aged cells, not shown) or as slow oscillations (young cells, Figure 5) reminiscent of the response induced by postprandial levels of CCK (14). This treatment abolished the polarization of the CCK response in both young and aged cells (not shown). However, in the case of ACh 8Br-cADPr blocked the signal polarity only in aged cells, whereas in young cells only reduced the speed of the $Ca^{2+}$ wave ($31.10 \pm 4.52$ pixels × $s^{-1}$, $n = 8$, $p < .01$ respect to control ACh-stimulated cells). This result suggests that the role of RyRs in the initiation of the response, as expected stronger for CCK than for ACh, is enhanced for the ACh response in aged cells.

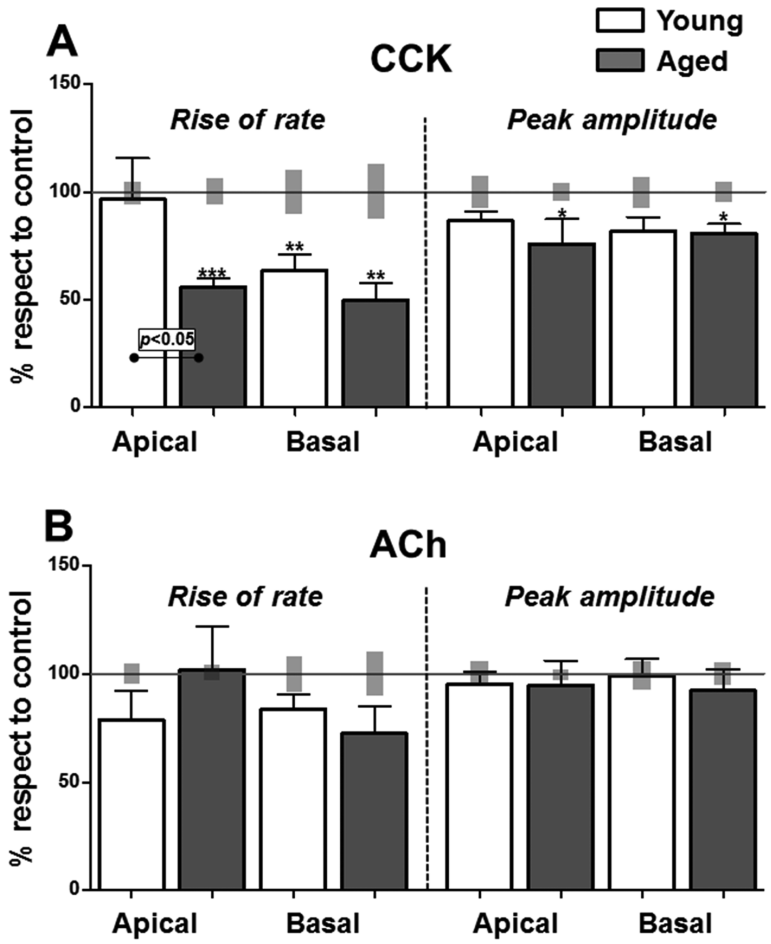


**Figure 4.** Effect of folimycin on local $[Ca^{2+}]_i$ responses to ACh and cholecystokinin (CCK) in young and aged pancreatic acinar cells. The histograms show mean ± SEM values of the rate of rise and the amplitude of the CCK (**A**) and ACh (**B**) responses normalized to control values for each preparation. The shadow boxes around the 100% line represent the ± SEM values of the control mean values (100%, omitted for the sake of clarity). One-way ANOVA for CCK rate of rise: $F = 2.84$, $p < .05$; for CCK amplitude $F = 2.28$, $p < .05$. $^*p < .05$, $^{**}p < .01$, and $^{***}p < .005$ respect to control. $n = 3$–8 for CCK, 6–9 for ACh.

## Discussion

Our study shows that aging of pancreatic acinar cells induces a differential modification of the propagation of $[Ca^{2+}]_i$ signals. This alteration is likely associated to mitochondrial depolarization and to modifications in the role of intracellular stores in the generation and spreading of the $Ca^{2+}$ signal.

Besides their metabolic role, mitochondria are considered a key factor in the regulation of $Ca^{2+}$ signals (22,23) and redox balance (24), and therefore the mitochondrial alterations observed in aged cells have been proposed to explain the biology of aging (25,26). We have previously shown in exocrine pancreas (1) and smooth muscle cells (27) that mitochondria of aged individuals are partially depolarized compared to young cells. Our finding that mitochondrial inhibitors reproduce in young cells the differential effects of aging on two $Ca^{2+}$ releasing agonists strongly supports mitochondrial depolarization as a causal process for age-related alterations in $Ca^{2+}$ signals. Although this is, to our knowledge, the first report of this differential effect in a single cell type, a mechanistic precedent could underlie the apparently contradictory results of the present study on $Ca^{2+}$ signal propagation. So, while in glial and smooth muscle cells mitochondria seem to support propagation (28,29), similar to ACh response in pancreatic acinar cells (15), other reports indicate a restrictive role for mitochondria in the spreading of the signal (14,30,31).

The differential effect is likely related to differences in the mechanisms initiating the response to ACh and to CCK. Although both use $IP_3R$, RyR, and acidic stores to generate local and global responses, CCK has been reported to rely mainly on endoplasmic reticulum RyR receptors (activated by cADPr) and on NAADP (that binds the two pore channels placed at endosomes) to activate the trigger area (9,32). On the contrary, ACh seems to depend specifically on $IP_3$ synthesis (9) to initiate the signal. Mitochondria modulate $Ca^{2+}$ signals due to close contact with the releasing channels of the internal calcium stores (22,23). In our experimental model mitochondrial $Ca^{2+}$ uptake restricts the initial propagation of RyR-based signals [our CCK data and also (14,30,31)] but facilitates it for IP3R-based responses [our ACh data and (15)]. This could be explained if the high $[Ca^{2+}]_i$ microdomains close to $Ca^{2+}$ channels generated during $Ca^{2+}$ signals produce feedback inhibition of $IP_3R$ (restricting the ACh signal) and potentiation of RyR (potentiating the CCK signal). Thus, local mitochondrial $Ca^{2+}$ uptake fosters the ACh $Ca^{2+}$ wave (by limiting the inhibitory $Ca^{2+}$ feedback on $IP_3R$) and restricts the CCK $Ca^{2+}$ release (by inhibiting the positive $Ca^{2+}$ feedback on RyR).

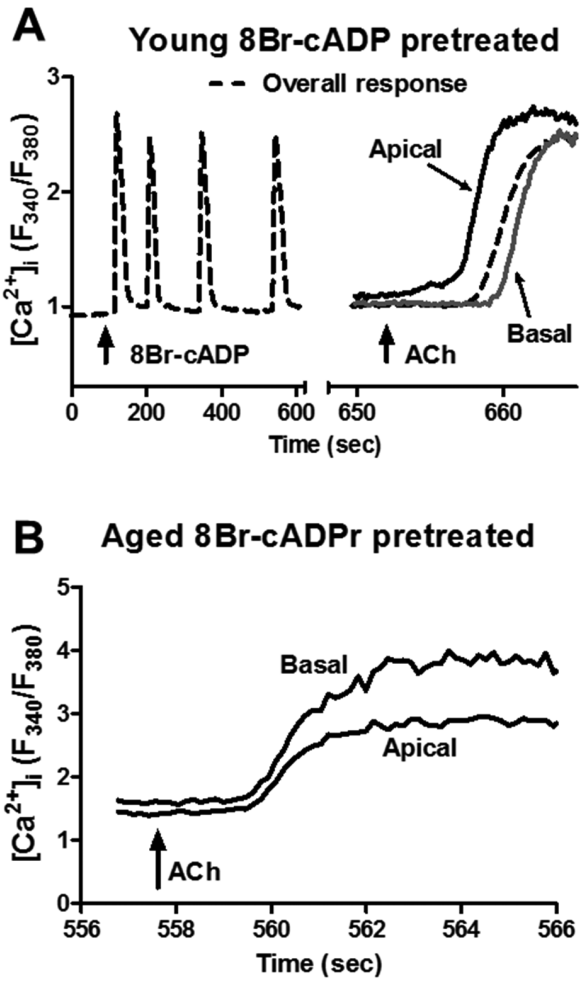


**Figure 5.** Effect of 8Br-cADPr (50 μM) pretreatment on 10 μM ACh-induced calcium signal in young and aged mouse pancreatic acinar cells. The top graph shows the cyclic $Ca^{2+}$ increase induced by 8Br-cADPr during the pretreatment period in the overall cytosol sampled at slow rate (dashed line). The final segment of the graph represents the apical and basal responses during the high frequency sampling period initiated at the ACh application. Note that the polarization of the signal was absent in aged (**B**) but not in young (**A**) cells. Representative of three independent preparations.

An additional mechanism for alteration of the CCK response (RyR-based) is the finding of Gant and colleagues (4) that aging enhances RyR-mediated $Ca^{2+}$ release in neurones, in keeping with enhanced RyR activity due to oxidation by mitochondrial oxidants (33) in cardiac cells. Moreover, it is tempting to speculate that mitochondrial depolarization in aged cells (and in young cells treated with mitochondrial inhibitors) enhances $Ca^{2+}$ propagation by restricting the mitochondrial $Ca^{2+}$ uptake through the RyR present in mitochondrial inner membrane (34).

It is likely that other age-related alterations contribute to impairment of $Ca^{2+}$ signals. On one hand, recent work shows that aging disrupts the physical association between mitochondrial membrane and RyR, thereby inhibiting the mitochondrial import of $Ca^{2+}$ released through RyR (35). On the other hand, several lines of evidence suggest that the mTOR signaling network, the main senescence signaling pathway, impairs $Ca^{2+}$ signals. First, the $Ca^{2+}$ releasing channels are modulated by this pathway (36,37) and aging modifies $Ca^{2+}$ release in parallel to changes in FKBP12 (37,38), a protein regulatory of the mTOR complex. Second, although there is no information on mTOR changes in aged exocrine pancreas, it has been shown that aging inhibits the PI3K/Akt pathway, a main regulator of mTOR (39). Third, in aged salivary glands impairment of $Ca^{2+}$ signals is prevented by caloric restriction (40), which inhibits mTOR pathway and is the best prolongevity treatment.

Regarding the origin of mitochondrial depolarization, it is likely the result of several factors: activation of mitochondrial permeability transition pore, alteration of DNA and other mitochondrial components (due to oxidative imbalance) and a progressive accumulation of depolarized mitochondria due to loss of mitophagy (the mechanism to remove defective mitochondria) (41,42).

In addition to the consequences of mitochondrial alterations, the present work indicates that aging induces two modifications of the subcellular mechanisms of the $[Ca^{2+}]_i$ response: (a) an increase of the dependence of CCK response on acidic stores, and (b) an increase of the role of RyR in the initiation of ACh response. Regarding the former, the effect of folimycin at the apical pole (Figure 4A) suggests that aging enhances the role of an acidic calcium store at the initiation area of the $Ca^{2+}$ wave (Figures 3 and 4). This is in keeping with the presence of a high sensitivity $Ca^{2+}$ pool at the secretory pole of this cell type (20,43), and with age-induced changes in the lysosomal-endosomal compartment (25) that could lead to modifications of $Ca^{2+}$ signals. Actually, a recent study reports that induction of lysosome proliferation enhances $Ca^{2+}$ signals (44).

The modifications found in our study can produce a derangement of the spatially restricted subcellular signals expected to occur in this cell type during physiological stimulation (9). Further research is needed to unveil how this change contributes to the secretory, replicative and metabolic changes associated to senescence.

## Funding

This study was supported by Ministerio de Ciencia e Innovación, Spain (BFU2011-24365); Red Temática de Investigación en Envejecimiento y Fragilidad (RETICEF RD12/0043/0016 and RETICEF RD06/0013/1007); Fondo Europeo de Desarrollo Regional (FEDER); and Junta de Extremadura, Spain (GR10009).

## Acknowledgments

The authors cordially thank the technicians Fatima Barriga and Maria Pura Delgado for their valuable assistance.

## References

1. Camello-Almaraz C, Gomez-Pinilla PJ, Pozo MJ, Camello PJ. Age-related alterations in $Ca^{2+}$ signals and mitochondrial membrane potential in exocrine cells are prevented by melatonin. *J Pineal Res*. 2008;45:191–198. doi:10.1111/j.1600-079X.2008.00576.x
2. Gomez-Pinilla PJ, Pozo MJ, Camello PJ. Aging differentially modifies agonist-evoked mouse detrusor contraction and calcium signals. *Age (Dordr)*. 2011;33:81–88. doi:10.1007/s11357-010-9163-7
3. Gomez-Pinilla PJ, Pozo MJ, Baba A, Matsuda T, Camello PJ. $Ca^{2+}$ extrusion in aged smooth muscle cells. *Biochem Pharmacol*. 2007;74:860–869. doi:10.1016/j.bcp.2007.06.037
4. Gant JC, Sama MM, Landfield PW, Thibault O. Early and simultaneous emergence of multiple hippocampal biomarkers of aging is mediated by $Ca^{2+}$-induced $Ca^{2+}$ release. *J Neurosci*. 2006;26:3482–3490. doi:10.1523/JNEUROSCI.4171-05.2006

5. Behringer EJ, Vanterpool CK, Pearce WJ, Wilson SM, Buchholz JN. Advancing age alters the contribution of calcium release from smooth endoplasmic reticulum stores in superior cervical ganglion cells. *J Gerontol A Biol Sci Med Sci*. 2009;64:34–44. doi:10.1093/gerona/gln053
6. Del CC, Ostrovskaya O, McAllister CE, et al. Effects of aging on $Ca^{2+}$ signaling in murine mesenteric arterial smooth muscle cells. *Mech Ageing Dev*. 2006;127:315–323. doi:10.1016/j.mad.2005.12.001
7. Howlett SE, Grandy SA, Ferrier GR. Calcium spark properties in ventricular myocytes are altered in aged mice. *Am J Physiol Heart Circ Physiol*. 2006;290:H1566–H1574. doi:10.1152/ajpheart.00686.2005
8. Knot HJ, Laher I, Sobie EA, et al. Twenty years of calcium imaging: cell physiology to dye for. *Mol Interv*. 2005;5:112–127.
9. Petersen OH, Tepikin AV. Polarized calcium signaling in exocrine gland cells. *Annu Rev Physiol*. 2008;70:273–299. doi:10.1146/annurev.physiol.70.113006.100618
10. Kasai H, Augustine GJ. Cytosolic $Ca^{2+}$ gradients triggering unidirectional fluid secretion from exocrine pancreas. *Nature*. 1990;348:735–738.
11. Ashby MC, Craske M, Park MK, et al. Localized $Ca^{2+}$ uncaging reveals polarized distribution of $Ca^{2+}$-sensitive $Ca^{2+}$ release sites: mechanism of unidirectional $Ca^{2+}$ waves. *J Cell Biol*. 2002;158:283–292. doi: 10.1083/jcb.200112025
12. Sneyd J, Tsaneva-Atanasova K, Bruce JI, Straub SV, Giovannucci DR, Yule DI. A model of calcium waves in pancreatic and parotid acinar cells. *Biophys J*. 2003;85:1392–1405. doi:10.1016/S0006-3495(03)74572-X
13. Tinel H, Cancela JM, Mogami H, et al. Active mitochondria surrounding the pancreatic acinar granule region prevent spreading of inositol trisphosphate-evoked local cytosolic Ca(2+) signals. *EMBO J*. 1999;18:4999–5008. doi:10.1093/emboj/18.18.4999
14. 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. doi:10.1016/S0006-2952(01)00830-9
15. González A, Schulz I, Schmid A. Agonist-evoked mitochondrial $Ca^{2+}$ signals in mouse pancreatic acinar cells. *J Biol Chem*. 2000;275:38680–38686. doi:10.1074/jbc.M005667200
16. Camello-Almaraz C, Pariente JA, Salido G, Camello PJ. Differential involvement of vacuolar H(+)-ATPase in the refilling of thapsigargin- and agonist-mobilized Ca(2+) stores. *Biochem Biophys Res Commun*. 2000;271:311–317. doi:10.1006/bbrc.2000.2633
17. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9:676–682. doi:10.1038/nmeth.2019
18. Voronina SG, Barrow SL, Gerasimenko OV, Petersen OH, Tepikin AV. Effects of secretagogues and bile acids on mitochondrial membrane potential of pancreatic acinar cells: comparison of different modes of evaluating DeltaPsim. *J Biol Chem*. 2004;279:27327–27338. doi:10.1074/jbc.M311698200
19. González A, Pariente JA, Salido GM, Camello PJ. Intracellular pH and calcium signalling in rat pancreatic acinar cells. *Pflugers Arch*. 1997;434:609–614. doi:10.1007/s004240050443
20. Gerasimenko JV, Sherwood M, Tepikin AV, Petersen OH, Gerasimenko OV. NAADP, cADPR and IP3 all release $Ca^{2+}$ from the endoplasmic reticulum and an acidic store in the secretory granule area. *J Cell Sci*. 2006;119(Pt 2):226–238. doi:10.1242/jcs.02721
21. Menteyne A, Burdakov A, Charpentier G, Petersen OH, Cancela JM. Generation of specific Ca(2+) signals from Ca(2+) stores and endocytosis by differential coupling to messengers. *Curr Biol*. 2006;16:1931–1937. doi:10.1016/j.cub.2006.07.070
22. Csordás G, Hajnóczky G. SR/ER-mitochondrial local communication: calcium and ROS. *Biochim Biophys Acta*. 2009;1787:1352–1362. doi:10.1016/j.bbabio.2009.06.004
23. Rizzuto R, De Stefani D, Raffaello A, Mammucari C. Mitochondria as sensors and regulators of calcium signalling. *Nat Rev Mol Cell Biol*. 2012;13:566–578. doi:10.1038/nrm3412
24. Camello-Almaraz C, Gomez-Pinilla PJ, Pozo MJ, Camello PJ. Mitochondrial reactive oxygen species and Ca2+ signaling. *Am J Physiol Cell Physiol*. 2006;291:C1082–C1088. doi:10.1152/ajpcell.00217.2006
25. Brunk UT, Terman A. The mitochondrial-lysosomal axis theory of aging: accumulation of damaged mitochondria as a result of imperfect autophagocytosis. *Eur J Biochem*. 2002;269:1996–2002. doi:10.1046/j.1432-1033.2002.02869.x
26. Seo AY, Joseph AM, Dutta D, Hwang JC, Aris JP, Leeuwenburgh C. New insights into the role of mitochondria in aging: mitochondrial dynamics and more. *J Cell Sci*. 2010;123(Pt 15):2533–2542. doi:10.1242/jcs.070490
27. Gomez-Pinilla PJ, Gomez MF, Swärd K, et al. Melatonin restores impaired contractility in aged guinea pig urinary bladder. *J Pineal Res*. 2008;44:416–425. doi:10.1111/j.1600-079X.2007.00544.x
28. Olson ML, Chalmers S, McCarron JG. Mitochondrial $Ca^{2+}$ uptake increases $Ca^{2+}$ release from inositol 1,4,5-trisphosphate receptor clusters in smooth muscle cells. *J Biol Chem*. 2010;285:2040–2050. doi:10.1074/jbc.M109.027094
29. Simpson PB, Russell JT. Mitochondria support inositol 1,4,5-trisphosphate-mediated $Ca^{2+}$ waves in cultured oligodendrocytes. *J Biol Chem*. 1996;271:33493–33501. doi:10.1074/jbc.271.52.33493
30. Walsh C, Barrow S, Voronina S, Chvanov M, Petersen OH, Tepikin A. Modulation of calcium signalling by mitochondria. *Biochim Biophys Acta*. 2009;1787:1374–1382. doi:10.1016/j.bbabio.2009.01.007
31. Hajnóczky G, Hager R, Thomas AP. Mitochondria suppress local feedback activation of inositol 1,4, 5-trisphosphate receptors by Ca2+. *J Biol Chem*. 1999;274:14157–14162. doi:10.1074/jbc.274.20.14157
32. Yamasaki M, Thomas JM, Churchill GC, et al. Role of NAADP and cADPR in the induction and maintenance of agonist-evoked Ca2+ spiking in mouse pancreatic acinar cells. *Curr Biol*. 2005;15:874–878. doi:10.1016/j.cub.2005.04.033
33. Cooper LL, Li W, Lu Y, et al. Redox modification of ryanodine receptors by mitochondria-derived reactive oxygen species contributes to aberrant Ca2+ handling in ageing rabbit hearts. *J Physiol*. 2013;591(Pt 23):5895–5911. doi:10.1113/jphysiol.2013.260521
34. Jakob R, Beutner G, Sharma VK, et al. Molecular and functional identification of a mitochondrial ryanodine receptor in neurons. *Neurosci Lett*. 2014;575:7–12. doi:10.1016/j.neulet.2014.05.026
35. Fernandez-Sanz C, Ruiz-Meana M, Miro-Casas E, et al. Defective sarcoplasmic reticulum-mitochondria calcium exchange in aged mouse myocardium. *Cell Death Dis*. 2014;5:e1573. doi:10.1038/cddis.2014.526
36. MacMillan D. FK506 binding proteins: cellular regulators of intracellular $Ca^{2+}$ signalling. *Eur J Pharmacol*. 2013;700:181–193. doi:10.1016/j.ejphar.2012.12.029
37. Martín-Cano FE, Camello-Almaraz C, Hernandez D, Pozo MJ, Camello PJ. mTOR pathway and $Ca^{2+}$ stores mobilization in aged smooth muscle cells. *Aging (Albany NY)*. 2013;5:339–346.
38. Gant JC, Chen KC, Norris CM, et al. Disrupting function of FK506-binding protein 1b/12.6 induces the $Ca^{2+}$-dysregulation aging phenotype in hippocampal neurons. *J Neurosci*. 2011;31:1693–1703. doi:10.1523/JNEUROSCI.4805-10.2011
39. Takahashi H, Okamura D, Starr ME, Saito H, Evers BM. Age-dependent reduction of the PI3K regulatory subunit p85α suppresses pancreatic acinar cell proliferation. *Aging Cell*. 2012;11:305–314. doi:10.1111/j.1474-9726.2011.00787.x
40. Olsen JG, Salih MA, Harrison JL, et al. Modulation by food restriction of intracellular calcium signaling in parotid acinar cells of aging Fischer 344 rats. *J Gerontol A Biol Sci Med Sci*. 1997;52:B152–B158. doi:10.1093/gerona/52A.3.B152
41. Mammucari C, Rizzuto R. Signaling pathways in mitochondrial dysfunction and aging. *Mech Ageing Dev*. 2010;131:536–543. doi:10.1016/j.mad.2010.07.003
42. Paradies G, Paradies V, Ruggiero FM, Petrosillo G. Changes in the mitochondrial permeability transition pore in aging and age-associated diseases. *Mech Ageing Dev*. 2013;134:1–9. doi:10.1016/j.mad.2012.12.006
43. Camello C, Pariente JA, Salido GM, Camello PJ. Role of proton gradients and vacuola H(+)-ATPases in the refilling of intracellular calcium stores in exocrine cells. *Curr Biol*. 2000;10:161–164. doi:10.1016/S0960-9822(00)00313-4
44. Dickinson GD, Churchill GC, Brailoiu E, Patel S. Deviant nicotinic acid adenine dinucleotide phosphate (NAADP)-mediated $Ca^{2+}$ signaling upon lysosome proliferation. *J Biol Chem*. 2010;285:13321–13325. doi:10.1074/jbc.C110.112573