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Research Article

Change in the Characteristics of Ca^{2+} Signaling in Pancreatic Acinar Cells in Culture

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Abstract. In this study we studied Ca^{2+} responses to different stimuli in pancreatic acinar cells subjected to culture conditions. Cells were isolated from adult mice pancreas and were subjected to culture conditions along one week. Changes in intracellular free Ca^{2+} concentration were monitored by single cell fluorescence analysis of fura-2-loaded cells. Mitochondrial distribution was analyzed by confocal microscopy study of MitoTracker Green FM-loaded cells. Expression of amylase-containing cytoplasmic vesicles was analyzed by confocal microscopy study of cells transfected with a plasmid encoding amylase linked to a green fluorescent protein. Cell viability was analyzed employing the AlamarBlue test. Our results show that pancreatic cells in culture retain a mitochondrial network and amylase-positive vesicles. However, cells dropped their ability to mobilize Ca^{2+} in response to activation of cell membrane receptors. Ca^{2+} mobilization in response to the sarcoendoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin was not altered. Cell viability was not affected by treatment with cholecystokinin, but was reduced in the presence of thapsigargin or hydrogen peroxide. We conclude that primary culture of pancreatic cells may be a suitable model to be used in studies where the involvement of mechanisms linked to the activation of specific cell membrane receptors is not required.

Keywords Exocrine pancreas, cell culture, cell signaling, cell viability, calcium

1. Introduction

The main function of the exocrine pancreas is the secretion of digestive enzymes, conveyed in an alkaline HCO_3^- and Cl^- rich fluid. This process occurs via mechanisms which depend on the generation of Ca^{2+} signals in response to secretagogues [1]. Ca^{2+} mobilization in response to agonists depict different spatiotemporal patterns that are controlled by different intracellular Ca^{2+} stores [2].

In vitro models of exocrine pancreas are useful for the study of Ca^{2+} signaling, pancreatic differentiation, secretion mechanisms or cell injury for example. In order to get a thorough knowledge of the physiology of the exocrine pancreas, and due to the importance of the pathological processes affecting this tissue (inflammation, cancer, etc.), an array of studies have been performed employing freshly isolated pancreatic acinar cells or cell lines in culture, mostly tumoral. Regarding cell culture, it would be interesting tofind a cellular

model, non-tumoral, on which we could carry out studies that allow us to better understand the physiology of this tissue and, additionally, the basis of the pancreatic pathophysiological processes. Thus, it is interesting to investigate whether it is possible to prepare primary cultures of pancreatic acinar cells, and to gather information regarding its utility for cell biology studies.

In this line, different methods have been developed for culturing pancreatic acinar cells. Oliver et al. [3] employed reconstituted basement membrane gel with epidermal growth factor, dexamethasone, insulin and secretagogues; Brannon et al. [4] perfomed studies employing serum-free medium with albumin, epidermal growth factor, dexamethasone and HEPES; and Bendayan et al. [5] carried out cell culture studies on a basal lamina (basement membrane) matrix. It has been suggested that pancreatic cells in culture maintain their differentiated morphology and polarity, contain secretory granules and secrete amylase in response to secretagogues [6]. Furthermore, the cultured acinar cells show morphologic evidences for autophagic processes [7, 8]. Recently, Guo et al. [9] have used monolayer culture of pancreatic acinar cells to study proliferation.

Conversely, it has been shown that a combination of both extracellular matrix and secretagogues is not enough for the maintenance of well-differentiated pancreatic acinar cells in culture [10]. Despite adult mouse pancreatic acinar cells divide in primary culture, during growth cells lose their differentiated architecture; additionally, cells form isolated branched tubular structures lined by a single cell layer, and display decreased expression of secretory proteins. Moreover, cell growth is followed by some degree of morphological redifferentiation after reaching confluency. This study indicates that adult acinar cells can retrodifferentiate to a more duct-like cell. In this sense, cultured primary mouse acinar cells express the acinar antigen for the first days, but during the growth phase fewer cells expressed it and, in contrast, most expressed the duct antigen.

The protein/DNA ratio, light and electron microscopic appearance, amylase release, and the presence of duct and acinar cell markers in mouse acinar cells cultures have also been evaluated [11, 12]. The results show that acinar/ductular tissue gives rise to ductular cells in culture by some combination of acinar cell death and/or transdifferentiation to a ductular phenotype, accompanied by proliferation of these cells and preexisting ductular cells.

Similarly, mouse pancreatic stellate cells undergo a phenotypic transformation from a quiescent state to a myofibroblast-like phenotype in culture [13].

Further research on this field was carried out employing porcine pancreatic acinar cells. The interest was based on a very different expression of receptors for gut peptides in porcine and humans compared to that found in rodents. In this case, the primary culture of acinar cells from pig pancreas maintains the same morphological characteristics and great viability; but, on the other hand, the enzyme activity decreases after several days [14]. In primary cultures of human (fetal) acinar cells similar results have been observed, depicting a reduction of amylase and lipase activity [15].

Another attempt to obtain functionally competent normal mouse acinar cells for long-term in vitro experimentation is that described by Blauer et al. [16], consisting of explants cultures under a gas-liquid interphase, followed by a secondary subculture on tissue culture plastic. Explants retained their pancreatic cytoarchitecture for 2 days. Nevertheless, most studies point out that cells growing out from pancreatic acini display a reduced expression of acinar cell markers, as shown recently [9].

Actually, different long-term culture models for non-transformed pancreatic acini exist. In the present study we have evaluated whether primary cultures of pancreatic acinar cells, obtained from adult mice, maintain the characteristics of Ca^{2+} signaling in response to several stimuli (cholecystokinin, acetylcholine, or thapsigargin), to assess whether they may be a suitable model to study pancreatic physiology.

2. Materials and Methods

2.1. Animals and chemicals. Adult male *Swiss* mice (4−6 weeks, weighing between 25 and 30 g), were used in the present study. Animals were obtained from the animal house of the University of Extremadura (Caceres, Spain). They were handled humanely and killed in accordance with the institutional Bioethical Committee. Collagenase was obtained from Worthington Biochemical Corporation (Lakewood, NJ, U.S.A.). Acetylcholine, (Tyr[SO_3H]^{27}) Cholecystokinin fragment 26−33 amide (CCK-8) and thapsigargin were obtained from Sigma Chemicals Co. (Madrid, Spain). AlamarBlue was purchased from AbD serotec (bioNova Científica, Madrid, Spain). Medium 199, horse serum, fura-2-acetoxymethyl ester (fura-2-AM) and MitoTracker Green FM were obtained from Invitrogen (Barcelona, Spain). Fetal bovine serum (FBS) was purchased from HyClone (Thermo Scientific, Erembodegen, Belgium). Penicillin/Streptomycin was obtained from BioWhittaker (Lonza, Basel, Switzerland). Transfection reagent Trans-IT-LT1 was purchased from Mirus (MoBiTec, Germany). All other chemicals used were of analytical grade and were obtained from Sigma Chemicals (Madrid, Spain).

2.2. Preparation of isolated pancreatic acinar cells and cell cultures. Isolation of pancreatic acinar cells from adult mice was performed following previously described methods [17]. The tissue was incubated in Na-HEPES buffer supplemented with collagenase (100 Units/mL) during 10 minutes. Enzymatic digestion was followed by mechanical dissociation of the cells, as described above. Following centrifugation at 30 ×g for 5 min at 4°C, cells were resuspended in Na-HEPES buffer without collagenase. Cell viability was not

significantly changed by the isolation procedure, as assayed by trypan blue exclusion test, and was greater than 95%.

Preparation of cultures was made following previously described methods [18]. Briefly, the pancreas was placed in a physiological Na–HEPES solution containing: 130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 10 mM HEPES, 0.01% trypsin inhibitor (soybean), and 0.2% bovine serum albumin (pH = 7.4 adjusted with NaOH). This buffer was supplemented with 30 units/mL collagenase CLSPA from Worthington.

The tissue was incubated for 50 min at 37°C under constant oxygenation. This enzymatic digestion of the tissue was followed by gently pipetting the cell suspension through tips of decreasing diameter for mechanical dissociation of the cells. Following centrifugation at 30 × g for 5 min at 4°C, cells were resuspended in culture medium.

Culture medium consisted of Medium 199 supplemented with 4% horse serum, 10% FBS, antibiotics (0.1 mg/ml streptomycin, 100 IU penicillin) and 1mM $NaHCO_3$. This medium was prepared under sterile conditions. Finally, cells were seeded ($\approx 10^5$ cells) on glass coverslips placed in independent dishes (35 mm diameter) and grown in culture medium in a humidified incubator at 37°C and 5% CO_2.

The experiments were done employing different batches of cells, obtained from different preparations (pancreas).

2.3. Determination of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_c$). Isolated mouse pancreatic acinar cells, resuspended in Na–HEPES buffer, or cells in culture, were incubated in the presence of fura- 2/AM (4 μM) at room temperature (23–25°C) for 40 min. Afterwards, cells suspension was centrifuged at 30 × g for 5 min at 4°C and resuspended in Na-HEPES buffer without trypsin inhibitor and BSA. In the case of cells growing on coverslips, once the incubation period with fura-2/AM had finished, the extracellular medium was discarded and replaced with fresh Na-HEPES buffer.

For monitoring of changes of fura-2-dependent fluorescence, related to changes in $[Ca^{2+}]_c$, small aliquots of the suspension of dye-loaded cells were transferred to a coverslip mounted on an experimental perfusion chamber. The coverslip with cultured cells was mounted on the perfusion chamber. In either case, and in experiments performed separately, the perfusion chamber was then placed on the stage of an epifluorescence inverted microscope (Nikon Diaphot T200, Melville, NY, USA).

During the experiments, the cells were continuously superfused with a control Na-Hepes buffer containing (in mM): 140 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, 10 glucose (pH adjusted to 7.4).

For fura-2 fluorescence changes determination, an image acquisition and analysis system for video microscopy was employed (Hamamatsu Photonics, Hamamatsu, Japan). Cells were alternatively excited with light from a xenon arc lamp passed through a high-speed monochromator (Polychrome IV, Photonics, Hamamatsu, Japan) at 340/380 nm. Fluorescence emission at 505 nm was detected using a cooled digital CCD camera (Hisca CCD C-6790, Hamamatsu, Japan) and recorded using dedicated software (Aquacosmos 2.5, Hamamatsu Photonics, Hamamatsu, Japan). All fluorescence measurements were made from areas considered individual cells.

All stimuli were dissolved in the extracellular Na-HEPES buffer, and applied directly to the cells in the perfusion chamber.

2.4. Localization of mitochondria. Localization of mitochondria was assayed by incubation of cultured cells in the presence of MitoTracker Green FM. Loading of cells with MitoTracker Green FM (100 nM) was performed at room temperature (23–25°C) for 30 min. After incubation, the extracellular medium was replaced by fresh Na-HEPES buffer. This dye has been employed as a mitochondrial marker [19].

For monitoring fluorescence signals, coverslips with dye loaded cells were placed in a perfusion chamber on the stage of a confocal Nikon Eclipse TE300 microscope (Nikon Instruments Inc.) and continuously superfused with Na–Hepes buffer. Fluorescence images from cells loaded with Mito Tracker Green FM were obtained employing a confocal laser scanning system Bio-Rad MRC 1024 (American Laser Corp, Salt Lake City, UT, USA). Using a × 60 oil immersion objective fluorescence, images of 256 × 256 pixels with a resolution of 0.287 μm/pixel were recorded every 4 s.

Excitation light at 488 nm from a 100-mW argon laser was employed. Emitted fluorescence was collected through a 522/35-nm band-pass filter employing a photomultiplier. Laser intensity was reduced to 1–3% with neutral density filters to reduce photobleaching. The software used for the imaging was Laser Sharp MRC-1024 Version 3.2 (Bio-Rad, Deisenhofen, Germany).

2.5. Transfection procedure and GFP-amylase fluorescence detection. Localization of granules. Rat-amylase DNA was kindly provided by Dr. Robert Blum as amylase-GFP-N3. Amylase sequence was inserted into GFP-C1 vector using Hind III / Klenow-blunted and BamH I restriction sites, creating a GFP-Amylase DNA construct. The reading frames of the GFP-amylase was verified by using Western blot detection of the GFP-amylase protein.

A standard transfection method was employed. In brief, cells (one week in culture) were transfected with 1 microgram DNA per 35 mm Petri dish using the transfection reagent Trans-IT-LT1 and cultured for 2−3 days under standard cell culture conditions before use. Fluorescence signal detection was carried out employing the confocal laser scanning system described above, with excitation light at 488 nm. Emitted

fluorescence was collected through a 522/35-nm band-pass filter.

2.6. Cell viability assay. Analysis of cell survival under the different treatments applied was performed using Alamar-Blue test as previously described [20]. For this purpose stable cultures (cells had reached confluence) were employed. Stimuli were added to the cells at the desired concentration. Data of cell viability show the mean reduction of AlamarBlue expressed in percentage ± S.E.M. (n) with respect to control (non-stimulated) cells, where n is the number of independent experiments.

3. Results

3.1. Acinar cells in culture display a mitochondrial network and contain amylase-positive vesicles. Pancreatic acinar cells were grown as described in material and method section. With time of culture, cells formed a confluent monolayer, flattened and with increased cell size compared to freshly isolated cells. Figure 1 shows representative images of cells growing at different stages of culture (A, day of preparation; B, 24 hours; C, 72 hours; and D, one week of culture). Localization of mitochondria was assayed by incubation of confluent cells in the presence of MitoTracker Green FM, a dye that selectively accumulates inside these organelles [19]. Figure 2 shows a representative fluorescence image of pancreatic cells in culture (one week) labeled with MitoTracker Green FM. We could observe a network of mitochondria, which appeared distributed all through the cytosol.

In addition, pancreatic cells in culture (one week) express cytosolic vesicles consistent with zymogens. Cells in culture were transfected with a plasmid containing DNA encoding GFP-amylase. It was possible to detect expression of the protein 24 h to 48 h after transfection. A cytosolic homogeneous green fluorescence could be observed in cells transfected with DNA encoding the fluorescent protein GFP (Figure 3A). However, punctuate green fluorescence could be observed in cells transfected with GFP-amylase-encoding DNA, consistent with cytosolic vesicles containing the enzyme (Figure 3B).

3.2. Pancreatic acinar cells in culture lose their ability to mobilize intracellular Ca^{2+} in response to the activation of plasma membrane receptors by physiological secretagogues. In this study we were interested in evaluating the effect of physiological secretagogues on pancreatic acinar cells in culture, in comparison with the responses observed in freshly isolated cells.

The cells, at different stages of culture (0 hours of culture –day of cell preparation-, 24 hours, 48 hours, 72 hours, 96 hours, and after one week in culture) were incubated with different stimuli. Agonist concentrations used were 1 nM and 20 pM in the case of CCK, and 100 nM in the case of ACh.

Table 1: Percentage of cells responding to stimuli.

Days of culture	CCK 1 nM	CCK-8 20 pM	ACh 100 nM	Tps 1 μM
0	100	99	96	100
1	59	63	66	100
2	46	78	69	78
3	40	55	22	83
4	26	39	11	88
One week	0	0	0	100

In freshly prepared cells, 0 hours of culture, stimulation with 1 nM CCK (48 of 48 total cells studied, $n = 5$ experiments) or 100 nM ACh (71 of 74 total cells studied, $n = 5$ experiments) induced a transient mobilization of Ca^{2+} that consisted of an increase followed by a slow reduction toward a value close to the resting level (Figure 4A and B, respectively). In the case of stimulation of cells with 20 pM of CCK, the response depicted a typical oscillatory pattern in $[Ca^{2+}]_c$ (80 of 81 total cells studied, $n = 5$ experiments; Figure 4C).

When cells grown in culture between 24 and 96 hours were stimulated with CCK (1 nM) or ACh (100 nM), we observed changes in $[Ca^{2+}]_c$ that did not follow the typical response pattern shown by pancreatic acinar cells at day zero of culture. Figure 5 shows examples of secretagogue-evoked Ca^{2+} responses of pancreatic cells in culture for 48 hours (CCK-8 1 nM: 16 of 35 total cells studied, $n = 6$ experiments; ACh 100 nM: 19 of 28 total cells studied, $n = 6$ experiments; CCK-20 pM: 17 of 22 total cells studied, $n = 5$ experiments). In cells cultured for 1 week (cell cultures had reached confluence) we did not observe changes in $[Ca^{2+}]_c$ in response to secretagogues (CCK-8 1 nM: 0 cells of 49 total cells studied, $n = 3$ experiments; ACh 100 nM: 0 cells of 78 total cells studied, $n = 4$ experiments ; CCK-8 20 pM: 0 cells of 61 total cells studied, $n = 4$ experiments).

The analysis of the responses obtained shows that the number of cells that mobilize Ca^{2+} in response to physiological secretagogues decreases along the stage (day) of culture. After one week in culture cells did not show Ca^{2+} mobilization in response to stimulation neither with CCK-8 nor with ACh (Table 1).

3.3. Pancreatic acinar cells in culture retain their ability to mobilize intracellular Ca^{2+} in response to stimuli non-linked to plasma membrane receptors, like thapsigargin. In this set of experiments we studied the Ca^{2+}-mobilization ability of agents whose action is not mediated by the activation of specific receptors at the cell membrane. As a model, we employed thapsigargin (Tps). Tps, the most potent selective sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, is often used to inhibit this pump [21].

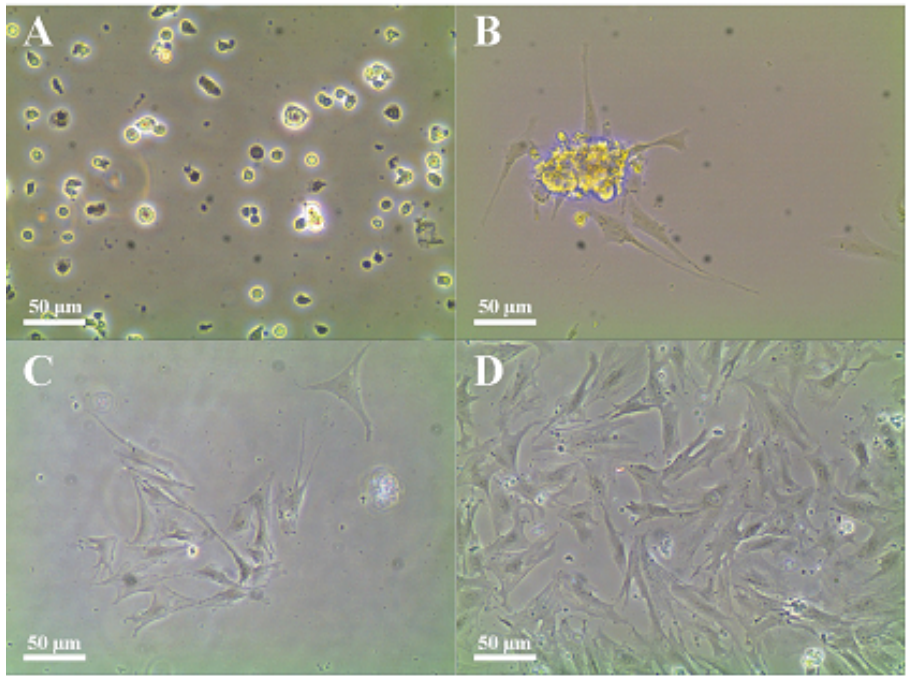

Figure 1: **Pancreatic acinar cells growing at different stages of culture**. (A) Picture taken the day of preparation. (B) Image taken at 24 hours of culture. (C) Cells grown for 72 hours. (D) One week of culture. Viability of cells was not decreased under culture conditions and in the absence of stimulus. Images are representative of 5 different preparations.

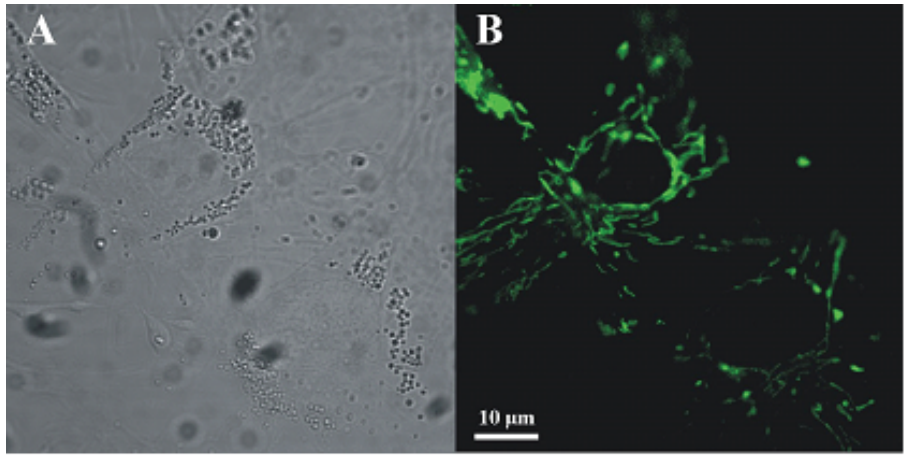

Figure 2: **Confocal microscopy studies of pancreatic cells in cultre loaded with the fluorescent probe Mito-Tracker Green FM.** The picture shows a representative fluorescence image of pancreatic cells in culture labeled with MitoTracker Green FM. The dye selectively accumulates inside mitochondria. Bright fluorescent spots forming a consistent network could be detected all through the cytosolic area. Viability of cells was not decreased under culture conditions and in the absence of stimulus. The experiments were performed after the cells had grown for 7 days ($n = 3$ independent preparations).

It has been shown that the functional Ca^{2+} stores can be depleted by inhibitors of the SERCA.

In the presence of 1 μM Tps, a transient increase in $[Ca^{2+}]_c$ was observed, which then decreased and returned towards the prestimulation level (128 of 128 total cells studied, $n = 6$ experiments; Figure 6A). The pattern of Ca^{2+} mobilization remained to a great extent unaffected by culture of cells, i.e., pancreatic cells in culture depicted the typical Ca^{2+} mobilization in response to Tps. Figure 6B and 6C show the Ca^{2+} response observed following addition of Tps to pancreatic cells cultured for 72 hours (25 cells of 30 total cells studied, $n = 4$ experiments) and 96 hours (57 cells of 64 total cells studied; $n = 5$ experiments).

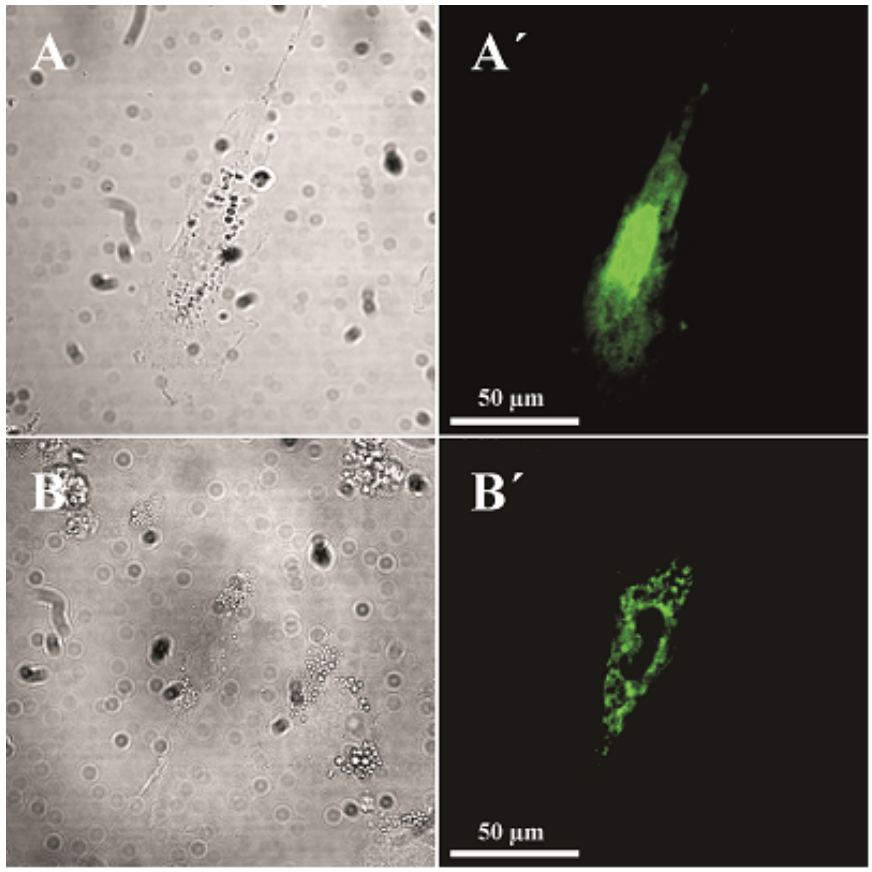

Figure 3: **Pancreatic cells in culture express cytosolic vesicles consistent with zymogens.** Cells in culture were transfected with a plasmid containing a DNA GFP-amylase, and confocal laser scanning microscopy was employed to detect GFP-derived fluorescence. Viability of cells was not decreased under culture conditions. (A) A cytosolic homogeneous green fluorescence could be observed in cells transfected with wild GFP-encoding DNA. (A′) Transmitted light image of cell shown in A. (B′) Green fluorescence distribution, consistent with trapped fluorescent protein in cytosolic vesicles, could be observed in cells transfected with GFP-amylase-encoding DNA. (Bμ) Transmitted light image of cell shown in B. (transfection was performed after day 7 of cell culture; $n = 4$ independent preparations).

In this set of experiments, the analysis of the responses obtained shows that the number of cells that mobilize Ca^{2+} in response to Tps did not decrease as dramatically as it had happened in the experiments in which the responses to CCK and ACh were evaluated. Conversely, the percentage of cells showing Ca^{2+} mobilization remained largely unchanged along the stage (day) of culture (Table 1).

3.4. Study of cell viability. Finally, we evaluated whether the treatment of cells with CCK-8 has any effect on cell viability. Cells, growing on independent dishes, were incubated for 24-96 h in the presence of no stimulus (control cells), 1 nM CCK-8 or 1 μM Tps. For comparisons, cells were incubated in the presence of 100 μM H_2O_2, an oxidant with proven negative effects on cell viability.

Viability of cells did not change significantly in the presence of CCK-8 compared to control (unstimulated) cells, which was considered 100% (Figure 7A). However, cell survival dropped after incubation of cells in the presence of Tps (Figure 7B). Similarly, cell viability decreased in the presence of H_2O_2 (Figure 7C).

4. Discussion

Several models for long-term culture of pancreatic acinar cells exist, and they have been employed to study different physiological processes; however, there are not clear evidences regarding Ca^{2+} mobilization by this cell type under culture conditions, provided that Ca^{2+} is a vitally important second messenger in the pancreas.

The analysis of intracellular Ca^{2+} signaling is a useful strategy in the study of cellular function. This ion participates in the process of enzyme secretion involved in digestion of food. Moreover, impairment of Ca^{2+} signaling mechanisms is the basis of many pathological processes affecting the pancreas [22].

The aim of our study was to determine whether primary cultures of pancreatic acinar cells can be used as a cellular

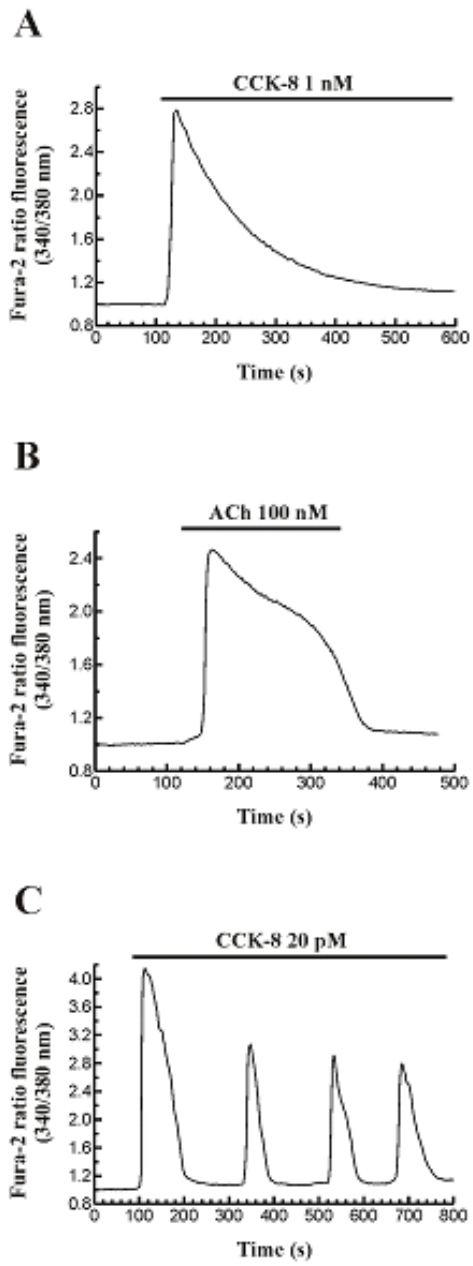

Figure 4: **Monitorization of the effect of different agonists on $[Ca^{2+}]_c$ in freshly prepared pancreatic acinar cells.** Cells were isolated as described in material and methods section, and then checked at day 0 (i.e., cells were not subjected to culture) for Ca^{2+} responses after stimulation with physiological agonists. (A) Time-course of changes in $[Ca^{2+}]_c$ after stimulation with 1 nM CCK-8. (B) Ca^{2+} response observed after stimulation of cells with 100 nM ACh. (C) Typical oscillatory pattern of Ca^{2+} mobilization in response to stimulation of freshly prepared pancreatic acinar cells with 20 pM CCK-8. The experiments were performed in the presence of Ca^{2+} in the extracellular medium. Viability of cells was not decreased under culture conditions and in the absence of stimulus. Stimuli were added to the cells on the day in which the tests for Ca^{2+} mobilization were performed. The horizontal bar indicates the time during which the stimulus was applied to the cells. Traces are representative of 48–71 cells analyzed in 5 independent experiments for each treatment.

model to investigate the role of Ca^{2+} in pancreatic physiology.

Our results indicate that pancreatic acinar cells in culture show a decrease in Ca^{2+} mobilization in response to typical exocrine pancreatic secretagogues, such as CCK and ACh. A possible explanation for this finding could be a loss of cell differentiated architecture, as reported by De Lisle and Logsdon [10]. However, maintenance of cell structural organization in culture remains controversial.

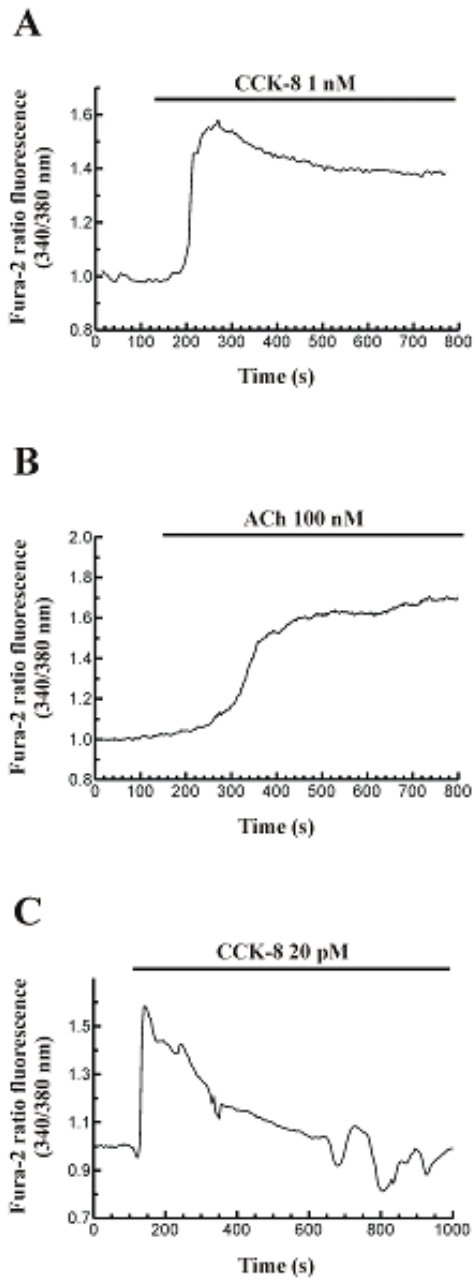

Figure 5: **Monitorization of the effect of different agonists on $[Ca^{2+}]_c$ in pancreatic acinar cells cultured for 48 hours.** Pancreatic acinar cells were allowed to grow for 48 hours. Then cells were loaded with fura-2 and fluorescence was analyzed after stimulation of cells with 1 nM CCK-8 (A), 100 nM ACh (B) or 20 pM CCK-8 (C). The changes in $[Ca^{2+}]_c$ did not follow the typical response pattern shown by pancreatic acinar cells at day zero of culture. Viability of cells was not decreased under culture conditions and in the absence of stimulus. Stimuli were added to the cells on the day in which the tests for Ca^{2+} mobilization were performed. Traces are representative of 22–35 cells analyzed in 5-6 independent experiments for each treatment.

Bendayan et al. [5] showed that pancreatic acinar cells in culture reaggregated into acini-like structures and retained their differentiated morphology, as well as their ability to secrete enzymes. This has been confirmed in a later work [6].

Other studies have evaluated cell morphology, enzyme secretion and molecular markers, and reported transformation of pancreatic cells in culture, in agreement to De Lisle and Logsdon findings [10]. The latter reported expression of a well organized rough ER and small apical vesicles, but also

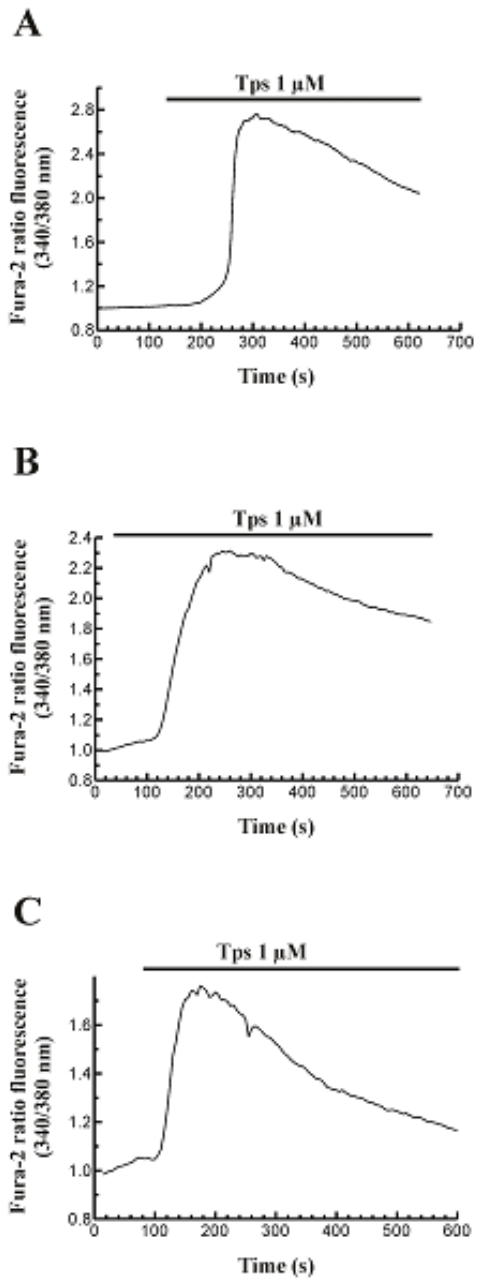

Figure 6: **Effect of thapsigargin (Tps) on $[Ca^{2+}]_c$.** (A) Time-course of changes in $[Ca^{2+}]_c$ in freshly prepared pancreatic acinar cells after stimulation with 1 μM Tps. (B) Ca^{2+} response observed following addition of Tps to pancreatic acinar cells cultured for 72 hours. (C) Effect of Tps on $[Ca^{2+}]_c$ in pancreatic acinar cells grown for 96 hours. The experiments were performed in the presence of Ca^{2+} in the extracellular medium. Viability of cells was not decreased under culture conditions and in the absence of stimulus. Stimuli were added to the cells on the day in which the tests for Ca^{2+} mobilization were performed. The horizontal bar indicates the time during which Tps was applied to the cells. Traces are representative of 30–128 cells analyzed in 4–6 independent experiments for each treatment.

described changes in cell-specific antigens that were paralleled by cell type associated morphological characteristics of pancreatic cells in culture.

In this line, it has been shown that pancreatic acinar cells in culture grow forming epithelial islands, and retain the zymogen granules and ER structure; however, cells lose part of the secretory machinery and express cytokeratin 7, suggesting that pancreatic cells in culture switch to an intermediate stage between acinar and ductal cells [23].

Moreover, it has been suggested that pancreatic tissue gives rise to ductular cells in culture by some combination

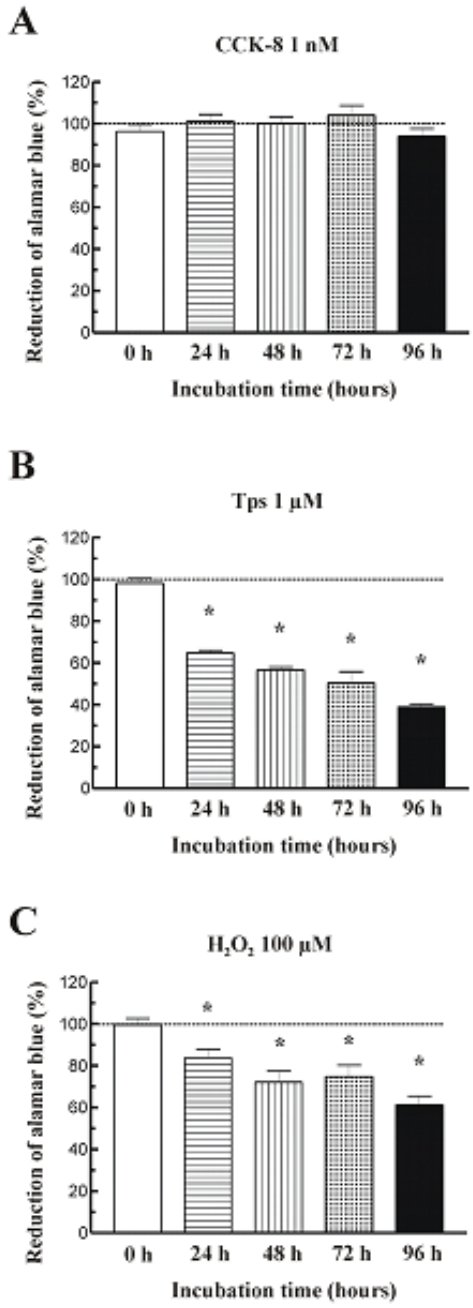

Figure 7: **Analysis of viability of pancreatic acinar cells in culture.** Cell viability under the different treatments applied was analyzed studying AlamarBlue reduction, as described in "Material and methods". (A) Incubation of cells in the presence of 1 nM CCK-8 did not alter cell viability significantly along the incubation period. (B) Effect of incubation of cells with 1 μM Tps on cell viability. (C) H_2O_2 (100 μM) led to a decrease in cell viability compared to non-stimulated cells. The horizontal dotted line represents the viability of non-stimulated, which was considered 100%. Graphics are representative of 4 independent experiments for each treatment (*, $P < 0.001$ for treatments *vs* non-stimulated cells).

of acinar cell death and/or transdifferentiation to a different phenotype [11, 12].

Recently, it has been also shown that cells depict a reduced expression of acinar cell markers, including digestive enzymes and Mist1. The expression of ductal and embryonic markers (for example cytokeratin 7, β-catenin, E-cadherin, pdx-1, and nestin) was also increased [9].

Indeed, despite expressing amylase-containing cytosolic vesicles, we could not detect any enzyme secretion in response to secretagogues under our experimental conditions

(data not shown). A former study has shown that, after 48 hours of culture, cells contain altered zymogen granules undergoing regression. Moreover, their analyses indicated that the zymogen granules were degraded by the lysosomes [7].

We did not observe an absolute absence of granules along the time of culture under our experimental conditions. However, our results agree to previous studies in the observation of an impaired enzyme secretion when pancreatic acinar cells are subjected to culture.

Conversely, Brannon et al. [4] showed that cells secreted amylase in response to the secretagogue carbamyl choline; however, secretion was not observed in response to caerulein, insulin, somatostatin, and dexamethasone. The effect of agonists on enzyme secretion changed depending on culture conditions, as was studied at day three of culture.

Here we show that cells mobilized Ca^{2+} in response to agonists when the studies were carried out within the first four days of culture. Ca^{2+} mobilization was not observed after day five of culture and following, despite the viability of cells was not decreased. However, although cells showed Ca^{2+} responses, the changes in $[Ca^{2+}]_c$ after two days of culture did not follow the typical response pattern shown by pancreatic acinar cells at day zero of culture (fresh cells). Bearing in mind the importance of Ca^{2+} mobilization for enzyme secretion, this might be the reason for the absence of amylase secretion that we have observed, supporting the findings reported by other groups.

In spite of the absence of Ca^{2+}-mobilization after stimulation of cells with physiological agonists, our results show that a 100% of cells exhibited Ca^{2+} mobilization after stimulation of cells with Tps. Ca^{2+} mobilization could even be observed in stable and confluent cell cultures (over one week of culture). Additionally, changes in cell viability could be observed in the presence of Tps or H_2O_2, but not when cells in culture were incubated in the presence of CCK. Therefore, this cellular model can be employed to study signaling pathways that do not depend on the activation of cell membrane receptors.

In this regard, cultures of pancreatic acinar cells were successfully employed to study the flux of potassium ions through membrane channels [18], or the flux of Ca^{2+} through intracellular channels activated by the Ca^{2+}-mobilizing substances inositol-1,4,5-trisphosphate and cyclic ADP-ribose [24]. And we have shown in the present work that cells were easily transfected with GFP-amylase-encoding DNA.

It is important to keep in mind that the morphology and probably also the function of acinar cells changes by time in culture. Probably there might also be some stellate cells with potential stem cell function in the cell isolates, that could be a source for un-differentiated rapidly dividing precursor cells, and which are overgrowing slowly [13]. In addition, we cannot discard that in culture it could take place some combination of acinar cell death and/or transdifferentiation to a different phenotype, accompanied by proliferation of these cells (discussed above).

However, it could be worth to study the basis of this transformation process, because chronic damage to the pancreas, as it occurs in pancreatitis, involves morphologic changes in acinar cells. In the disease, cells are transformed into metaplastic ductal cells, increasing the risk of developing neoplastic processes.

Recently, it has been suggested that monolayer culture can serve as a model to study acinar cell proliferation similar to regeneration after pancreatitis in vivo [9]. In this study CCK enhanced cellular spreading, stimulated MAPK signaling and DNA synthesis.

Culture of pancreatic acinar cells can be useful in the field of genetic engineering and molecular culture. For example, recombinant adenoviral vectors were employed to transfer and express genes in pancreatic acinar cells in culture [23, 25].

In comparison to other studies that we have performed, in general, we did not observe differences in the responses of cells isolated from adult compared to cells obtained from new born animals (data not shown). Thus, it seems feasible that the transformation that cells undergo is independent of animal maturation status (i.e., adult *vs* new born). Nonetheless, we have observed that cultures of cells obtained from new born animals depict an increased mitotic index. Thus, we think that cultures prepared with cells obtained form new born animals might represent a more appropriate model for its use in research.

5. Conclusion

We did not observe Ca^{2+} mobilization by pancreatic acinar cells in long-term culture after stimulation with physiological secretagogues, such as CCK-8 and ACh. The absence of response might be probably due to partial or total loss of cell membrane receptors, or to the loss of their link to cytosolic signaling cascades. From this point of view, this cell model might not be suitable for the study of processes in which such receptors are involved. Conversely, cells in culture do mobilize Ca^{2+} in response to drugs whose mechanism of action is not mediated by the activation of cell membrane receptors, and that rather exert a direct action, such as Tps. This fact suggests using this cell model to study intracellular events that are not linked to cell membrane receptor-initiated cascades. In this line, cellular Ca^{2+} responsiveness should be checked when Ca^{2+}-dependent pathways are to be studied.

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