Asynchronous impairment of calcium homoeostasis in different acinar cells after pancreatic duct obstruction in rat

Aránzazu URUÑUELA*, Manuel A. MANSO*, Ana Mª DE LA MANO*, Sara SEVILLANO*, Alberto ORFAO† and Isabel DE DIOS*
*Department of Physiology and Pharmacology, Edificio Departamental, Campus Miguel de Unamuno s/n, 37007 Salamanca, Spain, and †Flow Cytometry Service, Edificio Departamental, Campus Miguel de Unamuno s/n, 37007 Salamanca, Spain

ABSTRACT

Current evidence suggests that alterations within acinar cells are responsible for the development of acute pancreatitis. After inducing acute pancreatitis in rats by pancreatic duct obstruction, we analysed, using flow cytometry, the progressive changes in cytosolic Ca\(^{2+}\) concentrations in individual acinar cells from the earliest stages to 48 h after obstruction to investigate whether parallel alterations in the homoeostasis of Ca\(^{2+}\) could be defined in the different acinar cells throughout the evolution of pancreatitis. Morphological alterations of the pancreas, related to the severity of the disease at different stages, were observed by electron microscopy. Hyperamylasaemia and progressively more severe alterations, such as vacuolization, dilatation of endoplasmic reticulum, accumulation of zymogen granules and reorientation towards basolateral membrane, were observed during the first 12 h after pancreatic obstruction. A significant increase in cytosolic Ca\(^{2+}\) concentration was measured at these stages in a particular type of acinar cells (R1) differentiated by flow cytometry with low forward scatter (FSC), whereas another representative group of cells (R2) with higher FSC values were able to maintain resting cytosolic Ca\(^{2+}\) concentrations up to 24 h after obstruction. Longer periods of pancreatic duct obstruction induced disturbances in Ca\(^{2+}\) homoeostasis in all acinar cells. A similar increase in cytosolic Ca\(^{2+}\) load was reached in both R1 and R2 cells when acute pancreatitis was completely developed. In conclusion, the homoeostasis of Ca\(^{2+}\) in acinar cells is asynchronously impaired during the development of acute pancreatitis: cells with higher FSC (R2) appear to be more resistant than R1 cells.

INTRODUCTION

Ca\(^{2+}\) is a key intracellular messenger in the control of pancreatic secretion [1–3]. In response to physiological doses of secretagogues, it is directly involved in the regulated exocytosis of zymogen granules through the apical pole of the acinar cells [2,4]. However, a disruption of normal apical enzyme secretion occurs in acute pancreatitis [5,6]. As a result, pancreatic secretion appears diminished both in clinical [7] and experimental [8–10] pancreatitis. This feature suggests that an alteration in the stimulus–secretion coupling can play an important role in the pathogenesis of pancreatitis. Acute pancreatitis is generally considered to be an autodigestive disease that results from premature activation of digestive enzymes [11–13]. Although the mechanisms that lead to intrapancreatic enzyme activation have not been elucidated, studies in vitro have revealed that high intracellular Ca\(^{2+}\)
concentrations were required for the premature activation of trypsinogen within acinar cells, which received short-term stimulation with supramaximal doses of cholecystokinin (CCK) [14] or its analogue, caerulein [15,16]. These reports emphasize the importance of Ca^{2+} at early stages of pancreatitis.

Two different subsets of acinar cells have been reported by us in previous studies [17–19] that were termed R1 and R2. They can be clearly differentiated on the basis of light scatter properties; R1 cells display lower forward and side scatter than R2 cells. Differences in the density of saccharidic terminals in the glycoconjugates of plasma membrane were also found between the types of acinar cells. In the present study, experimental acute pancreatitis has been induced in rats by pancreatic duct obstruction in order to analyse the cytosolic Ca^{2+} load in individual acinar cells at different stages of pancreatitis, from the earliest to later stages, 48 h, after obstruction, and to explore whether Ca^{2+} homoeostasis is homogeneously impaired in all acinar cells as a consequence of pancreatitis. Parallel electron microscopy studies of the pancreas were also carried out to observe ultrastructural alterations in acinar cells that could be related to the abnormalities in cytosolic Ca^{2+} concentrations.

MATERIALS AND METHODS

Chemicals

Buprenorphine, collagenase type XI, soya-bean trypsin inhibitor, amino-acid mixture, BSA, and Fluo-3 acetoxy-methyl ester (Fluo-3/AM) were supplied from Sigma (Madrid, Spain). Standard analytical grade laboratory reagents were obtained from Merck (Frankfurt, Germany).

Surgical procedure

Male Wistar rats, each weighing 250–300 g, were used. After a 12 h fast, the rats were anaesthetized with ether, and acute pancreatitis was induced by dissection and ligation of the bile pancreatic duct at the distal part, close to its exit into the duodenum. Afterwards, the abdomen was closed with 6% polyester fibre sutures, and the rats were placed in their cages with free access to food and water. Sham-operated rats were used as controls; the bile pancreatic duct was dissected, but not ligated in these animals. Post-operative analgesia was maintained in all animals by subcutaneous injections of buprenorphine (0.3 mg/kg per 8 h).

Animal groups

Rats were divided randomly into different groups. Controls and rats with pancreatic duct obstruction for 1.5 h, 3 h, 6 h, 12 h, 24 h and 48 h were prepared. Different animals from each group were used either for electron microscopy studies of pancreas sections or flow cytometric measurements of free cytosolic Ca^{2+} in isolated acinar cells. The study protocol was approved by the Ethics Committee of the University of Salamanca.

Collection of samples

After 12 h fasting, the rats were anaesthetized with sodium pentobarbital (3 mg/100 g of body mass). Blood samples were taken by cardiac puncture to determine plasma amylase activity according to the method of Noelting and Bernfeld [20]. Finally, the entire pancreas was removed for electron microscopy analysis or isolation of pancreatic cells.

Electron microscopy studies

Portions of pancreatic tissue were examined by electron microscopy. Samples were prefixed with 0.1 M cacodylate buffer, pH 7.4, containing 2% glutaraldehyde at 4 °C for 2 h, and then post-fixed with 2% OsO_{4} in the same buffer, were dehydrated in ethanol and finally embedded in Epon. Ultrathin sections were prepared, stained with uranylacetate, as well as lead citrate, and examined with a Zeiss electron microscope (EM 900). Multiple-blinded sections were evaluated and representative findings were used for photo-reprint (see Figure 2).

Preparation of isolated cells

Under anaesthesia with sodium pentobarbital (3 mg/100 g of body mass, intraperitoneally), a median laparotomy was made, the bile duct was ligated at its exit from the liver and the main pancreatic duct was cannulated at its exit into the duodenum to perfuse 5 ml of 25 mM Hepes solution, pH 7.4, containing collagenase (40 units/ml), 0.1 mg/ml soya-bean trypsin inhibitor, 100 mM NaCl, 5 mM KCl, 1 mM MgCl_{2}, 1 mM CaCl_{2}, 14 mM D-glucose, 2 mM glutamine, 2% (w/v) BSA and 2% (w/v) amino-acid mixture. The solution was pre-balanced with 95% O_{2} and 5% CO_{2}, and all further incubations were performed with this gas phase. The pancreas was digested at 37 °C in a shaking water bath (200 cycles/min) for 20 min and washed with fresh collagenase solution every 5 min. Following gentle pipetting through tips of decreasing diameter (3–1 mm), cells were filtered through a double layer of muslin gauze and centrifuged at 30 g for 30 s at 4 °C. The supernatant was collected and centrifuged at 500 g for 5 min at 4 °C. Then the supernatant was discarded and the cell pellet resuspended in Hepes buffer without collagenase, and centrifuged again at 500 g for 5 min at 4 °C. Finally, the pellet was resuspended in Hepes buffer without collagenase at a concentration of 10^{5} cells/ml.
Measurement of cytosolic free Ca\(^{2+}\) in acinar cells

Pancreatic acinar cells were loaded with the Ca\(^{2+}\)-sensitive fluorochrome Fluo-3, according to a modification of the method of Vandenbergh and Ceuppens [21]. Briefly, a suspension of 100 \(\mu l\) of isolated acinar cells was incubated with 5 \(\mu M\) Fluo-3/AM at 37 °C for 30 min in the dark. Next, an equal volume of Hepes buffer (pH 7.4) was added, and the cells were incubated for a further 20 min followed by three washing cycles. Thereafter, the green fluorescence (Fl1) intensity that correlated with the cytosolic Ca\(^{2+}\) concentrations was determined using a dual laser FACS caliber flow cytometer equipped with a doublet discrimination module (Becton Dickinson, San José, CA, U.S.A.). The argon-ion laser, which emitted light at 488 nm and 15 mW, was used. Calibration of the instrument was performed on a daily basis using Calibrite beads (Becton Dickinson). Leucocytes infiltrating the pancreas were excluded on the basis of their unique high-light scatter properties [forward or low light scatter (FSC)/side or 90° light scatter (SSC)]. The labelling of cells with specific antisera against digestive enzymes [17] was used to assess the purity of cells in the samples. Gated cells (99%; cells selected by their FSC and SSC properties, as well as their fluorescence intensity) were stained positively for pancreatic enzymes and were considered acinar cells. At each stage studied, cells from sham-operated animals and from rats with pancreatic duct obstruction were measured in parallel. The Paint-a-Gate 3.0 software programme (Becton Dickinson) was used for data acquisition and analysis. In each experiment at least 10000 cells were analysed. Changes in the cytosolic free Ca\(^{2+}\) concentrations of rats with pancreatic duct obstruction were expressed as percentages compared with the control values obtained each day.

Statistical analysis

Results are expressed as means ± S.E.M. ANOVA, followed by Dunnett’s test, was applied in order to establish whether the differences between controls and rats with pancreatic obstruction were statistically significant. \(P\) values lower than 0.05 were considered to be statistically significant.

RESULTS

Plasma amylase levels significantly (\(P < 0.001\)) increased in rats with pancreatic duct obstruction that had been maintained for 3 h, 6 h, 12 h and 24 h, then returned to control values by 48 h (Figure 1).

Increasingly severe histological alterations were found in the pancreas throughout the 48 h following pancreatic duct obstruction (Figure 2). Electron microscopy images revealed initial oedema, macrophage infiltration, vacuolization and dilatated rough endoplasmic reticulum (RER) in the pancreas sections of rats with pancreatic duct obstruction for 1.5 h (Figure 2A). These alterations became greater 3 h and 6 h after ligature (Figures 2B and 2C). At these periods, reorientation of zymogen granules to basolateral membrane of acinar cells and discharge into the interstitial space were also observed. Hypergranulation, huge dilatation of the RER, oedema, and a greater number and size of cytoplasmic vacuoles were found 12 h after pancreatic duct obstruction (Figure 2D). The oedema persists in the pancreas 24 h after ligation (Figure 2E), and condensed karyoplasm in some nuclei and a prominent distension of the cisterns of the RER were also observed. Some apoptotic nuclei with pyknotic chromatin found against the nuclear membrane were observed in some sections together with normal nuclei. Pancreas sections of rats with pancreatic duct obstruction over 48 h (Figure 2F) showed intracellular disorganization and severe nuclear alterations, and apoptosis together with normal and shrunken nuclei were observed.

Figure 3 shows a representative example of dot plots of the flow cytometric distribution of acinar cells from control rats according to FSC (forward or low light scatter angle) compared with SSC (side or 90° light scatter) (Figure 3A), and FSC compared with Fluo-3/AM labelling (Figure 3B). Histograms (Figures 3C and 3D) represent the proportion and distribution of cells according to FSC (Figure 3C) and Fluo-3/AM labelling (Figure 3D). Negative fluorescence of unlabelled cells is shown in Figure 3E. Two populations of cells can be distinguished on the basis of FSC: the major population is referred to as R1 and the minor as R2. Taking into account that FSC is a parameter directly related to...
size [22], R2 cells are larger than R1. From the distribution, a high degree of heterogeneity according to the Fluo-3/AM labelling can be inferred, especially in R1 cells. Accordingly, the mean fluorescence (FL) value of R2 cells displayed a significantly higher free cytosolic \( \text{Ca}^{2+} \) concentration compared with R1 cells.

Results of the measurements of cytosolic free \( \text{Ca}^{2+} \) in the different animal groups using the fluorescent dye Fluo-3/AM are represented in Figure 4. When total cells were considered, a significant increase \( (P < 0.01) \) in the mean fluorescence value was found in rats with pancreatic duct obstruction from 3–48 h after pancreatic duct ligation. However, the analysis of R1 and R2 populations revealed that whereas a rise in cytosolic \( \text{Ca}^{2+} \) load was observed in R1 cells from early stages, normal intracellular \( \text{Ca}^{2+} \) levels were maintained in R2 cells up to 12 h after ligation. Pancreatic duct obstruction for 24 h and 48 h induced significant \( (P < 0.01) \) increases in the cytosolic \( \text{Ca}^{2+} \) concentrations in both R1 and R2 cells.
DISCUSSION

Acute pancreatitis is an inflammatory disease that results in pancreatic cell injury and progressive autodigestion of the pancreas [23–25]. The pathophysiology is not well known and although the premature activation of trypsinogen within acinar cells appears to play a critical role in the onset of pancreatitis [13,26], other pathogenic factors are involved in the development and evolution of the disease. Many reports have established a relationship between changes in cellular Ca\(^{2+}\) homoeostasis and pancreatitis. Hypercalcemia is a known aetiological factor of acute pancreatitis in humans [27,28] and experimental models [29,30]. Recently, elegant experiments in vitro have shown that an increase of intracellular Ca\(^{2+}\) levels is required for the premature activation of trypsinogen in acinar cells [14,15] and isolated acini [16] exposed to supramaximal doses of CCK or its analogue, caerulein. These studies [14–16] report relevant results about what is happening during the early stages of this pancreatitis model that mimics pancreatitis induced in vivo, using the main pancreatic secretagogue, whose action mechanism, which is clearly established, is associated with intracellular Ca\(^{2+}\) increase.

We have induced acute pancreatitis in rats by bile pancreatic duct obstruction, a model that represents a useful experimental corollary of gallstone-induced acute pancreatitis in humans. The study was designed to explore whether the Ca\(^{2+}\) intracellular rise also appears in in vivo-induced pancreatitis and it could represent a common feature for different types of pancreatitis. On the other hand, since protease activation is highly dependent on the concentration [31] and duration of the Ca\(^{2+}\) signal [16], intracellular Ca\(^{2+}\) concentrations have been analysed on different types of acinar cells not only at the earliest stages of pancreatitis but also during the evolution of the disease while the pancreatic duct remains obstructed.

Our results in the present study clearly show a progressive formation of cytoplasmic vacuoles after pancreatic duct obstruction, a common feature in early stages of both experimental and clinical pancreatitis [32]. Accumulation of zymogen granules and their release through the basolateral membrane of acinar cells into interstitial space was also observed during the first 12 h of pancreatic obstruction, a finding that provides a morphological basis for the progressive increase in amylase plasma levels during this period. All these events occur in parallel with a significant increase in the cytosolic free Ca\(^{2+}\) concentrations of most acinar cells. An association between increased cytosolic Ca\(^{2+}\) and cytoskeletal disruption has been suggested previously [33,34], a finding that could explain the accumulation and abnormal trafficking of zymogen granules observed in acute pancreatitis. In accordance with previous studies in which...
importance of the cytosolic Ca\textsuperscript{2+} increase in intra-acinar trypsinogen activation, our results suggest that not all acinar cells would be equally affected, as R2 cells, the largest cells, are more resistant to the pancreatic injury induced by pancreatic duct obstruction, because, although a high trypsinogen load is displayed by R2 cells from the earliest stages of pancreatitis [17], it could not be activated if the cytosolic Ca\textsuperscript{2+} increase did not occur in parallel. R1 and R2 cells are not restricted to a particular area of the pancreas, but they are spread throughout the head, body and tail of the gland [38]. The mechanism that prevents alterations in Ca\textsuperscript{2+} homoeostasis in R2 cells at early stages of pancreatitis cannot be explained at present. The source of the increase in cytosolic Ca\textsuperscript{2+} concentrations in acute pancreatitis is the release of Ca\textsuperscript{2+} from Ca\textsuperscript{2+} stores and the subsequent influx from the extracellular space [15]. The mechanism for the refilling of Ca\textsuperscript{2+} stores and the Ca\textsuperscript{2+} efflux outside the cell, which is responsible for maintaining resting intracellular Ca\textsuperscript{2+} levels, could normally work in R2 cells, because they are capable of generating sufficient ATP for the pumping of Ca\textsuperscript{2+}. Inhibition of the Ca\textsuperscript{2+}-regulating-ATPase of the endoplasmic reticulum and the plasma membrane has been associated with mitochondrial damage, as a consequence of oxidative stress [34,36]. Electron microscopy revealed a wide range in the degree of damage from one cell to the other, a finding that could explain the differential effect of acute pancreatitis on homoeostasis of Ca\textsuperscript{2+} in R1 and R2 cells.

At later stages of pancreatitis, all acinar cells showed significantly increased cytosolic Ca\textsuperscript{2+} concentrations. Pancreatic duct obstruction maintained for 24 h and 48 h was also able to alter cytosolic Ca\textsuperscript{2+} levels in R2 cells, increasing the values as much as it did in R1 cells. Although a diminished amount of trypsinogen is stored in acinar cells at this stage of pancreatitis [17], their activation would account for the total of acinar cells, thus allowing the spread of proteolytic digestion throughout the pancreas. In fact, the morphological study revealed extensive cell injury in the different pancreatic sections at these stages. As has been reported previously [17–19,37,38] in this model of pancreatitis, apoptosis was also observed, which is a type of cell death associated with increased intracellular Ca\textsuperscript{2+} concentration [39].

According to the results in the present study, the increase in cytosolic Ca\textsuperscript{2+} does not appear at early stages of acute pancreatitis as a general event in all acinar cells. The analysis by flow cytometry of a large number of individual cells (10000) has revealed for the first time that a representative group of acinar cells does not show the same pattern of disturbance in Ca\textsuperscript{2+} homoeostasis as a consequence of pancreatitis. Asynchronous impairment of the intracellular Ca\textsuperscript{2+} homoeostasis after pancreatic duct obstruction might be considered in different acinar cells. A portion of cells (R1) seems to be more sensitive to the damage induced by duct obstruction. Because this cell

**Figure 4** Changes in free cytosolic Ca\textsuperscript{2+} measured by flow cytometry using Fluo-3/AM (as described in Materials and methods section)

Controls (C) (n = 44) and rats with obstruction for 1.5 h (n = 6), 3 h (n = 8), 6 h (n = 6), 12 h (n = 8), 24 h (n = 8) and 48 h (n = 7). Values are expressed as the means + S.E.M. of percentages compared with the control values. ANOVA test followed by Dunnett’s test showed statistically significant differences compared with the controls (**P < 0.01, ***P < 0.001).
population becomes unable to maintain resting Ca\textsuperscript{2+} levels at the earliest stages, activation of trypsinogen could specifically spread out from R1 cells. The R2 cell population could not be altered by removing the pancreatic duct obstruction during the first 12 h of obstruction.

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