

Increased calcium influx in the presence of ethanol in mouse pancreatic acinar cells

Angel Del Castillo-Vaquero, Ginés M. Salido and Antonio González

Department of Physiology (Cell Physiology Research Group), University of Extremadura, Cáceres, Spain

INTERNATIONAL
JOURNAL OF
EXPERIMENTAL
PATHOLOGY

Summary

The effects of alcohol on Ca^{2+} signalling remains poorly understood. Here we have investigated the effects of acute ethanol exposure on Ca^{2+} influx in mouse pancreatic acinar cells. Cells were loaded with fura-2 and the changes in fluorescence were monitored by spectrofluorimetry and imaging analysis. Stimulation of cells with 20 pM cholecystokinin evoked an oscillatory pattern in $[\text{Ca}^{2+}]_c$, both in the presence and in the absence of extracellular Ca^{2+} . Stimulation of cells with cholecystokinin in the presence of 50 mM ethanol led to a transformation of physiological oscillations into a single transient increase in $[\text{Ca}^{2+}]_c$. This effect was observed when Ca^{2+} was present in the extracellular medium, and did not appear in its absence. Addition of 1 mM CaCl_2 to the extracellular medium, following release of Ca^{2+} from intracellular stores by stimulation of cells with 1 nM cholecystokinin or 1 μM thapsigargin in the absence of extracellular Ca^{2+} , was followed by an increase in $[\text{Ca}^{2+}]_c$. Ca^{2+} influx was increased in the presence of 50 mM ethanol. The anti-oxidant cinnamtannin B-1 (10 μM) or inhibition of alcohol dehydrogenase by 4-MP (1 mM), significantly reduced Ca^{2+} influx evoked by cholecystokinin in the presence of ethanol. In summary, intoxicating concentrations of ethanol may lead to over stimulation of pancreatic acinar cells by cholecystokinin. This might be partially explained by the generation of reactive oxygen species and an increased Ca^{2+} entry in the presence of ethanol. Potentially ethanol might lead to Ca^{2+} overload, which is a common pathological precursor that is implicated in pancreatitis.

Keywords

calcium, cholecystokinin, ethanol, pancreas, reactive oxygen species

Received for publication:
7 May 2009

Accepted for publication:
15 September 2009

Correspondence:

Dr. Antonio González
Department of Physiology
Faculty of Veterinary Sciences
University of Extremadura
Avenida Universidad s/n
E-10071 Cáceres (Spain)
Tel.: +34 927 257000 (ext. 51377)
Fax: +34 927 257110
E-mail: agmateos@unex.es

Cholecystokinin acts in a concentration-dependent fashion on its binding sites in pancreatic acinar cells, and results in the generation of different second messengers in the signal cascades that lead to enzymatic secretion (González *et al.* 1999). The activation of phospholipase C-linked receptors by cholecystokinin produces an increase in the concentration of

inositol 1,4,5-trisphosphate (IP_3) in the cytosol. IP_3 in turn releases calcium (Ca^{2+}) from cytoplasmic stores leading to an increase in cytosolic free calcium concentration ($[\text{Ca}^{2+}]_c$) (Streb *et al.* 1983). Ca^{2+} signals are not only a result of release from intracellular stores but also a coordinate influx from the extracellular space (Putney 1988), Ca^{2+} extrusion

across the plasma membrane (Carafoli 1991) as well as Ca^{2+} uptake into intracellular organelles (González *et al.* 1997a). A rise in $[\text{Ca}^{2+}]_c$ is an important early signal by which physiological secretagogues elicit the release of digestive enzymes from pancreatic acinar cells.

In pancreatic acinar cells, cholecystokinin regulates secretion via generation of repetitive local cytosolic Ca^{2+} signals in the apical pole. In addition, the spatiotemporal pattern of agonist-induced Ca^{2+} signals is of critical importance for exocytosis of enzymes (Habara & Kanno 1994).

Cytosolic Ca^{2+} overload may result from excessive loss of Ca^{2+} from the intracellular stores by its release through specific channels and by the inhibition of Ca^{2+} pumps, and/or followed by entry of extracellular Ca^{2+} (Petersen & Sutton 2006; González *et al.* 2008). Therefore, it is important to know how Ca^{2+} homeostasis is regulated because, despite being one of the initial steps involved in cellular function, abnormally elevated $[\text{Ca}^{2+}]_c$ is a shared phenomenon that could induce trypsin premature activation (Ding *et al.* 2006). This is a previous step that can trigger acute pancreatitis and necrosis (Ward *et al.* 1995; Petersen & Sutton 2006).

Although cholecystokinin is a major physiological regulator of secretion by the exocrine pancreas, an over stimulation can cause injury to the pancreas which may lead to dysfunction of the gland and even to the activation of death signalling pathways involving caspases (Saluja *et al.* 1999; Gukovskaya *et al.* 2002).

It has been long recognized that a significant percentage of patients with pancreatitis often presents a history of excessive alcohol consumption. Alcohol is involved in approximately 40% of cases of acute pancreatitis (Kristiansen *et al.* 2008; Ellis *et al.* 2009). Nevertheless, the mechanisms underlying alcohol-derived deleterious effects are not completely understood.

The exocrine pancreas can metabolize ethanol mainly via an oxidative pathway involving the enzymes alcohol dehydrogenase (ADH) and cytochrome P4502E1, although a non-oxidative pathway involving fatty acid ethyl ester synthases has also been proposed (Criddle *et al.* 2004). Metabolism of ethanol by pancreatic acinar cells and the consequent generation of toxic metabolites are postulated to play an important role in the development of alcohol-related pancreatic injury (Petersen & Sutton 2006; Lawencia *et al.* 2009). In these terms, ethanol may induce its deleterious effects in pancreatic acinar cells through the generation of oxidative and non-oxidative metabolites (Criddle *et al.* 2006; González *et al.* 2006; Palmieri *et al.* 2007).

One of the early events leading to alcoholic pancreatitis seems to be the effect of ethanol on stimulus-secretion coupling mechanisms. It has been suggested that ethanol acts to sensitize

the pancreas to the deleterious effects of other stimuli such as the physiological agonist cholecystokinin octapeptide (CCK-8), which leads to an inflammatory response and pancreatitis. This effect is in part mediated by augmenting activation of proinflammatory factors (Pandol *et al.* 2003), although a decrease in the levels of prostaglandin E2 by ethanol could be involved in alcohol-induced injury in the pancreas too (Sigmund *et al.* 2003). Furthermore, it has been proposed that ethanol induces generation of oxygen radicals in pancreatic acinar cells (Wittel *et al.* 2003; González *et al.* 2006; Lawencia *et al.* 2009), impairs CCK-8-evoked secretion of digestive enzymes (González *et al.* 2006) and leads to a delayed or reduced Ca^{2+} extrusion from the cytosol towards the extracellular space or into the cytosolic stores (González *et al.* 2008).

Despite the great amount of research carried out to understand the action of ethanol on cell physiology, the involvement of Ca^{2+} metabolism in ethanol-evoked effects needs to be further studied, because most cellular activity is initiated by changes in $[\text{Ca}^{2+}]_c$. In this study, we have investigated the early effects of acute ethanol exposure on CCK-8-evoked Ca^{2+} influx in mouse pancreatic acinar cells. Our objective is to shed more light on the effects of ethanol on CCK-8 physiological actions. The findings will contribute to a better understanding of the mechanisms by which ethanol causes pancreatic disorders.

Materials and methods

Animals and chemicals

Adult male *Swiss* mice were used for this study. Mice were humanely handled and killed in accordance to the institutional Bioethical Committee. Fura-2-AM was obtained from Invitrogen (Spain) and cinnamannin B-1 from Alexis (Switzerland). All other materials used were obtained from Sigma Chemicals Co. (Spain).

Preparation of isolated pancreatic acinar cells

A suspension of mouse pancreatic single cells and small acini (2–4 cell clusters) was prepared by collagenase treatment following a previously described method (González *et al.* 1997a). Briefly, the pancreas was incubated in the presence of collagenase for 10 min at 37 °C. 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. After centrifugation, cells were resuspended in a buffer without collagenase. With this isolation procedure, single cells, as well as small clusters consisting of up to five cells, were obtained.

Throughout the preparation procedure, as well as during dye loading, we employed a physiological solution containing: 130 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1 mM MgCl₂, 1.2 mM KH₂PO₄, 10 mM glucose, 10 mM Hepes, 0.01% trypsin inhibitor (soybean) and 0.2% bovine serum albumin (pH = 7.4 adjusted with NaOH). During fluorescence determinations, trypsin inhibitor and bovine serum albumin were not included in the medium. In experiments where Ca²⁺ free medium is indicated, Ca²⁺ was omitted from the extracellular solution and 250 μM EGTA was added to the cells in the cuvette.

The viability of cell suspensions, either of cells obtained after the isolation procedure and at the end of the experimental treatments, was assayed by trypan blue exclusion test. Cell viability was not significantly changed and was greater than 95%. After loading with fluorescent dyes, cells were kept at 4 °C until use and the experiments were performed within the next 4 h.

Dye loading and determination of intracellular free Ca²⁺ concentration ([Ca²⁺]_c)

Freshly isolated mouse pancreatic acinar cells were loaded with fura-2-acetoxymethyl ester (4 μM) at room temperature (23–25 °C) for 40 min as previously established (González et al. 1999).

Monitorization of changes in fluorescence signals was performed by placing aliquots of dye-loaded pancreatic acinar cells into a cuvette in a fluorescence spectrophotometer (Cary Eclipse, mod. EL 0210-6479 from Varian, USA). Cells were continuously stirred and experiments were performed at 37 °C. All stimuli were dissolved in the extracellular Na-Hepes buffer without Ca²⁺, and were directly added into the cuvette to yield the final concentration required.

For determination of [Ca²⁺]_c fura-2 loaded pancreatic acinar cells were alternatively excited at 340/380 nm and fluorescence emission was detected at 505 nm.

Several values of the Ca²⁺ response were calculated: (1) the plateau of [Ca²⁺]_c, calculated once [Ca²⁺]_c had reached a stable level after Ca²⁺ influx; (2) the amount of initial Ca²⁺ influx was estimated using the integral of the rise in [Ca²⁺]_c for 90 s after addition of CaCl₂; (3) to calculate the initial rate of Ca²⁺ elevation after the addition of Ca²⁺ to the medium, the traces were fitted to the equation $y = A + Kx$, where K is the slope. This analysis was performed for the first 30 s of the rising phase of Ca²⁺ entry process. Results for [Ca²⁺]_c are expressed in nM ± SEM (n), where n is the number of independent experiments, following the calibration method proposed by Grynkiewicz et al. (1985).

In single cell experiments, monitorization of Ca²⁺-dependent fluorescence signals was carried out by placing small aliquots of cell suspensions into a cover slip mounted on an experimental perfusion chamber, and placed on the stage of an epifluorescence inverted microscope (Nikon Diaphot T200; Melville, NY, USA). The cells were continuously superfused with Na-Hepes buffer. For fluorescence change 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) at 340/380 nm. Fluorescence emission at 505 nm was detected using a cooled digital CCD camera (Hisca CCD C-6790, Hamamatsu) and recorded using dedicated software (Aquacosmos 2.5; Hamamatsu Photonics). All fluorescence measurements were made from areas considered individual cells. In this series of experiments, results are expressed as the ratio of fluorescence emitted at 340 nm and 380 nm excitation wavelengths.

All stimuli were dissolved in the extracellular Na-Hepes buffer and applied directly to the cells in the perfusion chamber. Experiments were performed at room temperature (23–25 °C).

We can discard an effect of ethanol on fura-2-emission fluorescence that could induce artefacts in the determination of [Ca²⁺]_c. In our experimental conditions, the concentration of ethanol employed did not influence fura-2-derived fluorescence. Furthermore, no apparent morphological changes of pancreatic cells that could introduce errors in Ca²⁺ measurements in our experimental conditions were observed (data not shown).

Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test, and only P -values <0.05 were considered statistically significant. For individual comparisons and statistics between individual treatments, we employed Student's t test and only P values <0.05 were considered statistically significant.

Results

CCK-8-evoked changes in [Ca²⁺]_c and effect of ethanol in single cell studies

In the presence of extracellular Ca²⁺, stimulation of fura-2-loaded cells with 20 pM CCK-8 led to an oscillatory pattern in [Ca²⁺]_c. This pattern of Ca²⁺ mobilization was

observed both in the presence ($n = 5$ experiments/26 cells studied; Figure 1a) and in the absence ($n = 4$ experiments; 25 cells studied; Figure 1b) of extracellular Ca^{2+} , although the frequency and amplitude of $[\text{Ca}^{2+}]_c$ spikes were reduced in the absence of external Ca^{2+} . These patterns of changes in $[\text{Ca}^{2+}]_c$ have been observed previously (González *et al.* 1997b, 1999).

To study the effects of ethanol on CCK-8-evoked Ca^{2+} signals, we have chosen a 50 mM ethanol concentration. It is a concentration that falls within the range of alcohol levels found in the blood of intoxicated humans (Lamarche *et al.* 2004). In addition, this concentration of ethanol induces Ca^{2+} mobilization and ROS generation, and clearly affects Ca^{2+} transport mechanisms and amylase secretion in response to CCK-8 in pancreatic acinar cells (González *et al.* 2006, 2008).

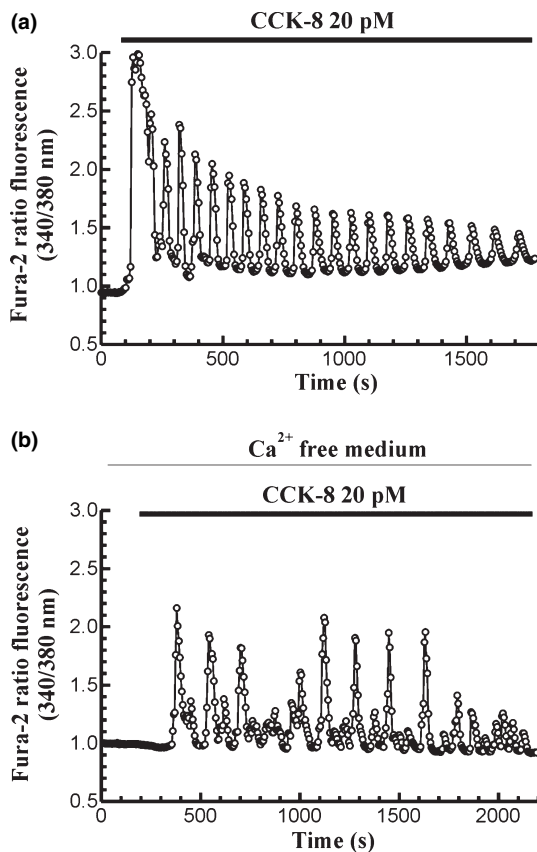


Figure 1 Time-course of changes in $[\text{Ca}^{2+}]_c$ in response to CCK-8. Fura-2 loaded pancreatic acinar cells were stimulated with 20 pM CCK-8 in the presence (a) and in the absence (b) of Ca^{2+} in the extracellular medium. The horizontal bars indicate the time during which CCK-8 was applied to the cells. The traces are typical of 4–5 independent experiments.

In a set of experiments, we evaluated the effect of ethanol on CCK-8-evoked Ca^{2+} signals. In the presence of extracellular Ca^{2+} , perfusion of cells with 20 pM CCK-8 induced oscillations in $[\text{Ca}^{2+}]_c$ as we have shown. The amplitude of oscillations progressively decreased after 15 min in the presence of the agonist in the extracellular solution. Under these conditions, inclusion of 50 mM ethanol in the perfusion medium induced a potentiation of CCK-8-evoked oscillations in $[\text{Ca}^{2+}]_c$, which is observed as an increase in the amplitude of Ca^{2+} spikes in the 55.64% of cells ($n = 9$ experiments/74 cells of 133 total cells studied; Figure 2a). However, when pancreatic acinar cells were challenged with 20 pM CCK-8 in the absence of Ca^{2+} in the extracellular medium, ethanol treatment did not induce any change on CCK-8-evoked oscillations in $[\text{Ca}^{2+}]_c$ ($n = 9$ experiments, 41 total cells studied; Figure 2b).

On the other hand, when 50 mM ethanol was first applied to the cells in the presence of Ca^{2+} in the perfusion medium, an oscillatory mobilization of $[\text{Ca}^{2+}]_c$ was observed. In the presence of ethanol (50 mM), perfusion of cells with 20 pM CCK-8 evoked a single transient change in $[\text{Ca}^{2+}]_c$, similar to that evoked by a supramaximal concentration of CCK-8 in the majority of cells (89.56%) ($n = 9$ experiments/103 cells of 115 total cells studied; Figure 2c).

However, ethanol failed to increase CCK-8-evoked mobilization of Ca^{2+} in the absence of external Ca^{2+} , i.e. inclusion of 20 pM CCK-8 in the perfusion medium after stimulation of cells with 50 mM ethanol evoked the typical oscillatory pattern of $[\text{Ca}^{2+}]_c$ in the majority of cells (86.48% of cells) ($n = 3$ experiments, 32 cells of 37 total cells studied; Figure 2d).

Changes in $[\text{Ca}^{2+}]_c$ in response to thapsigargin and effect of ethanol in cell-suspension studies

As the maintenance of secretagogue-evoked oscillations in $[\text{Ca}^{2+}]_c$ highly depends on the replenishment of cytosolic Ca^{2+} stores, i.e. endoplasmic reticulum (ER), by Ca^{2+} entry from the extracellular space, and the potentiation effect of ethanol was only observed when Ca^{2+} was present in the external medium, it was of interest to analyse the effect of ethanol on Ca^{2+} influx.

Thapsigargin (Tps), the most potent selective sarcoendoplasmic reticulum Ca^{2+} -ATPase inhibitor, is often used to inhibit this pump (Nielsen *et al.* 1995). This will release Ca^{2+} from the ER and consequently will activate Ca^{2+} influx.

Stimulation of cells with Tps in the absence of extracellular Ca^{2+} , led to a transient change in $[\text{Ca}^{2+}]_c$, consisting of

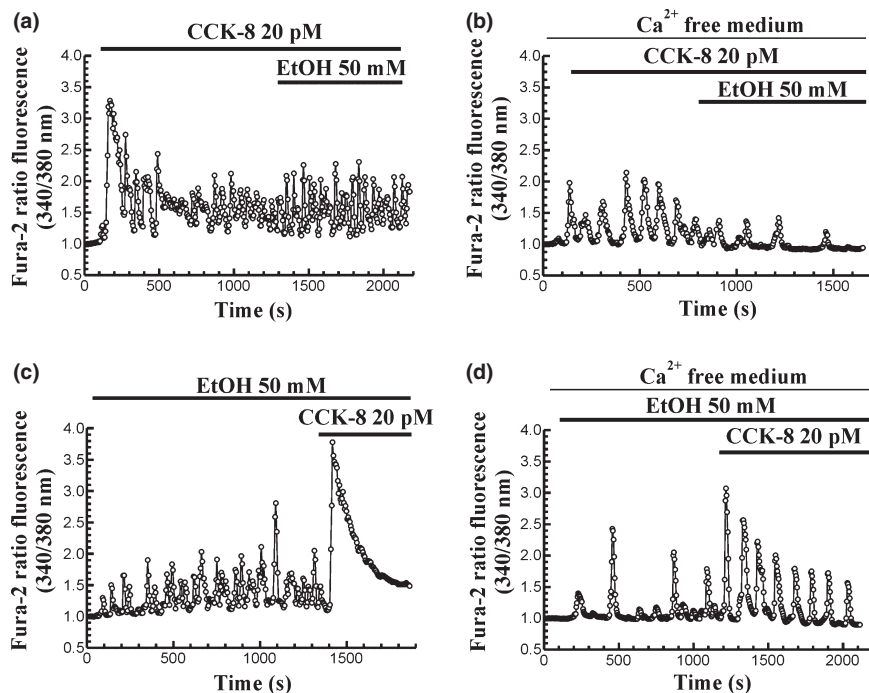


Figure 2 Time-course of CCK-8-evoked changes in $[Ca^{2+}]_c$ in the presence of ethanol. (a) Fura-2 loaded pancreatic acinar cells were perfused with 20 pM CCK-8 in the presence of Ca^{2+} in the extracellular medium. Following CCK-8 stimulation, 50 mM ethanol (EtOH) was included in the perfusion medium. (b) The same protocol was performed in the absence of Ca^{2+} in the extracellular space (medium containing 250 μ M EGTA). (c) Pancreatic acinar cells were perfused with 50 mM ethanol (EtOH) in the presence of Ca^{2+} in the extracellular medium. In the presence of ethanol, 20 pM CCK-8 was included in the perfusion medium. (d) The same protocol was performed in the absence of Ca^{2+} in the extracellular medium (containing 250 μ M EGTA). The horizontal bars indicate the time during which CCK-8 and ethanol were applied to the cells. The traces are typical of 3–9 independent experiments.

an initial increase followed by a decrease of $[Ca^{2+}]_c$ towards a value close to the prestimulation level. Subsequent addition of $CaCl_2$ (1 mM) to the external medium resulted in a sustained increase in $[Ca^{2+}]_c$, indicative of Ca^{2+} entry into the cells. The time-course of changes in $[Ca^{2+}]_c$ in response to Tps can be observed in Figure 3a.

On the other hand, pretreatment of pancreatic acinar cells with 50 mM ethanol for 1 min, prior to Tps (1 μ M) stimulation, significantly increased Ca^{2+} influx following emptying of cytosolic stores by the inhibitor, compared with the values obtained in the presence of CCK-8 alone.

Pretreatment of cells with 50 mM ethanol significantly increased the initial rate of $[Ca^{2+}]_c$ elevation ($3.10 \pm 0.22 \Delta$ nM/s, $n = 7$ vs. $1.77 \pm 0.42 \Delta$ nM/s, $n = 8$; $P < 0.05$), the calculated amount of Ca^{2+} entry (10710 ± 1042 nM, $n = 7$ vs. 7440 ± 1060 , $n = 8$; $P < 0.05$) and the plateau of $[Ca^{2+}]_c$ (429.60 ± 39.91 nM, $n = 7$ vs. 310.40 ± 36.81 , $n = 8$; $P < 0.05$), compared with the effect of Tps alone.

The time course of changes in $[Ca^{2+}]_c$ after addition of 1 mM $CaCl_2$ to the cells in the presence of Tps or Tps plus ethanol is shown in Figure 3b.

Changes in $[Ca^{2+}]_c$ in response to CCK-8 and effect of ethanol in cell-suspension studies

To better analyse the effects of ethanol on CCK-8 evoked Ca^{2+} mobilization, we performed a series of experiments in which cells were stimulated with the peptide. These experiments differ from the former ones in the presence of Tps, in the sense that all Ca^{2+} pumps are active, and therefore there is no influence of an inhibited pump on the Ca^{2+} signal observed.

Throughout these studies, pancreatic acinar cells were stimulated with 1 nM CCK-8 alone or in the presence of 50 mM ethanol, in the absence of extracellular Ca^{2+} (medium containing 250 μ M EGTA). This concentration of CCK-8 successfully releases Ca^{2+} from intracellular stores in a Ca^{2+} free medium, and therefore decreases the content of

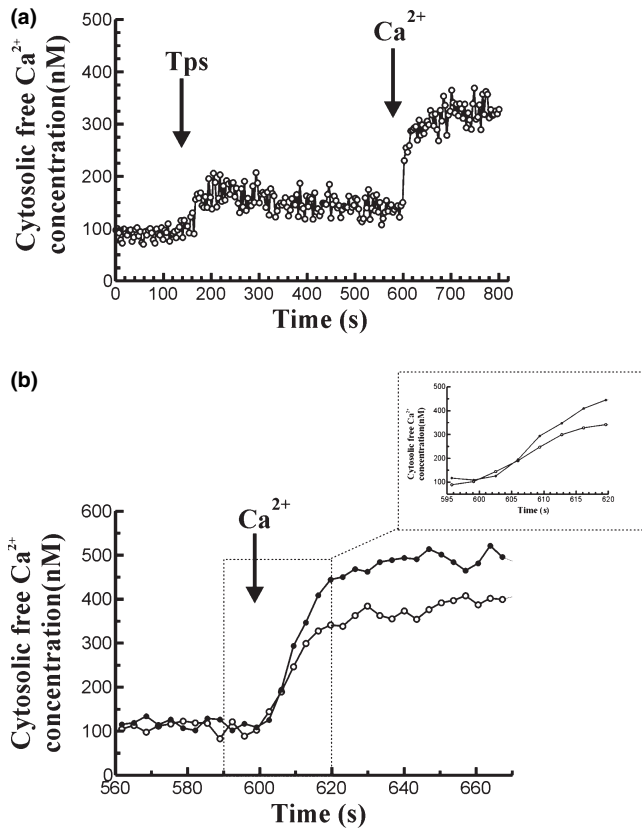


Figure 3 Changes in $[Ca^{2+}]_c$ in response to thapsigargin and stimulated Ca^{2+} influx in mouse pancreatic acinar cells. (a) Cells were stimulated with $1 \mu M$ Tps in the absence of Ca^{2+} in the extracellular medium ($250 \mu M$ EGTA). At the desired time point $1 mM$ $CaCl_2$ was added to the cells in the cuvette. The arrow indicates the time point at which Tps and $CaCl_2$ were added. The trace is typical of eight such independent experiments. (b) Following a previous stimulation of mouse pancreatic acinar cells with $1 \mu M$ Tps in a Ca^{2+} free medium ($250 \mu M$ EGTA) to release of Ca^{2+} from intracellular stores, $1 mM$ $CaCl_2$ was added to the cuvette to induce Ca^{2+} influx. Ca^{2+} influx was monitored in the presence of Tps alone (open circles) or in the presence of $50 mM$ ethanol (full circles). The arrow indicates the time point at which $1 mM$ $CaCl_2$ was added to the cells. The traces are typical of 7–8 such independent experiments (inset: the time scale has been changed for a better observation of the changes in the rate of Ca^{2+} influx under the different treatments).

Ca^{2+} in cytosolic stores. As a consequence, store operated Ca^{2+} entry will take place. Thus, it can be employed to study activation of Ca^{2+} influx in pancreatic acinar cells (González *et al.* 1997b, 2002).

Stimulation of cells with $1 nM$ CCK-8 in the absence of extracellular Ca^{2+} , led to a transient change in $[Ca^{2+}]_c$, consisting of an initial increase followed by a decrease of

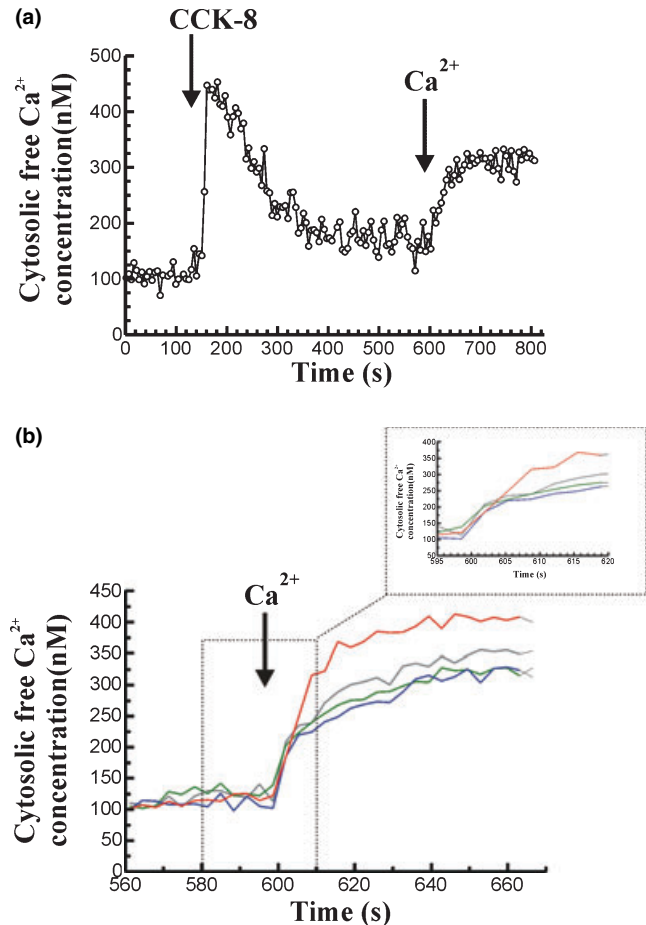


Figure 4 Changes in $[Ca^{2+}]_c$ in response to CCK-8 and stimulated Ca^{2+} influx in mouse pancreatic acinar cells. (a) Cells were stimulated with $1 nM$ CCK-8 in the absence of Ca^{2+} in the extracellular medium ($250 \mu M$ EGTA). At the desired time point, $1 mM$ $CaCl_2$ was added to the cells in the cuvette. The arrow indicates the time point at which CCK-8 and $CaCl_2$ were added. The trace is typical of 14 such independent experiments. (b) Following a previous stimulation of mouse pancreatic acinar cells with $1 nM$ CCK-8 in a Ca^{2+} free medium ($250 \mu M$ EGTA) to release of Ca^{2+} from intracellular stores, $1 mM$ $CaCl_2$ was added to the cuvette to induce Ca^{2+} influx. Ca^{2+} influx was monitored in the presence of CCK-8 alone (blue line), in the presence of $50 mM$ ethanol (red line), in the presence of $10 \mu M$ cinnamtannin B-1 (grey line) or in the presence of the $1 mM$ of the ADH inhibitor 4-MP (green line). The arrow indicates the time point at which $1 mM$ $CaCl_2$ was added to the cells. The traces are typical of 7–14 such independent experiments. (Inset: the time scale has been changed for a better observation of the changes in the rate of Ca^{2+} influx under the different treatments).

$[Ca^{2+}]_c$ towards a value close to the prestimulation level. Subsequent addition of $CaCl_2$ ($1 mM$) to the external medium resulted in a sustained increase in $[Ca^{2+}]_c$, indicative

of store-operated Ca^{2+} entry into the cells. The initial rate of $[\text{Ca}^{2+}]_c$ elevation was $2.36 \pm 0.26 \Delta \text{nM/s}$ ($n = 14$), the calculated amount of Ca^{2+} entry was $4886 \pm 298 \text{ nM}$ ($n = 14$) and the plateau of $[\text{Ca}^{2+}]_c$ was $320.80 \pm 19.42 \text{ nM}$ ($n = 14$). The time course of changes in $[\text{Ca}^{2+}]_c$ following stimulation of cells with 1 nM CCK-8, and after Ca^{2+} influx, is shown in Figure 4a.

Treatment of pancreatic acinar cells with 50 mM ethanol for 1 min, prior to CCK-8 (1 nM) stimulation, significantly increased Ca^{2+} influx following emptying of cytosolic stores by the agonist, compared with the values obtained in the presence of CCK-8 alone. In the presence of ethanol, the initial rate of $[\text{Ca}^{2+}]_c$ elevation was $3.26 \pm 0.14 \Delta \text{nM/s}$ ($n = 12$), the calculated amount of Ca^{2+} entry was $7822 \pm 733 \text{ nM}$ ($n = 12$) and the plateau of $[\text{Ca}^{2+}]_c$ was $378.60 \pm 13.22 \text{ nM}$ ($n = 12$), which were significantly increased compared with the effect of CCK-8 alone ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively). The time course of changes in $[\text{Ca}^{2+}]_c$ after addition of 1 mM CaCl_2 to the cells in the presence of ethanol is shown in Figure 4b.

Effect of alcohol dehydrogenase inhibition on ethanol-induced changes in Ca^{2+} influx in response to CCK-8

Alcohol dehydrogenase plays an important role in the metabolism of alcohol. The following set of experiments was carried out to evaluate whether the effects of ethanol on Ca^{2+} influx are direct or not. To characterize the differences in Ca^{2+} responses between the control experiments with CCK-8 alone and those performed in the presence of ethanol, it was of interest to investigate the changes in Ca^{2+} influx after inhibition of ADH. These experiments were performed on cell suspensions.

Under these experimental conditions, cells were preincubated for 30 min in the presence of the ADH inhibitor 4-methylpyrazole (4-MP) (Cheung *et al.* 2003; Dudka *et al.* 2005; Lawrencja *et al.* 2009).

The increase evoked by 50 mM ethanol on the initial rate of $[\text{Ca}^{2+}]_c$ elevation, the calculated amount of Ca^{2+} entry and the plateau of $[\text{Ca}^{2+}]_c$, induced by emptying of cytosolic stores by 1 nM CCK-8, were significantly inhibited by preincubation of pancreatic acinar cells with 1 mM 4-MP (Table 1). These differences were not statistically significant compared with the values obtained following emptying of cytosolic stores by stimulation of pancreatic acinar cells with 1 nM CCK-8 alone. The time course of changes in $[\text{Ca}^{2+}]_c$ after addition of 1 mM CaCl_2 to the cells in the presence of 4-MP is shown in Figure 4b.

Effect of ethanol-induced ROS generation on CCK-8-evoked Ca^{2+}

To evaluate whether the effects of ethanol are mediated by a secondary ROS generation, we investigated the changes in Ca^{2+} influx in the presence of the antioxidant cinnamtannin B-1. This substance is a naturally occurring A-type proanthocyanidin, which belongs to a class of polyphenols that is widely distributed throughout the plant kingdom. These compounds have long been investigated as a result of the antioxidant functions (Yamakoshi *et al.* 1999; Bagchi *et al.* 2003; Ariga 2004; Bouaziz *et al.* 2007). These experiments were performed on cell suspensions too.

When Ca^{2+} influx was analysed after stimulation of pancreatic acinar cells with 1 nM CCK-8 in the presence of 10 μM cinnamtannin B-1 (30 min preincubation), and after 1 min preincubation with 50 mM ethanol, we observed a statistically significant inhibition of the increase evoked by 50 mM ethanol on the initial rate of $[\text{Ca}^{2+}]_c$ elevation, the calculated amount of Ca^{2+} entry and the plateau of $[\text{Ca}^{2+}]_c$ induced by emptying of cytosolic stores by 1 nM CCK-8 (Table 1). These differences were not statistically significant compared with the values obtained following emptying of cytosolic stores by stimulation of pancreatic acinar cells with 1 nM CCK-8 alone. The time course of changes in $[\text{Ca}^{2+}]_c$

Table 1 Changes in Ca^{2+} influx in response to CCK-8 alone and in the presence of ethanol, and effect of alcohol dehydrogenase inhibition by 4-MP and cinnamtannin B-1

	CCK-8	CCK-8/EtOH	CCK-8/EtOH/4-MP	CCK-8/EtOH/CinB
Rate of $[\text{Ca}^{2+}]_c$ elevation ($\Delta \text{nM/s}$)	2.36 ± 0.26	3.25 ± 0.14 $n = 12^*$	2.47 ± 0.11 $n = 7^{\dagger\dagger}$	2.58 ± 0.19 $n = 10^{\dagger}$
Ca^{2+} entry (nM/90 s)	4886 ± 298	7822 ± 733 $n = 12^{**}$	5102 ± 1064 $n = 7^{\dagger}$	4954 ± 1094 $n = 10^{\dagger}$
Plateau $[\text{Ca}^{2+}]_c$ (nM)	320.80 ± 19.42	378.60 ± 13.22 $n = 12^*$	319.10 ± 15.95 $n = 7^{\dagger}$	340.80 ± 12.44 $n = 10^{\dagger}$

Pancreatic acinar cells were stimulated, in the absence of extracellular Ca^{2+} , with 1 nM CCK-8 alone, in the presence of 50 mM ethanol (EtOH), after preincubation in the presence of 1 mM of the alcohol dehydrogenase inhibitor 4-MP or following preincubation in the presence of 10 μM of the antioxidant cinnamtannin B-1 (CinB). Following CCK-8 stimulation, 1 mM CaCl_2 was added to the extracellular medium and the initial rate of Ca^{2+} elevation, the calculated amount of Ca^{2+} entry and the plateau of $[\text{Ca}^{2+}]_c$ induced by emptying of cytosolic stores were calculated ($*P < 0.05$; $**P < 0.01$ vs. CCK-8; $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ vs. CCK-8 plus EtOH).

after addition of 1 mM CaCl_2 to the cells in the presence of cinnamtannin B-1 is shown in Figure 4b.

Discussion

Alcohol consumption has long been associated with cell damage. Many studies on ethanol toxicity have been conducted following middle or long-term exposition to ethanol (Siegmond *et al.* 2003; Bhopale *et al.* 2006; Fortunato *et al.* 2006; McVicker *et al.* 2007; Wu *et al.* 2008). However, attention on the early steps initiated by ethanol is missing. Furthermore, although relevant studies have been performed on the effect of ethanol on the oxidative state of cellular components and/or apoptosis (Saluja *et al.* 1999; Gukovskaya *et al.* 2002; Fortunato *et al.* 2006; Palmieri *et al.* 2007), its effects on Ca^{2+} homeostasis have not been dealt with in depth.

To date, it is not clear whether the deleterious actions of ethanol on cellular physiology could result from the production of toxic derivatives from alcohol metabolism or from a direct action of alcohol on cellular structures, which would be morphologically and functionally altered (Bhopale *et al.* 2006; González *et al.* 2006; Petersen & Sutton 2006; Palmieri *et al.* 2007; Szabo *et al.* 2007; Prosser *et al.* 2008; Wu *et al.* 2008).

In this study, we have further investigated the early effects of an acute ethanol exposure on CCK-8-evoked Ca^{2+} signals in mouse pancreatic acinar cells, because Ca^{2+} signalling is of critical importance for CCK-8-evoked responses in the exocrine pancreas. We show that stimulation of pancreatic acinar cells with a physiological concentration of CCK-8 (20 pM) evoked an oscillatory pattern of $[\text{Ca}^{2+}]_c$, and that ethanol increases the amplitude of CCK-8-evoked oscillations in $[\text{Ca}^{2+}]_c$. As the potentiation effect was only observed in the presence of external Ca^{2+} , a stimulated Ca^{2+} influx in the presence of ethanol might underlie the increased Ca^{2+} signalling. Our results support previously reported works, which propose that ethanol-induces a sensitization of pancreatic acinar cell to low doses of CCK-8, that by itself does not cause pancreatitis (Pandol *et al.* 2003).

Our observations cannot be explained by a decrease of the viability of cell suspensions as a result of the experimental treatments. If ethanol should cause a high cell death ratio, a higher Ca^{2+} influx should therefore be observed. In our hands, the viability of cells was not affected by ethanol treatment. In addition, we can exclude an alteration of cell membrane fluidity and permeability that could potentially affect ion channels and other transporters. Under our experimental conditions, the shape of cells was not affected by the treatments (tested under microscopy observation) and, furthermore, the cells

maintained their ability to respond to agonists and to effectively transport Ca^{2+} , as we have previously reported (González *et al.* 2006; Salazar *et al.* 2008).

We have further studied the mechanisms by which ethanol evokes its deleterious effects in pancreatic acinar cells, to clarify whether the effects of ethanol are direct or mediated through its metabolization, and if ROS are somehow involved. The inhibition of alcohol metabolization would increase those effects that are resulting from a direct action of ethanol, whereas it would block those effects of ethanol that would be resulting from its metabolites.

Through our study, we have employed the ADH inhibitor 4-MP. This compound substantially decreased ethanol effects on connective tissue growth factor mRNA expression in mouse pancreatic stellate cells (Lawrencia *et al.* 2009). Inhibition of ADH by 4-MP is concentration-dependent, with a range of action from 1 μM up to 1000 μM in different tissues (Xie & Hurley 1999; Cheung *et al.* 2003; Dudka *et al.* 2004, 2005). Thus, the concentration of the ADH inhibitor, we have employed falls within the concentration range successfully employed to inhibit ethanol metabolization by this enzyme. Our results show that the effect of ethanol on Ca^{2+} influx was significantly reduced in the presence of ADH inhibition. Therefore, alcohol metabolization is necessary for ethanol-evoked stimulation of Ca^{2+} influx. However, the exact mechanisms by which ethanol evokes its effects need to be further clarified.

Ethanol has been proposed to induce its deleterious effects in pancreatic acinar cells through the generation of ROS (González *et al.* 2006; Palmieri *et al.* 2007). Therefore, treatment of cells with the antioxidant cinnamtannin B-1 would block those effects of ethanol because of the generation of ROS. As expected, preincubation of pancreatic acinar cells in the presence of this antioxidant inhibited ethanol-stimulated Ca^{2+} influx. This supports the hypothesis that ethanol metabolization could lead to ROS generation which, in turn, would affect Ca^{2+} transport mechanisms, and is in agreement with our previous findings.

Therefore, we can hypothesize that ethanol has an indirect action, mediated through ROS generation, on CCK-8-evoked changes in $[\text{Ca}^{2+}]_c$, which consists of a stimulated Ca^{2+} influx from the extracellular space. This will in turn present its consequences on cellular function. According to our observations, we have shown in a previous work that ethanol induces ROS production in mouse pancreatic acinar cells (González *et al.* 2006). Our findings are in agreement with others showing that ethanol can induce several cellular reactions which result in a modification of cellular red-ox status, and lead to overproduction of ROS (Wittel *et al.* 2003; Gonthier *et al.* 2004; Palmieri *et al.* 2007).

The close relationship between impairment in Ca^{2+} homeostasis (i.e. abnormal elevated $[\text{Ca}^{2+}]_c$) and pancreatitis has been previously reported (Ward *et al.* 1995; Criddle *et al.* 2006; Petersen & Sutton 2006). The factors that precipitate pancreatitis, for example hyperstimulation of cells or excessive ROS generation, cause excessive release of Ca^{2+} or damage to the integrity of the mechanisms that restore low resting levels of $[\text{Ca}^{2+}]_c$. Our results show clear effects of ethanol on Ca^{2+} mobilization, which are minimized by inhibition of oxidative metabolism of ethanol by alcohol dehydrogenase, and by an antioxidant, suggesting ROS generation. Nevertheless, ethanol can be metabolized by non-oxidative pathways in pancreatic acinar cells too, and thus we cannot exclude that non-oxidative metabolites of ethanol exert an action on Ca^{2+} metabolism (Criddle *et al.* 2004, 2006). On the other hand, the relationship between ROS generation and Ca^{2+} -overload is so close that it is difficult to assess which one leads to the other, because either high levels of $[\text{Ca}^{2+}]_c$ lead to ROS generation (Granados *et al.* 2004) and/or excessive ROS production induces Ca^{2+} -overload (González *et al.* 2002, 2005).

In summary, our findings show that ethanol, at intoxicating concentrations, leads to a greater Ca^{2+} mobilization by a physiological concentration of CCK-8. This behaviour depends on a stimulated Ca^{2+} influx in the presence of ethanol. Furthermore, inhibition of ethanol metabolism by alcohol dehydrogenase, or preincubation of cells in the presence of the antioxidant cinnamtannin B-1, reverted ethanol-evoked effects on CCK-8-induced Ca^{2+} influx, which shows that ROS generation by ethanol metabolism is involved in the process. Altogether, this leads to higher levels of $[\text{Ca}^{2+}]_c$ following stimulation of cells with CCK-8. Ethanol will consequently lead to Ca^{2+} accumulation within the cytosol, creating a situation potentially leading to cytosolic Ca^{2+} overload, which is a common pathological precursor that mediates pancreatitis.

Acknowledgements

This work was supported by Junta de Extremadura-FEDER (PRI08A018). Angel Del Castillo-Vaquero was granted a fellowship by the University of Extremadura. The authors would like to thank Mrs. Mercedes Gómez Blázquez for her excellent technical support.

Conflict of Interest

The authors declare that there are no conflicts of interest with other people or organizations that could inappropriately influence (bias) the present work.

References

- Ariga T. (2004) The antioxidative function, preventive action on disease and utilization of proanthocyanidins. *Biofactors* **21**, 197–201.
- Bagchi D., Sen C.K., Ray S.D. *et al.* (2003) Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract. *Mutat. Res.* **523–524**, 87–97.
- Bhopale K.K., Wu H., Boor P.J., Popov V.L., Ansari G.A., Kaphalia B.S. (2006) Metabolic basis of ethanol-induced hepatic and pancreatic injury in hepatic alcohol dehydrogenase deficient deer mice. *Alcohol* **39**, 179–188.
- Bouaziz A., Salido S., Linares-Palomino P.J. *et al.* (2007) Cinnamtannin B-1 from bay wood reduces abnormal intracellular Ca^{2+} homeostasis and platelet hyperaggregability in type 2 diabetes mellitus patients. *Arch. Biochem. Biophys.* **457**, 235–242.
- Carafoli E. (1991) Calcium pump of the plasma membrane. *Physiol. Rev.* **71**, 129–153.
- Cheung C., Davies N.G., Hoog J.O., Hotchkiss S.A.M., Smith Pease C.K. (2003) Species variations in cutaneous alcohol dehydrogenases and aldehyde dehydrogenases may impact on toxicological assessments of alcohols and aldehydes. *Toxicology* **184**, 97–112.
- Criddle D.N., Raraty M.G., Neoptolemos J.P., Tepikin A.V., Petersen O.H., Sutton R. (2004) Ethanol toxicity in pancreatic acinar cells: mediation by nonoxidative fatty acid metabolites. *Proc. Natl Acad. Sci. U.S.A.* **101**, 10738–10743.
- Criddle D.N., Sutton R., Petersen O.H. (2006) Role of Ca^{2+} in pancreatic cell death induced by alcohol metabolites. *J. Gastroenterol. Hepatol.* **21**, Suppl. 3, S14–7.
- Ding Y.X., Yang K., Chin W.C. (2006) Ethanol augments elevated- $[\text{Ca}^{2+}]_c$ induced trypsin activation in pancreatic acinar zymogen granules. *Biochem. Biophys. Res. Commun.* **350**, 593–597.
- Dudka J., Burdan F., Madej B. *et al.* (2004) Effect of selected alcohol dehydrogenase inhibitors on the human heart lactate dehydrogenase activity – an *in vitro* study. *Acta Physiol. Hung.* **91**, 235–241.
- Dudka J., Burdan F., Szumillo J. *et al.* (2005) Effect of selected alcohol dehydrogenase inhibitors on human hepatic lactate dehydrogenase activity – an *in vitro* study. *J. Appl. Toxicol.* **25**, 549–553.
- Ellis M.P., French J.J., Charnley R.M. (2009) Acute pancreatitis and the influence of socioeconomic deprivation. *Br. J. Surg.* **96**, 74–80.
- Fortunato F., Deng X., Gates L.K. *et al.* (2006) Pancreatic response to endotoxin after chronic alcohol exposure: switch from apoptosis to necrosis? *Am. J. Physiol.* **290**, G232–G241.
- Gonthier B., Signorini-Allibe N., Soubeyran A., Eysseric H., Lamarche F., Barret L. (2004) Ethanol can modify the effects

- of certain free radical-generating systems on astrocytes. *Alcoholism* 28, 526–533.
- González A., Camello P.J., Pariente J.A., Salido G.M. (1997a) Free cytosolic calcium levels modify intracellular pH in rat pancreatic acini. *Biochem. Biophys. Res. Commun.* 230, 652–656.
- González A., Pariente J.A., Salido G.M., Camello P.J. (1997b) Intracellular pH and calcium signalling in rat pancreatic acinar cells. *Pflügers Arch. – Eur. J. Physiol.* 434, 609–614.
- González A., Schmid A., Sternfeld L., Krause E., Salido G.M., Schulz I. (1999) Cholecystokinin-evoked Ca^{2+} waves in isolated mouse pancreatic acinar cells are modulated by activation of cytosolic phospholipase A2, phospholipase D, and protein kinase C. *Biochem. Biophys. Res. Commun.* 261, 726–733.
- González A., Schmid A., Salido G.M., Camello P.J., Pariente J.A. (2002) XOD-catalyzed ROS generation mobilizes calcium from intracellular stores in mouse pancreatic acinar cells. *Cell. Signal.* 14, 153–159.
- González A., Granados M.P., Salido G.M., Pariente J.A. (2005) H_2O_2 -induced changes in mitochondrial activity in isolated mouse pancreatic acinar cells. *Mol. Cell. Biochem.* 269, 165–173.
- González A., Núñez A.M., Granados M.P., Pariente J.A., Salido G.M. (2006) Ethanol impairs CCK-8-evoked amylase secretion through Ca^{2+} -mediated ROS generation in mouse pancreatic acinar cells. *Alcohol* 38, 51–57.
- González A., Pariente J.A., Salido G.M. (2008) Ethanol impairs calcium homeostasis following CCK-8 stimulation in mouse pancreatic acinar cells. *Alcohol* 42, 565–573.
- Granados M.P., Salido G.M., Pariente J.A., González A. (2004) Generation of ROS in response to CCK-8 stimulation in mouse pancreatic acinar cells. *Mitochondrion*, 3, 285–296.
- Grynkiewicz G., Poenie M., Tsien R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440–3450.
- Gukovskaya A.S., Gukovsky I., Jung Y., Mouria M., Pandol S. (2002) Cholecystokinin induces caspase activation and mitochondrial dysfunction in pancreatic acinar cells. *J. Biol. Chem.* 277, 22595–22604.
- Habara Y. & Kanno T. (1994) Stimulus-secretion coupling and Ca^{2+} dynamics in pancreatic acinar cells. *Gen. Pharmacol.* 25, 843–850.
- Kristiansen L., Grønbaek M., Becker U., Tolstrup J. S. (2008) Risk of pancreatitis according to alcohol drinking habits: a population-based cohort study. *Am. J. Epidemiol.* 168, 932–937.
- Lamarche F., Gonthier B., Signorini N., Eysseric H., Barret L. (2004) Impact of ethanol and acetaldehyde on DNA and cell viability of cultured neurons. *Cell Biol. Toxicol.* 20, 361–374.
- Lawrencia C., Charrier A., Huang G., Brigstock D.R. (2009) Ethanol-mediated expression of connective tissue growth factor (CCN2) in mouse pancreatic satellite cells. *Growth Factors* 27, 91–99.
- McVicker B.L., Tuma D.J., Casey C.A. (2007) Effect of ethanol on pro-apoptotic mechanisms in polarized hepatic cells. *World J. Gastroenterol.* 13, 4960–4966.
- Nielsen S.F., Thastrup O., Pedersen R., Olsen C.E., Christensen S.B. (1995) Structure-activity relationships of analogues of thapsigargin modified at O-11 and O-12. *J. Med. Chem.* 38, 272–276.
- Palmieri V.O., Grattagliano I., Palasciano G. (2007) Ethanol induces secretion of oxidized proteins by pancreatic acinar cells. *Cell Biol. Toxicol.* 23, 459–464.
- Pandol S.J., Gukovski I., Satoh A., Lugea A., Gukovskaya A.S. (2003) Emerging concepts from the mechanism of alcoholic pancreatitis from experimental models. *J. Gastroenterol.* 38, 623–628.
- Petersen O.H. & Sutton R. (2006) Ca^{2+} signalling and pancreatitis: effects of alcohol, bile and coffee. *Trends Pharmacol. Sci.* 27, 113–120.
- Prosser R.A., Mangrum C.A., Glassb J.D. (2008) Acute ethanol modulates glutamatergic and serotonergic phase shifts of the mouse circadian clock *in vitro*. *Neuroscience* 152, 837–848.
- Putney J.W. (1988) A model for receptor-regulated calcium entry. *Cell Calcium* 7, 1–12.
- Salazar M., Pariente J.A., Salido G.M., Gonzalez A. (2008) Ethanol induces glutamate secretion by Ca^{2+} mobilization and ROS generation in rat hippocampal astrocytes. *Neurochem. Int.* 52, 1061–1067.
- Saluja A.K., Bhagat L., Lee H.S., Bhatia M., Frossard J.L., Steer M. (1999) Secretagogue-induced digestive enzyme activation and cell injury in rat pancreatic acini. *Am. J. Physiol.* 276, G835–G842.
- Siegmund E., Weber H., Kasper M., Jonas L. (2003) Role of PGE2 in the development of pancreatic injury induced by chronic alcohol feeding in rats. *Pancreatology* 3, 26–35.
- Streb H., Irvine R.F., Berridge M.J., Schulz I. (1983) Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5-trisphosphate. *Nature* 306, 67–69.
- Szabo G., Mandrekar P., Oak S., Mayerle J. (2007) Effect of ethanol on inflammatory responses. Implications for pancreatitis. *Pancreatology* 7, 115–123.
- Ward J.B., Petersen O.H., Jenkins S.A., Sutton R. (1995) Is an elevated concentration of acinar cytosolic free ionised calcium the trigger for acute pancreatitis? *Lancet* 346, 1016–1019.

- Wittel U.A., Bachem M., Siech M. (2003) Oxygen radical production precedes alcohol-induced acute pancreatitis in rats. *Pancreas* **26**, 74–80.
- Wu H., Bhopale K.K., Ansari G.A., Kaphalia B.S. (2008) Ethanol-induced cytotoxicity in rat pancreatic acinar AR42J cells: role of fatty acid ethyl esters. *Alcohol Alcohol.* **43**, 1–8.
- Xie P.T. & Hurley T.D. (1999) Methionine-141 directly influences the binding of 4-methylpyrazole in human sigma sigma alcohol dehydrogenase. *Protein Sci.* **8**, 2639–2644.
- Yamakoshi J., Kataoka S., Koga T., Ariga T. (1999) Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* **142**, 139–149.