Calcium signalling in the acinar environment of the exocrine pancreas: physiology and pathophysiology

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Key points
- Ca2+ signalling in different cell types in exocrine pancreatic lobules was monitored simultaneously and signalling responses to various stimuli were directly compared.
- Ca2+ signals evoked by K+-induced depolarization were recorded from pancreatic nerve cells. Nerve cell stimulation evoked Ca2+ signals in acinar but not in stellate cells.
- Stellate cells are not electrically excitable as they, like acinar cells, did not generate Ca2+ signals in response to membrane depolarization.
- The responsiveness of the stellate cells to bradykinin was markedly reduced in experimental alcohol-related acute pancreatitis, but they became sensitive to stimulation with trypsin.
- Our results provide fresh evidence for an important role of stellate cells in acute pancreatitis. They seem to be a critical element in a vicious circle promoting necrotic acinar cell death. Initial trypsin release from a few dying acinar cells generates Ca2+ signals in the stellate cells, which then in turn damage more acinar cells causing further trypsin liberation.

Abstract
Physiological Ca2+ signals in pancreatic acinar cells control fluid and enzyme secretion, whereas excessive Ca2+ signals induced by pathological agents induce destructive processes leading to acute pancreatitis. Ca2+ signals in the peri-acinar stellate cells may also play a role in the development of acute pancreatitis. In this study, we explored Ca2+ signalling in the different cell types in the acinar environment of the pancreatic tissue. We have, for the first time, recorded depolarization-evoked Ca2+ signals in pancreatic nerves and shown that whereas acinar cells receive a functional cholinergic innervation, there is no evidence for functional innervation of the...
stellate cells. The stellate, like the acinar, cells are not electrically excitable as they do not generate Ca$^{2+}$ signals in response to membrane depolarization. The principal agent evoking Ca$^{2+}$ signals in the stellate cells is bradykinin, but in experimental alcohol-related acute pancreatitis, these cells become much less responsive to bradykinin and then acquire sensitivity to trypsin. Our new findings have implications for our understanding of the development of acute pancreatitis and we propose a scheme in which Ca$^{2+}$ signals in stellate cells provide an amplification loop promoting acinar cell death. Initial release of the proteases kallikrein and trypsin from dying acinar cells can, via bradykinin generation and protease-activated receptors, induce Ca$^{2+}$ signals in stellate cells which can then, possibly via nitric oxide generation, damage more acinar cells and thereby cause additional release of proteases, generating a vicious circle.

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**Introduction**

Ca$^{2+}$ signalling studies on isolated pancreatic acinar cells (PACs) or small acinar cell clusters have led to a detailed understanding of the mechanisms underlying the primary intracellular Ca$^{2+}$ release elicited by physiological and pathological agents as well as the subsequent opening of store-operated Ca$^{2+}$ channels in the plasma membrane that accounts for the secondary Ca$^{2+}$ entry from the extracellular solution (Petersen & Tepikin, 2008; Petersen et al. 2017). Physiological, short-lasting and repetitive local Ca$^{2+}$ signals control acinar fluid and enzyme secretion (Petersen, 1992; Petersen & Tepikin, 2008), whereas sustained global elevations of the cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]), elicited by pathological agents, play a key role in the development of the acinar cell damage and death leading to acute pancreatitis (AP) (Gerasimenko et al. 2014). Most of the work on PAC Ca$^{2+}$ signalling has been carried out on isolated mouse cells, but the key results have been confirmed in studies on isolated human PACs (Murphy et al. 2008; Liang et al. 2017). A limited amount of work on acinar cell Ca$^{2+}$ signalling in pancreatic segments has confirmed that the basic character of such signals, as established in isolated cell studies, is also valid in the intact pancreas (Ashby et al. 2003).

PACs dominate the exocrine pancreatic tissue (Bolender, 1974), but there are other important cell types. In addition to the acinar fluid secretion, there is a ductal secretion process whereby a HCO$^{3-}$-rich fluid is produced, which is important for neutralizing in the gut the acid secretion from the stomach (Hegyi & Petersen, 2013). Ca$^{2+}$ signals in the pancreatic duct cells play an important role in the control of HCO$^{3-}$ secretion, and excessive Ca$^{2+}$ signal generation, as in the acinar cells, causes Ca$^{2+}$ overload and toxicity (Maleth & Hegyi, 2014).

More recently, Ca$^{2+}$ signalling and ion channels have been studied in pancreatic stellate cells (PSCs) (Fels et al. 2016; Ferdek et al. 2016; Gryshchenko et al. 2016; Nielsen et al. 2017; Storck et al. 2017). The role of these cells in normal physiology is unclear, but they have long been suspected of contributing to the fibrosis occurring in chronic pancreatitis as well as pancreatic cancer (Ferdek & Jakubowska, 2017; Pang et al. 2017). In the normal pancreas, PSCs can be observed as thin elongated structures situated at the acinar periphery, very close to the basal surface of the PACs (Gryshchenko et al. 2016). In spite of the close proximity of PSCs and PACs, they are not directly connected. Thus Ca$^{2+}$ signals specifically generated in PACs are not transmitted to neighbouring PSCs and vice versa (Gryshchenko et al. 2016). The principal physiological agents eliciting Ca$^{2+}$ signals in PACs are acetylcholine (ACh) and cholecystokinin (CCK), but they have no effect on PSCs (Gryshchenko et al. 2016). Bradykinin (BK) is the principal agent evoking Ca$^{2+}$ signals in normal PSCs (Gryshchenko et al. 2016), but this peptide has no direct effect on PACs (Gryshchenko et al. 2016). Furthermore, PACs and PSCs possess different bile acid transporters. Whereas taurocholate and cholate elicit Ca$^{2+}$ signals in PSCs, because they are taken up into these cells by Na$^{+}$-dependent transporters, these bile acids hardly evoke any Ca$^{2+}$ signals in the PACs. On the other hand, the bile acid tauroliothocholic acid sulphate (TLC-S) evokes clear Ca$^{2+}$ signals in PACs, but has no effect on PSCs (Ferdek et al. 2016).

In spite of the absence of evidence for any direct connection between neighbouring PACs and PSCs, there is indirect evidence showing that Ca$^{2+}$ signal generation in PSCs can have profound effects on PACs. Thus the level of PAC necrosis evoked by the bile acid TLC-S, which acts selectively on PACs, is markedly enhanced by stimulation with BK, which only acts on PSCs (Ferdek et al. 2016). Furthermore, the level of PAC necrosis elicited by a mixture of bile acids or by a fatty acid ethyl ester (FAEE), is markedly reduced by a BK type 2 receptor antagonist (Gryshchenko et al. 2016). Because Ca$^{2+}$ signals in PSCs generate nitric oxide (NO), whereas this is not the case in PACs, it is possible that the effects of PSC Ca$^{2+}$ signals on
PACs are mediated by NO diffusing from PSCs into PACs (Jakubowska et al. 2016).

Early studies by Scheele and Haymovits (1978, 1980) indicated that PACs are electrically excitable, as K+ depolarization evoked Ca2+-dependent enzyme secretion from guinea pig PACs, which could not be blocked by atropine. However, it turned out that the secretory response was due to the Ca2+-dependent release of a non-cholinergic, non-adrenergic neurotransmitter, probably vasoactive intestinal polypeptide (VIP) and its action on the PACs (Pearson et al. 1981a,b). It is now well established that PACs are electrically non-excitable, as they cannot fire action potentials, and do not possess voltage-activated Ca2+ channels (Petersen, 1992). The functional innervation of PACs by parasympathetic nerves is physiologically important and has been studied in some detail (Petersen, 1992), but it is unknown whether PSCs are functionally innervated.

PSCs can undergo significant transformations and this occurs in pancreatitis (Ferdek & Jakubowska, 2017; Pang et al. 2017), but it is not known how this would affect Ca2+ signal generation in PSCs in response to various stimuli. The aim of the study presented here was to provide a more complete description of cellular Ca2+ signalling events in and around the acinar units in the normal pancreas than has previously been available. Furthermore, we were interested in comparing PSC Ca2+ signalling properties in the pancreas from mice with experimental AP with those in the normal tissue, as any changes could have implications for our understanding of the mechanism underlying AP.

Our results demonstrate, that – in addition to observing Ca2+ signals in PACs and PSCs – it is possible to record Ca2+ signals from nerve cells in the peri-acinar environment. However, in contrast to the clear evidence for functional innervation of the PACs, we did not observe Ca2+ signals in PSCs in response to nerve stimulation. Experimental AP caused major changes in PSC Ca2+ signalling. In alcohol-related AP, induced by intraperitoneal injections of ethanol (1.35 g kg⁻¹) and palmitoleic acid (POA) (150 mg kg⁻¹), at 1 h intervals, preceded by injection of PBS, as previously described (Wen et al. 2015; Huang et al. 2017). Because it has been established that fatty acids and ethanol can react together inside cells to produce FAEEs (Criddle et al. 2006; Huang et al. 2014), we refer to this pancreatitis model as FAEE-AP (Wen et al. 2015; Huang et al. 2017). Control mice received injections of the PBS solution alone. Humane killing was 48 h after the last injection.

Histology
Pancreatic tissue was fixed in 4% formaldehyde and embedded in paraffin, and histological assessment was performed after haematoxylin and eosin staining of fixed pancreatic slices (4 μm thickness). Evaluation was performed on ≥10 random fields (magnification: ×200) by two blinded independent investigators grading (scale, 0–3) oedema, inflammatory cell infiltration and acinar necrosis as previously described (Van Laethem et al. 1996; Wen et al. 2015), calculating the means ± SEM (n = 3 mice per group).

Lobule preparation
Pancreatic lobules were isolated from the pancreas of adult normal mice (Gryshchenko et al. 2016) or from mice in which AP had been induced as described above. The pancreas was rapidly dissected, transferred to a collagenase Na+-Hepes-based solution and incubated for 5–6 min at 37°C. Thereafter, the tissue was kept in a standard medium with the following composition (in mM): NaCl, 140; KCl, 4.8; Hepes (KOH), 10; MgCl2, 1; CaCl2, 1; glucose, 10; pH 7.3. In experiments where the effects of omitting extracellular Ca2+ were investigated, CaCl2 was left out of the standard solution. In experiments where the effects of membrane depolarization were investigated, the medium contained 100 mM KCl and the NaCl concentration was reduced to 44.8 mM. Pancreatic lobules were then incubated with fluorescent dye following the
manufacturer’s description. All experiments on normal pancreatic lobules were carried out with fresh preparations attached to the coverslip of a perfusion chamber at room temperature (≈23°C). In experiments on lobules in which the effects of exposure to fatty acids and ethanol were investigated, the lobules were exposed to a medium containing POA (20 μM) and ethanol (12 mM) for 2.5 h before starting the experiments.

The pancreas is dominated quantitatively by exocrine cells, but also contains endocrine cells, in particular insulin-secreting β-cells. The endocrine cells are found in the islets of Langerhans and these can be identified as dense and discrete spherical or ovoid structures sharply delineated from the surrounding more translucent exocrine tissue (Dean & Matthews, 1970). We deliberately did not focus on these structures as it was our objective to specifically study Ca\(^{2+}\) signalling events in the acinar environment.

### Ca\(^{2+}\) measurements

Pancreatic lobules were loaded with 5 μM Fluo-4 acetoxymethyl ester (AM), for 20 min at room temperature. The tissue was transferred into a flow chamber and superfused with the Na\(^{+}\)-Hepes-based extracellular solution as described above. Cells were visualized using a Leica SP5 MPII two-photon confocal microscope, with an ×63 1.3 NA objective lens. Fluo-4 was excited with a 488 nm argon laser, at 1–4% power, and emitted light was collected at 500–580 nm. Generally, a series of images was recorded at 1–4% power, and emitted light was collected at 512 × 512 pixels resolution (at the speed of 1 frame s\(^{-1}\)), with an ×63 1.3 NA objective lens. Fluorescence signals were plotted as F/F₀ (F₀ is the initial level of fluorescence). In many experiments three-dimensional recording in time have been conducted (2–3 images per time point). Statistical analysis was performed using ANOVA or Student’s t-test.

### Results

#### General approach

Our general aim was to simultaneously study signalling in the various cell types to be found in the acinar environment in a live pancreatic lobule preparation. Figure 1 shows an example. As previously demonstrated (Gryshchenko et al. 2016), PSCs take up Ca\(^{2+}\)-sensitive fluorescent probes much more avidly than PACs, so the initial assumption – looking at the fluorescence intensity levels in the resting situation (Fig. 1Aii) – was that the bright cells represent PSCs. To check whether nerve cells were present and, if so, to test whether nerve stimulation could elicit Ca\(^{2+}\) signals in PACs or other cells, a solution with a high (100 mM) K\(^{+}\) concentration was introduced. As seen in Fig. 1Aiii, this caused a rise in [Ca\(^{2+}\)], in several relatively large cells, which must be the always quantitatively dominant PACs. Importantly, there was no rise in [Ca\(^{2+}\)], in the PSCs, but in one cell – partly ‘hidden’ by a PSC – there was a large Ca\(^{2+}\) signal. This cell is most likely a neuron (PN). The apparently unprovoked short-lasting Ca\(^{2+}\) signal in this cell occurring later in the experiment may be due to a spontaneous action potential or a short burst of action potentials. The assumption that the bright cells seen in Fig. 1Aii were PSCs was confirmed when these cells became significantly brighter, indicating rises in [Ca\(^{2+}\)], after stimulation with BK (1 nM) (Fig. 1Aiv). Finally, the lobule was stimulated by ATP (100 μM), which caused a rise in [Ca\(^{2+}\)], in the PACs and in a cell (green in the schematic diagram in Fig. 1Ai) that had not reacted to high K\(^{+}\) or BK exposure. The nature of this cell is unclear and it is therefore labelled X.

As mentioned in the Methods, the pancreas contains insulin-secreting β-cells, in addition to the quantitatively dominant exocrine cells. It has long been known that PACs possess insulin receptors and that insulin can affect PAC functions including Ca\(^{2+}\) signalling (Sankaran et al. 1981; Singh, 1985; Mankad et al. 2012; Samad et al. 2014). As described in the Methods, we did not explore Ca\(^{2+}\) signalling in or near the islets of Langerhans and it would therefore seem very unlikely that any of the cells in the acinar environment we investigated could be insulin-secreting β-cells or would be influenced by local insulin secretion. We nevertheless checked this by using the standard protocol for eliciting Ca\(^{2+}\) signals in β-cells, namely by testing the effect of elevating the external glucose concentration (Dean & Matthews, 1970) from 2 to 10 mM. As seen in Fig. 2, none of the peri-acinar cell types generated Ca\(^{2+}\) signals in response to glucose stimulation (see also further details in the sections below on PNs and X-cells).

### Ca\(^{2+}\) signals in pancreatic nerve cells (PNs)

It is clear from experiments of the type shown in Fig. 1 that there are cells other than PACs that respond to depolarization with Ca\(^{2+}\) signals. One possibility is that they are PNs and we therefore tested this hypothesis. The fluorescent dye FluoroGold has been demonstrated to undergo retrograde axonal transport and stains nerve cells (Naumann et al. 2000). Figure 3A–C shows the results from an experiment (n = 3) in which a FluoroGold-labelled cell (Fig. 3B) responded to membrane depolarization, elicited by a high-K\(^{+}\) (100 mM) solution, with an increase in [Ca\(^{2+}\)], (Fig. 3A). A non-FluoroGold-labelled PAC also produced a rise in [Ca\(^{2+}\)], presumably due to the action of ACh released from nerve endings (see below), whereas a PSC failed to respond (Fig. 3). We also undertook experiments in which the ultra-sensitive Ca\(^{2+}\) sensor GCaMP6 was expressed in mice by intravenous injection

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of adeno-associated virus AAV9.Syn.GCaMP6s targeted to neurons (Chen et al. 2013). As seen in Fig. 3Di–iii, a short-lasting high-K+ stimulation caused a substantial transient increase in [Ca2+]i (n = 7).

Many of the pancreatic cells that have neuron-like properties are located close to PSCs (see Fig. 1). In several cases (n = 14) we could observe Ca2+ signal propagation in PNs along the bodies and elongated parts of PSCs as a pathway through the lobules (Fig. 4A, B).

The rise in [Ca2+]i in PNs, elicited by K+-induced depolarization (Figs 1–5) could potentially be influenced by release of neurotransmitters from nerve cells not visualized in the segment under investigation and we therefore tested possible effects of various neurotransmitters (Figs 4 and 5). Figure 4C and D shows examples of Ca2+ signals in a PN and a PAC generated by exposure to a high-K+ solution. The PAC signal, as expected, was clearly not mediated by depolarization of the acinar cell membrane as it was abolished by atropine (n = 12), in agreement with the well-established cholinergic innervation of PACs (Petersen, 1992), whereas the Ca2+ signal in the PN could still be observed in the presence of this muscarinic antagonist (n = 17). ATP did not have any effects on PNs (n > 100; Fig. 4C, D), whereas this agent could, in several cases, produce Ca2+ signals in PACs and PSCs (Fig. 1), although not in the case shown in Fig. 4C and D. Ca2+ signals induced by the high-K+ solution in both PACs and PNs were reversibly abolished by removal of external Ca2+ (n = 4) (Fig. 4E, F). Ca2+ signals elicited by a high-K+ solution in PNs were not inhibited by the non-selective purinergic antagonist suramin (n = 5; Fig. 5B).

Several neurotransmitters elicited Ca2+ signals in PNs. Adrenaline (20 μM) evoked signals in 21 out of 35 PNs tested (Fig. 5A). This response was mediated by α- rather than β-receptors as the β-adrenergic agonist isoprenaline had no effect (n = 10) whereas the α-receptor agonists cirazoline (50 μM) and UK 14.304 (50 μM) could mimic the effect of adrenaline (n = 8). Ca2+ signals in PNs were also elicited by Substance P (10 μM) in 4 out of 7 neurons (Fig. 5C). Bombesin, which is known to elicit Ca2+ signals in PACs by interaction with receptors that are distinct from the CCK receptors (Deschodt-Lanckman et al. 1976; Iwatsuki & Petersen, 1978), evoked Ca2+ signals in 7 out of 8 PNs (Fig. 5E). On the other hand, PNs did not respond to BK (n > 100; Fig. 5D) or VIP (n = 4).

Figure 1. Simultaneous recordings of [Ca2+]i changes in response to various stimuli in four different cell types in a mouse pancreatic lobule
Ai, sketch of location of different cell types in the lobule: blue, PACs; orange/red, PSCs; purple, PN; green, unknown (X). Ai–v, fluorescence images in control and during stimulation with high K+ (100 mM), BK (1 mM) and ATP (100 μM). As also seen in the [Ca2+]i traces shown in B, PN and PACs displayed rises in [Ca2+]i, in response to membrane depolarization. PSCs responded to BK and both PACs and X responded to ATP. The colours of the traces in B match the coloured arrows in Ai.
In the hypothalamus there are neurons responsive
to glucose (Burdakov et al. 2005) and in the pancreas
the insulin-secreting β-cells have long been known to
depolarize and fire action potentials when challenged
with glucose above a certain threshold concentration
(Dean & Matthews, 1970; Dean et al. 1975; Atwater et al.
1978). We therefore tested whether the PNs in our pre-
paration would be sensitive to changes in the extracellular

glucose concentration. In these experiments the glucose
concentration in the fluid surrounding the lobules was
kept low (2 mM) for a prolonged period (15–30 min)
before exposure to 10 mM glucose. As seen in Fig. 5C, a
PN in which a high-K+ solution, as well as Substance P,
elicted Ca2++ signals failed to respond to stimulation with
10 mM glucose (n = 13).

PSCs are not electrically excitable but respond to
some neurotransmitters

As previously described, PSCs consistently generate Ca2++
signals when challenged with BK (Fig. 1; Ferdek et al. 2016;
Gryshchenko et al. 2016), but it is not known whether
they are functionally innervated. We never observed Ca2++
signals in PSCs when lobules were exposed to high-K+ solutions (n > 100). Figure 4E shows the result of an
experiment in which a high-K+ solution elicited Ca2++ signals in both a PN and a PAC without evoking a response from two PSCs, which both subsequently generated Ca2++ signals when stimulated by BK.

As previously shown (Gryshchenko et al. 2016), PSCs
could (Fig. 5EF), but did not always (Fig. 1), generate
Ca2++ signals in response to ATP (100 μM) stimulation.
Bombesin (1 μM) elicited Ca2++ signals in some PSCs (n = 8
out of 21 cells tested; Fig. 5E) and VIP (100 nM) could evoke
Ca2++ signals in 37 out of the 78 PSCs tested (Fig. 5F).

X-cells

As shown in Fig. 1 there is an unknown (X) cell type
that generates a substantial Ca2++ signal in response to
ATP stimulation (n > 100). In these ATP-sensitive X-cells,
high-K+ stimulation could in many, but not all, cases evoke
short-lasting Ca2++ signals (Fig. 5D), but once these cells
had been challenged with a high-K+ pulse, they needed a
long recovery time (>30 min) before they could respond
again (n = 9). In these cells, adrenaline (20 μM) could
evoke Ca2++ signals (Fig. 5B, n = 14 out of 33 cells tested),
but X-cells never responded to BK (n > 100) (Fig. 5D).
We also tested whether the X-cells were glucose-sensitive.
We used the same protocol as for the similar experiments
testing glucose sensitivity in PNs (Fig. 5C) (low – 2 mM –
basal glucose concentration and then a test pulse of 10 mM

glucose). In five experiments, X-cells that responded to
ATP stimulation with Ca2++ signals failed to generate any
increase in [Ca2++]i in response to 10 mM glucose (Fig. 2).

Alcohol-related AP changes the responsiveness of
PSCs

As described in the Introduction, it is known that PSCs
undergo morphological and functional changes during
pancreatitis (Ferdek & Jakubowska, 2017; Pang et al.
2017) and we were therefore interested in exploring
whether their responsiveness to BK would also change as
a result of this transformation. We investigated this in two
different ways. In one type of experiment, we induced
changes in the pancreatic lobule preparation, similar to

![Figure 2. Elevating the extracellular glucose concentration from 2 to 10 mm has no effect on [Ca^{2+}]_i in any of the peri-acinar cells](image-url)
those seen in AP, by exposing the tissue to a mixture of
ethanol and POA, which is known to generate palmitoleic
acid ethyl ester (POAEE) inside PACs (Laposata & Lange,
1986; Cridde et al. 2006; Huang et al 2014; 2017). In the
second type of experiment, we induced AP in mice in vivo,
by injections of ethanol and POA, and then removed
the pancreas to investigate Ca^{2+} signalling properties in
the lobule preparation.

Figure 6 summarizes the results from the in vitro series of
experiments. In the control lobules (no POA/ethanol) we
confirmed that PSCs respond to BK (1 nM) stimulation by
generating substantial Ca^{2+} signals and also confirmed
the previously reported result that trypsin does not elicit
Ca^{2+} signals (Gryshchenko et al. 2016). However, following
exposure to POA/ethanol, the cells produced substantial
Ca^{2+} signals in response to a concentration (50 nM) of
trypsin that had failed to elicit signals in the control
preparations (Fig. 6). As seen in Fig. 6, the effect of
trypsin was acute and reversible and could therefore not
be a consequence of cell death induced by digestion,
but must be a receptor-mediated (protease-activated
receptor) effect. Many PSCs may well have died during

Figure 3. K^+ depolarization evokes Ca^{2+} signals in labelled pancreatic neurons

\[ A, \text{K}^+ \text{ depolarization evoked a rise in [Ca}^{2+}] \text{ in a FluoroGold labelled PN (B) as well as in a PAC. C–ii, fluorescence images before (0 s) and during the high K^+ challenge (86 s) showing the evoked rise in [Ca}^{2+}] in the PN as well as the PAC, but with no change in the PSC. Ciii, transmitted light image of the field, also showing location of the different cells. Length of horizontal bar corresponds to 10 μm. D, ultrasensitive protein calcium sensor GCaMP6 was expressed in mouse pancreas by intravenous injection of adeno-associated virus AAV9.Syn.GCaMP6s (Penn Vector Core at University of Pennsylvania) targeted to neurons. K^+ depolarization evoked a significant rise in [Ca}^{2+}] (Di). As seen in Dii, at rest the PN had relatively low fluorescence (0 s) but this increased by a factor of three after depolarization with high-K^+ solution (Diii, 135 s). Length of horizontal bars in Dii and Diii corresponds to 5 μm. No other cells in the field of view displayed any changes in fluorescence intensity. In this experiment the only fluorescent probe present was GCaMP6.\]
the exposure to POA/ethanol, but the cells from which [Ca\(^{2+}\)]\(_i\) recordings were made were still viable, as seen by their ability to bring [Ca\(^{2+}\)]\(_i\) back to the control level after a short exposure to trypsin (Fig. 6B). The proportion of PSCs responding to trypsin in the AP lobules was markedly reduced by including the CRAC channel inhibitor GSK-7975A in the POA/ethanol solution used to generate AP (Fig. 6C). Because CRAC channel inhibition has been shown to reduce store-operated Ca\(^{2+}\) influx in both PACs and PSCs, this supports the idea previously proposed (Ferdek et al. 2016; Gryshchenko et al. 2016) that excessive Ca\(^{2+}\) signal generation in PACs as well as PSCs play a central role in the development of AP.

In the in vivo experiments, we verified that AP had been induced by evaluating pancreatic histology sections, comparing tissue from control mice with those that had been injected with POA/ethanol. Figure 7A–F summarizes these data. It can be seen that the overall histology score, the degree of oedema, the level of acinar necrosis and the extent of immune cell invasion were all markedly increased in the pancreatic tissue from the mice that had been injected with POA/ethanol as compared to the normal tissue. As seen in Fig. 7H, J and L and M, the PSCs in the AP mice, in contrast to the control mice (Fig. 7G, I, K and M), hardly responded to 1 nM BK, but in a number of PSCs Ca\(^{2+}\) signals in response to trypsin (10 nM) were observed (\(n = 8\) out of 38 cells tested,
Fig. 7H and N. Control PSCs did not respond to trypsin (Fig. 7G), as also previously reported (Gryshchenko et al. 2016). Similar to the effects of trypsin on PSCs in lobules exposed in vitro to POA/ethanol mixtures (Fig. 6B), the actions of this enzyme in this series of experiments (Fig. 7H and N) were also acute and reversible, indicating a receptor-mediated (protease-activated receptor) effect rather than a consequence of cell damage. Although many PSCs may have been destroyed by the actions of POA/ethanol in vivo, clearly those responding to trypsin were intact. In a few cases (n = 3 out of 59 cells tested), thrombin (5 mU ml−1), another protease, evoked Ca2+ signals in a PSC and bombesin also elicited a Ca2+ signal in a PN that responded to high-K+ stimulation. VIP (100 nM) evoked Ca2+ signals in two PSCs, but neither in a PAC nor in a X-cell, whereas ATP produced Ca2+ signals in all the cells (X, PAC and PSC). High K+ evoked Ca2+ signals in the X-cell and the PAC, but not in the PSCs.

Figure 5. The effects of various neurotransmitters on [Ca2+]i in the different cell types found in the lobules

A, the PN, but not the PSC, produces a Ca2+ signal in response to high-K+ stimulation and subsequently generates a Ca2+ signal in response to stimulation with adrenaline (20 μM). B, in the presence of the purinergic receptor antagonist suramin, high-K+ stimulation evokes a normal Ca2+ signal in the PN and a very short-lasting signal in the X-cell. The effect of ATP (100 μM) on the X-cell in the presence of the purinergic antagonist is very short-lasting compared to the effect of the same concentration of ATP later in the same cell after wash-out of suramin. Adrenaline (20 μM) evoked a train of Ca2+ spikes in the X-cell, but in this experiment only had a questionable effect on the PN. C, high K+ elicited Ca2+ signals in PAC and PN. Substance P (10 μM) evoked a Ca2+ signal in the PN, but not in the PAC. Glucose (10 mM) failed to evoke Ca2+ signals in both the PN and the PAC (in these experiments the standard solution contained only 2 mM glucose). D, ATP repeatedly evoked Ca2+ signals in X-cell and small signals in PAC, but not in PN. High-K+ stimulation evoked large Ca2+ signal in PN, but only a short-lasting signal in the X-cell. The ATP-elicited Ca2+ signal in the X-cell was not diminished during the period of high-K+ depolarization. BK did not evoke any effects in these three cells. E, ATP and bombesin (1 μM) evoked Ca2+ signals in a PSC and bombesin also elicited a Ca2+ signal in a PN that responded to high-K+ stimulation. F, VIP (100 nM) evoked Ca2+ signals in two PSCs, but neither in a PAC nor in a X-cell, whereas ATP produced Ca2+ signals in all the cells (X, PAC and PSC). High K+ evoked Ca2+ signals in the X-cell and the PAC, but not in the PSCs.
signals in PSCs in lobules from FAEE-AP mice, whereas this was not observed in control tissue, as also previously reported (Gryshchenko et al. 2016). Because Ca\(^{2+}\) signals in normal PSCs evoked by BK is due to activation of type 2 BK receptors (Gryshchenko et al. 2016), the desensitization to BK seen in AP (Fig. 7K–M) would appear to represent a specific desensitization of the type 2 receptors. This is supported by the finding that although PSCs in the FAEE-AP tissue failed to respond to a BK concentration (1 nM) that elicited a maximal Ca\(^{2+}\) signal in the control tissue, some PSCs from FAEE-AP lobules could produce Ca\(^{2+}\) signals when stimulated with a high concentration (1 \(\mu\)M) of a BK agonist specific for type 1 BK receptors (S-BK) (Fig. 7J and N; 8 cells out of 101 tested). In contrast, S-BK only evoked a Ca\(^{2+}\) signal in one PSC out of 118 tested in lobules from control mice. Figure 7N summarizes the results of the experiments comparing the responsiveness of PSCs to S-BK, thrombin and trypsin in control and FAEE-AP.

We evaluated quantitatively the reduced responsiveness of the PSCs to BK in AP by comparing concentration–response curves for BK-elicited Ca\(^{2+}\) signal generation in control and AP (Fig. 7K–M). The results show that the concentration–response curve was shifted markedly to the right in AP, as compared to the control values. Thus a BK concentration of 1 nM, which evokes a near-maximal Ca\(^{2+}\) signal in control PSCs (Gryshchenko et al. 2016; Fig. 7K and M, \(n = 11\)), hardly evoked any change in [Ca\(^{2+}\)]\(_i\) in the PSCs from the AP lobules (Fig. 7L and M, \(n = 12\)).

**Discussion**

In this study of Ca\(^{2+}\) signalling in the peri-acinar environment of the exocrine pancreas, we have for the first time been able to record Ca\(^{2+}\) signals from PNs, and demonstrated that PSCs are not electrically excitable and, in contrast to the PACs, do not appear to be functionally

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**Figure 6. Exposure of pancreatic lobules to a mixture of POA and ethanol induces PSC responsiveness to trypsin**

In A and B, the effects of trypsin (50 nM) and BK (1 nM) on [Ca\(^{2+}\)]\(_i\) in PSCs in a control lobule are compared with those in lobules that had been exposed to a mixture of POA (20 \(\mu\)M) and ethanol (12 mM) for 2.5 h. In control PSCs, trypsin (50 nM) only evoked a Ca\(^{2+}\) signal in 2 cells out of 28 tested, and not in the case shown in A, whereas the same concentration of trypsin evoked a clear Ca\(^{2+}\) signal in the PSC in a lobule that had been treated with POA and ethanol (\(n = 14\) out of 28 cells tested). C, summary of the results of the experiments illustrated in A and B, showing the marked increase in the percentage of PSCs responding to trypsin with Ca\(^{2+}\) signals after POA/ethanol exposure. In the presence of the CRAC channel inhibitor GSK-7975A (20 \(\mu\)M), the percentage of PSCs responding to trypsin in the POA/ethanol groups was markedly reduced (\(n = 12\) of 71 cells tested).
Figure 7. Functional changes in PSCs due to alcohol-induced AP (POA/ethanol in vivo mouse model – FAEE-AP)

A–F, the successful induction of AP was investigated by histological assessments of fixed pancreatic slices. Comparisons were made between pancreatic slices from control mice and FAEE-AP mice. Overall histology score (A), degree of oedema (B), extent of necrosis (C) and degree of inflammation (D) were recorded (**P < 0.01). Representative images of pancreatic histology sections from control (E) and FAEE-AP (F) mice are also shown (bars: 50 μm). In each case the number of independent experiments (from different mice) = 3 (but in each experiment >20 sections were examined; typically ~1000–2000 cells in each experimental group). G–J, representative [Ca^{2+}]i traces from PSCs in lobules from a control mouse (G, I) and a mouse with AP (H, J). In the control lobules (G, I), BK (1 nM) consistently evoked Ca^{2+} signals, whereas trypsin (10 nM) and the selective BK 1 receptor agonist Sar-[D-Phe8]-des-Arg8-bradykinin (S-BK) (1 μM) failed to do so. In the lobules isolated from mice with FAEE-AP,
BK (1 nM) failed to elicit Ca\textsuperscript{2+} signals, but trypsin (10 nM) and S-BK (1 \mu M) were able to elicit such signals. 

\textit{K–M}, quantitative evaluation of the change in PSC sensitivity to BK following induction of FAEE-AP. \textit{K} shows traces of BK-elicited [Ca\textsuperscript{2+}]\textsubscript{i} changes, all from one and the same PSC, in a control lobule, whereas \textit{J} shows the results, from one and the same PSC, in a lobule from an FAEE-AP mouse. The data from all experiments are summarized by the concentration–response curves in \textit{M} (\textit{n} = 6–10 for each point). \textit{N}, comparisons of the responsiveness of PSCs to S-BK (1 \mu M), thrombin (5 mM) and trypsin (10 nM) in control and FAEE-AP lobules.

We have also discovered that BK receptors have a role in regulating the balance between the anti-inflammatory and pro-inflammatory effects of endogenous mediators in injured PSCs (setting the stage for future studies). Our findings suggest that BK receptors play a key role in modulating the balance between the pro-inflammatory effects of endogenous mediators and the anti-inflammatory effects of endogenous mediators in injured PSCs. These findings may be relevant to understanding the role of BK receptors in regulating the balance between the pro-inflammatory and anti-inflammatory effects of endogenous mediators in injured PSCs.

Our results show that BK receptors may have a role in regulating the balance between the anti-inflammatory and pro-inflammatory effects of endogenous mediators in injured PSCs. The role of BK receptors in regulating the balance between the pro-inflammatory effects of endogenous mediators and the anti-inflammatory effects of endogenous mediators in injured PSCs warrants further investigation.

In conclusion, our results suggest that BK receptors play a role in regulating the balance between the pro-inflammatory effects of endogenous mediators and the anti-inflammatory effects of endogenous mediators in injured PSCs. The role of BK receptors in regulating the balance between the pro-inflammatory effects of endogenous mediators and the anti-inflammatory effects of endogenous mediators in injured PSCs warrants further investigation.
(Fig. 8) and provide fresh evidence in favour of the propositions made many years ago, but largely ignored, that inhibition of BK receptors could have benefits in the treatment of AP (Griesbacher et al. 1993; Hirata et al. 2002).

We have previously shown that CRAC channel inhibition markedly reduces the prolonged [Ca\textsuperscript{2+}] elevation due to the store-operated Ca\textsuperscript{2+} entry into the PSCs that follows the initial BK-elicited intracellular Ca\textsuperscript{2+} release (Gryshchenko et al. 2016). Since then it has been shown that PSCs possess Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (Storck et al. 2017) and it is therefore likely that initial Ca\textsuperscript{2+} release from intracellular stores would activate such channels, promoting store-operated Ca\textsuperscript{2+} entry due to the more favourable electrochemical gradient provided by the hyperpolarized plasma membrane. Inhibition of excessive Ca\textsuperscript{2+} signal generation in PACs and PSCs by partial blockade of CRAC channels is a promising therapeutic avenue in many inflammatory diseases (Parekh, 2010; Di Capite et al. 2011) including AP (Gerasimenko et al. 2013, 2014; Wen et al. 2015). Our new data (Fig. 6C), showing that CRAC channel inhibition largely prevents the increased responsiveness of PSCs to trypsin that occurs in AP-like conditions, provides fresh evidence in favour of CRAC channel inhibition as a potentially attractive treatment for AP.

![Figure 8](Image)
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Additional information

Competing interests

The authors declare no competing interests.

Author contributions

All experiments were carried out in the School of Biosciences at Cardiff University. All authors jointly conceived the project. OG and JVG carried out the experiments with help from SP, and these were then also analysed by OVG. JVG created the figures and OHP wrote the paper with significant intellectual input from all the other authors. All authors have approved the final version of the manuscript.

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**Translational perspective**

Our new data indicate that the pancreatic stellate cells play an important role in acute pancreatitis. They are key amplifying elements in a process resulting in necrotic acinar cell death. Initial release of proteases–including trypsin and kallikrein–from a small proportion of dying acinar cells generates Ca\(^{2+}\) signals in the stellate cells which then, probably via formation of the diffusible gas nitric oxide in these cells, causes more acinar necrosis which, via release of proteases from the further damaged acinar cells, causes additional stellate cell stimulation, thereby generating a vicious circle. These findings have potential therapeutic implications, as they indicate that interventions that would break this vicious circle could be helpful in the treatment of acute pancreatitis, a disease for which there is currently no authorized rational therapy. The key elements in the vicious circle promoting acinar necrosis would appear to be the actions of bradykinin, generated by the action of kallikrein, and trypsin on specific stellate cell receptors. These actions cause rises in the cytosolic Ca\(^{2+}\) concentration in the stellate cells, which activate the Ca\(^{2+}\)-sensitive enzyme nitric oxide synthase, producing the very diffusible nitric oxide, which in this situation appears to be toxic for the acinar cells. There are clear pharmacological interventions that could prove effective. Bradykinin receptor antagonists, antagonists of protease-activated receptors and inhibitors of nitric oxide synthase could all be helpful. Given that excessive Ca\(^{2+}\) signal generation in both stellate and acinar cells are critical elements, our previous proposal of treating acute pancreatitis with inhibitors of store-operated Ca\(^{2+}\) entry via the so-called CRAC channels, which has received further support from our new results, remains valid. However, it may well turn out that combination therapy with Ca\(^{2+}\) channel inhibitors and, for example, bradykinin receptor antagonists, could be particularly helpful and would allow relatively low doses of these agents to be used, thereby minimizing potential side effects.