**Intracellular rupture, exocytosis and actin interaction of endocytic vacuoles in pancreatic acinar cells: initiating events in acute pancreatitis**

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**Abbreviations:** CCK, cholecystokinin-8; diSulfo – Cyanine 5 carboxylic acid, diS-Cy5; EV, endocytic vacuole; FITCD, Fluorescein isothiocyanate–dextran 40000 MW; TLC-S, taurolithocholic acid 3-sulfate; LY, Lucifer yellow; TRD, dextran Texas Red 3000 MW neutral.

**Key Points:**

● Giant trypsin-containing endocytic vacuoles are formed in pancreatic acinar cells stimulated with inducers of acute pancreatitis.

● F-actin envelops endocytic vacuoles and regulates their properties.

● Endocytic vacuoles can rupture and release their content into the cytosol of the acinar cells.

● Endocytic vacuoles can fuse with the plasma membrane of acinar cells and exocytose their content.

**Abstract**

Intrapancreatic activation of trypsinogen is an early event in and hallmark of the development of acute pancreatitis. Endocytic vacuoles, which form by disconnection and transport of large post-exocytic structures, are the only resolvable sites of the trypsin activity in live pancreatic acinar cells. In this study we characterised dynamics of endocytic vacuole formation induced by physiological and pathophysiological stimuli and visualised a prominent actin coat that completely or partially surrounded endocytic vacuoles. An inducer of acute pancreatitis taurolithocholic acid 3-sulfate (TLC-S) and supramaximal concentrations of cholecystokinin (CCK) triggered formation of giant (more than 2.5μM in diameter) endocytic vacuoles. We discovered and characterised intracellular rupture of endocytic vacuoles and fusion of endocytic vacuoles with basal and apical regions of the plasma membrane. Experiments with specific protease inhibitors suggest that the rupture of endocytic vacuoles is unlikely to be induced by trypsin or cathepsin B. Perivacuolar filamentous actin (observed on the surface of approximately 30% of endocytic vacuoles) may play a stabilising role by preventing rupture of the vacuoles and fusion of the vacuoles with the plasma membrane. The rupture and fusion of endocytic vacuoles allow trypsin to escape the confinement of a membrane-limited organelle, gain access to intracellular and extracellular targets and initiate autodigestion of the pancreas, a crucial pathophysiological event.

**Introduction**

Pancreatic acinar cells secrete digestive enzymes and precursors of digestive enzymes (zymogens) by stimulated exocytosis (reviewed in (Williams, 2008; Yule, 2010; Thorn & Gaisano, 2012)). In the acinar cells zymogens are packaged into large secretory granules (also termed zymogen granules). Exocytosis induced by physiologically-relevant secretagogues cholecystokinin and acetylcholine in this cell type is mediated by Ca2+ signalling cascades (Petersen & Tepikin, 2008; Williams, 2008; Thorn & Gaisano, 2012; Liang *et al.*, 2017). Ca2+-dependent secretion of zymogens involves compound exocytosis in which secretory granules undergo fusion not only with the plasma membrane but also with each other, forming large post-exocytic structures (Nemoto *et al.*, 2004; Sherwood *et al.*, 2007; Thorn & Gaisano, 2012), which can disconnect from the plasma membrane forming endocytic vacuoles (EVs). Under physiological conditions exocytosis of zymogen granules is limited to the apical region of the cell; however, under pathophysiological conditions basolateral exocytosis can also occur (Cosen-Binker *et al.*, 2008). Actin filaments play an important role in regulating exocytosis in pancreatic acinar cells (Muallem *et al.*, 1995; Valentijn *et al.*, 2000; Nemoto *et al.*, 2004; Larina *et al.*, 2007; Jang *et al.*, 2012). In particular, actin has been shown to interact with post-exocytic structures (Jang *et al.*, 2012). Following exocytosis, zymogens secreted by the acinar cells are transported via the system of pancreatic ducts into the duodenum. This transport relies on the fluid secretion by pancreatic acinar and pancreatic ductal cells (reviewed in (Hegyi & Rakonczay, 2015; Pallagi *et al.*, 2015)). Under physiological conditions activation of zymogens occurs specifically in the intestine. However, during acute pancreatitis, some of the zymogens become activated inside the pancreas itself rather than the intestine (e.g. (Leach *et al.*, 1991; Hofbauer *et al.*, 1998)) initiating pancreatic autodigestion. Trypsin is the key digestive enzyme involved in this process; intrapancreatic trypsinogen activation (formation of trypsin) is an early step in the development of acute pancreatitis observed in both the clinical setting and experimental models of this disease (Gudgeon *et al.*, 1990; Hofbauer *et al.*, 1998; Otani *et al.*, 1998; Halangk *et al.*, 2000; Van Acker *et al.*, 2007; Dawra *et al.*, 2011; Pallagi *et al.*, 2011; Sendler *et al.*, 2017). The mechanism of trypsinogen activation in the pancreas is debated; a considerable body of evidence suggests involvement of cathepsin B in this process (Saluja *et al.*, 1997; Halangk *et al.*, 2000; Lerch & Halangk, 2006; Sendler *et al.*, 2017). Trypsinogen activation in pancreatic acinar cells is an important contributor to damage and death of the acinar cells *in vitro* and damage of pancreatic tissue in *in vivo* models (Ji *et al.*, 2009; Kereszturi & Sahin-Toth, 2009; Mareninova *et al.*, 2009; Dawra *et al.*, 2011; Gaiser *et al.*, 2011). Intracellular activation of trypsinogen has been described (Leach *et al.*, 1991; Hofbauer *et al.*, 1998; Otani *et al.*, 1998; Kruger *et al.*, 2000; Raraty *et al.*, 2000); however, the nature of the initiating organelle(s) is still debated. Functioning Vacuolar ATPase (V-ATPase) and acidic intraorganellar milieu are required for zymogen activation (Lerch *et al.*, 1993; Waterford *et al.*, 2005; Kolodecik *et al.*, 2009). Vacuolisation is a hallmark of the acinar cell damage triggered by inducers of acute pancreatitis (e.g. (Watanabe *et al.*, 1984; Hofbauer *et al.*, 1998; Otani *et al.*, 1998; Sherwood *et al.*, 2007; Mareninova *et al.*, 2009; Kim *et al.*, 2011). Both endocytic and non-endocytic vacuoles have been described (Voronina *et al.*, 2007). In our previous study we observed trypsinogen activation in endocytic vacuoles (Sherwood *et al.*, 2007) and this stimulated us to further investigate these cellular structures.

The initial aim of our study was to characterise the dynamics of the formation of endocytic vacuoles in physiological and pathological conditions. However, early in the project we serendipitously discovered three new phenomena: rupture, exocytosis and actination of the endocytic vacuoles. The project was consequently refocused on characterising these novel cellular events.

**Methods**

*Ethical approval and laboratory animals.*

Pancreata were obtained from adult (6–8 weeks old) male CD1mice (Charles River). The animals were humanely sacrificed by cervical dislocation (schedule 1 procedure) in accordance with the Animals (Scientific Procedures) Act (1986) under Establishment Licence 40/2408 and with approval by the University of Liverpool Animal Welfare Committee. Prior to experiments mice had *ad libitum* access to food and water.

*Chemicals*

Lucifer yellow (LY) and BZiPAR (fluorogenic probe for trypsin activity (Kruger *et al.*, 1998; Kruger *et al.*, 2000; Raraty *et al.*, 2000; Sherwood *et al.*, 2007)) were from ThermoFischer Scientific. Fluorescein isothiocyanate–dextran MW 40000 (FITCD) was from Sigma-Aldrich. Dextran Texas Red 3000 MW neutral (TRD) and other fluorescently- labelled dextrans were from ThermoFischer Scientific. Collagenase was from Worthington Biochemical (Lorne Laboratories). Thapsigargin was from Calbiochem. SiR-Actin kit was from Spirochrome. CA-074 and bafilomycin A1 were from Tocris Bioscience and CA-074Me was from Calbiochem. diSulfo - Cyanine5 carboxylic diS-Cy5acid (diS-Cy5) was from Cyandye LLC. CBQCA Atto-tag was from Setarech Biotech, and the rest of the chemicals (including DMSO, Benzamidine, CCK and TLC-S) were from Sigma-Aldrich.

*Solutions*

A standard extracellular solution used for cell isolation and measurements of vacuole formation contained: NaCl (140 mM); KCl (4.7 mM); MgCl2 (1.13 mM); CaCl2 (1 mM); D-glucose (10 mM); HEPES (10 mM). The solution was adjusted to pH 7.3 using NaOH. CCK and TLC-S were added to the standard extracellular solution to attain the concentrations specified in the description of individual experiments.

*Cell preparation, labelling and imaging*

The pancreatic acinar cells were isolated by digestion with purified collagenase (200 units/ml) as described previously (Chvanov *et al.*, 2015). Freshly isolated pancreatic acinar cells were dispersed in standard extracellular solution, plated on to poly-L-lysine-coated glass-bottomed Petri dishes from MatTek and maintained at 35°C. Extracellular solution was then removed and replaced with the incubation solution containing fluorescent probe(s) (e.g. LY or TRD) plus test compound (e.g. CCK) as specified in the description of individual experiments. In some experiments we utilised small pancreatic sections (approximately 1mm in liner dimensions), which were produced by cutting pancreas by surgical scalpel blade. The sections were then handled and labelled by the same procedures as the isolated cells. Only surface cells of the sections were imaged and analysed.

Images of cells and organelles were obtained by using a confocal laser scanning microscope Leica TCS SL or a Leica TCS SP2 (Leica Microsystems); axial resolution in our experiments was approximately 1 μm. Fluorescence of LY was excited using a 458 nm laser line; the emission was recorded at wavelengths 481-572 nm. Fluorescence of diS-Cy5 was excited using a 633 nm laser line; the emission was recorded at wavelengths 650-700 nm. Fluorescence of Texas Red – labelled dextrans (TRDs) was excited using 543 nm laser line; the emission was recorded at wavelengths 595-703 nm. Fluorescence of Tetramethylrhodamine-labelled dextrans (TMRDs) was excited using a 543 nm laser line; the emission was recorded at wavelengths 560-680nm. Fluorescence of Fluorescein isothiocyanate–dextran (FITCD) was excited using a 488 nm laser line; the emission was recorded at wavelengths 505-530 nm. In our experiments the fluorescently-labelled dextrans were used at concentrations that correspond to approximately 100 μM of the labelled fluorescent molecules (the number of fluorescent molecules associated with each molecule of dextran is different for dextrans of different molecular weight and can also vary between the batches of the labelled dextrans).

The following procedure was used for labelling trypsinogen: 100 mg of bovine trypsinogen and 8.2 mg of N-acetyl-cysteine (NAC) were dissolved in 5 ml of aqueous solution containing 2 mM of HEPES (pH 11.0 adjusted with NaOH). 10 mg CBQCA Atto-Tag was dissolved in the mixture containing 180 μl DMSO and 120 μl ethanol and slowly added dropwise to the stirred trypsinogen-NAC mixture. After 15 minutes the pH of the solution was adjusted to 7.0 with HCl and each 2.5 ml of the reaction mixture were eluted from pre-equilibrated PD-10 desalting column (Amersham Biosciences) by 3.5 ml of normal extracellular buffer, according to the manufacturer’s instructions. This solution was ready to be used in experiments with live cells (or could be frozen for storage). Fluorescence of CBQCA Atto-trypsinogen was excited using a 488 nm laser line; the emission was recorded at wavelengths 580-630 nm.

In experiments testing the accumulation of fluorescent probes with different molecular weights in endocytic vacuoles (EVs), cells were maintained in suspension containing the combinations of probes (LY and a fluorescently-labelled dextran, or diS-Cy5 and a fluorescently-labelled trypsinogen) plus CCK (10 pM or 10 nM) for 30 minutes at 35 oC. Cells were then placed in glass-bottomed Petri dish from MatTek, allowed to adhere, and then washed 3 times with standard extracellular solution to remove CCK and endocytic tracers. The cells-containing MatTek dish was then moved to the stage of an inverted confocal microscope for imaging. During the imaging cells were maintained in extracellular solution at 35oC.

In experiments involving simultaneous imaging of endocytic vacuoles and trypsin activity the cells were immersed in a solution containing 100 μM of dextran Texas Red 3000 MW neutral and100 μM BZiPAR. BZiPAR is a fluorogenic probe for trypsin activity; upon cleavage by trypsin it is first converted to fluorescent monoamine which is further cleaved by trypsin and releases a fluorescent molecule R110 (Leytus *et al.*, 1983) (see also <https://tools.thermofisher.com/content/sfs/manuals/mp06501.pdf>). Fluorescence of BZiPAR products was excited using a 488 nm laser line; the emission was recorded at wavelengths 500-530 nm.

To quantify the number of cells with ruptured vacuoles using increased cytosolic fluorescence the cells were incubated in the presence 100 μM of diS-Cy5 (and, in some cases, 100 μM of Texas Red dextran) for 2 hours at 35 oC. Specified agonists and/or inhibitors were added to the incubation media. The cells were then washed by perfusion to remove the fluorescent probes from the extracellular solution. After washing, fluorescent images of the cells were acquired and analysed by drawing the region of interest that included the cytosol of each individual cell, but excluded all stained vacuoles and plasma membrane (i.e. cell boundary resolvable on images). Next, the data of cytosolic fluorescence collected using this procedure were pooled together from all experimental groups (typically comprising 100-300 data points) to obtain a frequency distribution plot (example is shown in Fig. 3C). This frequency histogram was then approximated by Gaussian best fit (multi-peak). The first peak (the one closer to zero) was interpreted as the one representing the cells, in which there was no increase of cytosolic fluorescence due to EV rupture. After this the threshold of central value (corresponding to the maximal cell number) plus three half-width (three-sigma) of the first peak was calculated, and the cells whose cytosolic fluorescence was above the threshold were deemed as having EV rupture leading to cytosolic rise of fluorescence. The proportion of cells with increased cytosolic fluorescence was calculated for each specified experimental condition and compared to the appropriate control. If the first two peaks of the approximation did not separate (the value corresponding to the maximal amplitude plus the half-width of the first peak were more than the central value for the second peak), then such an experiment was excluded from further analysis.

To image F-actin distribution cells were infected with adenovirus containing LifeAct construct by adding adenovirus (107 pfu/ml) into a dish containing acinar cells and incubating for 10-12 hours at 35°C. Before performing experiments the adenovirus was washed off with extracellular solution. Cell expressing LifeAct were imaged on Zeiss LSM 710 at 35°C using excitation laser line 543 nm, emission was collected at 560-600 nm. Another approach for F-actin labelling involved SiR-actin. To reveal F-actin distribution using this probe cells were incubated in solution containing 1 μM SiR-actin and 10 μM Verapamil for 1 hour at 34.5°C prior to stimulation with supramaximal CCK. In these experiments cells were fixed after CCK stimulation by incubation with 1.8% PFA for 48 hours at +4°C, washed by PBS and imaged on Leica TCS SL using excitation laser line 633 nm, emission was collected at 650-795 nm.

 Results are reported as means ± SEM. Statistical comparisons were performed using the Student’s t-test or ANOVA followed by Dunnet’s test. Statistical significance was assumed for P<0.05.

**Results**

*Labelling, formation and sizes of endocytic vacuoles*

Endocytic vacuoles (EVs) are rapidly formed intracellular structures that can be labelled with membrane impermeant fluorescent dyes, including fluorescently labelled dextrans (Fig. 1). The numbers of vacuoles labelled by small molecules Lucifer yellow (LY) and diSulfo - Cyanine5 carboxylic acid (diS-Cy5) were similar to that labelled by fluorescent dextrans with molecular weights of 3kD and 10kD (Fig. 1A). Dextrans with molecular weights of 40kD and 70kD were partially excluded from the EVs (Fig. 1A) suggesting a relatively small diameter of the fusion pore. Similar partial exclusion was observed for the fluorescently labelled trypsinogen (Fig. 1A). Fluorescent probes LY, diS-Cy5 and Texas Red-labelled dextran with molecular weight 3kD (abbreviated as TRD) produced similar labelling of EVs and were used for detecting and investigating the properties of EVs in this study.

Supramaximal concentrations of CCK (500 pM and 10 nM) and toxic bile acid TLC-S (500 μM) induced vigorous vacuolisation (Fig. 1B). The number and, importantly, the size of the EVs induced by supramaximal CCK or TLC-S was substantially larger than that induced by the physiological concentration of CCK (10 pM) or observed in unstimulated cells (Fig. 1 B and C). Notably, EVs larger than 2.5 μm were almost exclusively observed in cells treated with supramaximal CCK or TLC-S (Fig. 1C).

*Rupture and actin interaction of endocytic vacuoles*

The large size of the EVs, formed as a result of pathologically relevant stimulation, allowed us to reveal the rupture of these organelles. Upon cell stimulation with supramaximal doses of CCK, some of the EVs ruptured inside the cells so that the probe escaped from the EVs into the cytosol and increased cytosolic fluorescence (Fig. 1D, n=30). The observation time necessary to resolve one rupture event was approximately 8 hours (approximately 480 minutes). To resolve 30 ruptures we imaged/analysed 220 vacuole-containing cells. The loss of the indicator from an EV could be either complete (Fig. 1D, n=17) or partial (n=13, not shown). In the majority of experiments, EV rupture was followed by blebbing of the plasma membrane (Fig. 1D) and cell death, suggesting that EV rupture is cytotoxic. The cell death occurred 27±4 min after the rupture (n= 30). In these experiments we have studied vacuoles with diameter more than 2.5 μm. The large size of the EVs undergoing rupture is essential for the positive identification of such events. The increase of cytosolic fluorescence, detectable during rupture of a large EV, strongly indicates classification of the event as a rupture. In this respect the large size of EVs formed by stimulation with supramaximal CCK or TLC-S is particularly important.

Endocytic vacuoles are the sites of trypsinogen activation in pancreatic acinar cells. Using combined application of TRD (to detect endocytic vacuoles) and BZiPAR (a fluorogenic probe for trypsin activity) we observed trypsinogen activation occurring prior to the rupture of an EV (Fig. 2A, n= 13). Notably we have observed vacuole formation and rupture in CCK-stimulated cells incubated with the trypsin inhibitor benzamidine (Fig. 2B, n=13). Importantly, 1mM benzamidine used in these experiments, eliminates resolvable trypsinogen activation in pancreatic acinar cells (Sherwood *et al.*, 2007). These experiments suggest that trypsin is not critically important for the rupture of EVs.

Similar results were obtained in experiments with the cathepsin B inhibitor CA074, which was designed by Towatari and colleagues to specifically inhibit this protease (Towatari *et al.*, 1991). In these experiments combination of CA074 (10 µM) with a more cell-permeable methyl ester form CA074-Me (1 µM) was applied at least 30min before the experiment and maintained in the extracellular solution for the duration of the experiment. This combination of CA074 and CA074-Me was abbreviated as CA074/Me in the further sections of this paper. In the cells stimulated by 10 nM CCK, CA074/Me did not prevent rupture of EVs (not shown, n=8), suggesting that cathepsin B is unlikely to be responsible for the rupture.

 Considerable confocal observation time (approximately 8 hours, comprising of 2-3 confocal sessions) was required to resolve a single rupture of an endocytic vacuole. These direct imaging experiments provided clear demonstration of the phenomenon but they were challenging, time consuming and not conducive to the study of the processes modulating the ruptures. We therefore developed and utilised a complementary technical approach to study possible roles of trypsin and cathepsin B in the rupture of the vacuoles. This approach relies on the fact that a rupture of an endocytic vacuole containing fluorescent probe results is an increase of cytosolic fluorescence (e.g. Fig. 1D). We have indeed observed a proportion of cells which had an intact plasma membrane but slightly increased cytosolic fluorescence with the spectral properties corresponding to those of the probes utilised in our experiments to study endocytic vacuoles (diS-Cy5 and/or TRD, see Fig. 3A and B). Importantly, the proportion of such cells increased upon CCK stimulation (Fig. 3C and D). Based on these observations, we inferred that the increase of cytosolic fluorescence is due to the rupture or leakage of endocytic vacuoles. In these experiments neither inhibition of trypsin (with 1mM of benzamidine) nor inhibition of cathepsin B (with CA-074/Me) produced statistically significant changes in the proportion of cells with the increased cytosolic fluorescence of diS-Cy5 (Fig. 3D). Inhibition of V-ATPase is another mechanism for supressing intracellular/intraorganellar zymogen activation (Waterford *et al.*, 2005; Kolodecik *et al.*, 2009), including trypsinogen activation in EVs (Sherwood *et al.*, 2007). In our experiments 100nM of bafilomycin A1, applied 30 minutes before CCK stimulation and maintained in the extracellular solution for the duration of the experiment, had no resolvable effect on the proportion of CCK-stimulated cells with the increased cytosolic fluorescence of diS-Cy5 (Fig.3D). Taken together these observations support the notion that trypsin and cathepsin B are unlikely to be involved in rupturing endocytic vacuoles.

Appearance of cytosolic diS-Cy5 fluorescence in CCK-stimulated cells with intact plasma membrane was also observed in experiments utilising small pancreatic tissue sections (Fig.4), which have not been subjected to collagenase treatment. These experiments indicate that the described phenomenon is not limited to enzymatically-isolated acinar cells or small acinar cell clusters.

 We observed that whilst some EVs are fragile and undergo rupture others are robust and can retain fluorescent probe for many hours. This apparent heterogeneity of the vacuoles suggested that the acinar cells contain a stabilising factor protecting some but not all vacuoles and that the loss of such a factor could be the mechanism behind the vacuole fragility and rupture. F-actin is an obvious candidate for this role, particularly considering the prominent role of F-actin in compound exocytosis of zymogen granules (Larina *et al.*, 2007).

We have expressed LifeAct (marker of F-actin) in pancreatic acinar cells and discovered an actin coat on EVs. In cells stimulated by 10nM CCK approximately 28% of EVs were actin coated (n=89; see Fig. 5). In approximately one third of these vacuoles the actin coat was incomplete or fenestrated (Fig. 5). Importantly a significant proportion of EVs had no actin coat (72%, n=89; see Fig. 4). Similar observations were made in cells stained with SiR-actin (a membrane permeant fluorogenic probe for actin (Lukinavicius *et al.*, 2014)). SiR-actin staining revealed both actin coated EVs (37% of EVs, n=119; Fig. 6A, upper row of images) and uncoated EVs (63% of EVs, n=119; Fig. 6A, lower row of images). Whilst both actin staining methods revealed similar phenomena the proportion of uncoated EVs was somewhat smaller for SiR-actin. Similarly to the staining with LifeAct, SiR-actin staining revealed non-uniformities and fenestrations of the actin coat on endocytic vacuoles (Fig. 6B). We next investigated the putative role of actin in the rupture (or rupture prevention) of endocytic vacuoles by using actin-modifying compounds Latrunculin-B and Jasplakinolide.

Latrunculin-B (10μM) application had no resolvable effect on the CCK-induced increase in the proportion of the cells with elevated diS-Cy5 fluorescence (not shown, n=12 cell preparations in both control group and in Latrunculin-B treated group).

At relatively low concentration (1μM) used in our experiments Jasplakinolide did not change the number of endocytic vacuoles per cell (not shown, n= 112 cells in control group and n=103 cells in Jasplakinolide- treated group). There was no statistically significant difference in the size of the vacuoles between control (1.81±0.07μm, n= 223 vacuoles) and Jasplakinolide-treated (1.89±0.07μm, n=239 vacuoles) groups (p=0.41). However, incubation with 1μM Jasplakinolide induced a moderate but statistically significant (p = 0.03) decrease in the proportion of cells with elevated cytosolic diS-Cy5 fluorescence (Fig.6C). Jasplakinolide is an actin-stabilising drug (Bubb *et al.*, 1994) and our finding suggests that the actin surrounding endocytic vacuoles may play a role in reinforcing these organelles and preventing their rupture or leakage. This notion was supported by the recording of a rare phenomenon - the vacuole rupturing through the narrow fenestration in the actin coat (Fig. 7 upper vacuole).

*Exocytosis of endocytic vacuoles*

Whilst studying the rupture of endocytic vacuoles we serendipitously discovered another mechanism allowing trypsin to escape from the confinement of an EV; this mechanism involves exocytosis of EVs. In order to systematically investigate the ability of EVs to undergo fusion with the plasma membrane we utilised sequential incubation of the cells in two cell-impermeable dyes: LY and TRD. The rationale of these experiments was that the exchange of fluorescent molecules between the EVs and the extracellular solution should definitively identify the fusion events. In these experiments cells formed LY-filled EVs during 30 minute incubation with LY in the presence of supramaximal CCK. The cells were then washed and again stimulated by supramaximal CCK in the presence of TRD. A number of new EVs filled with TRD were formed (Fig. 8A). Importantly, some of the LY-filled EVs, found in basolateral regions of the cell, were able to exchange the fluorescent probes with the extracellular solution (manifested by the loss of LY and the uptake of TRD), confirming fusion with the basolateral part of the plasma membrane (Fig. 8A, n=11). Like in the case of recording ruptures of EVs, the direct imaging of fusion of EVs was time consuming. Approximately 15.3 hours (920 minutes) of confocal observations time was required to resolve a single fusion event. In addition to the basolateral fusion events some of the LY-filled EVs fused with TRD-filled post-exocytic structures at the apical part of the cells indicating that EVs can also undergo secondary exocytosis in this region (Fig. 8B, n= 7).

**Discussion**

Pancreatic acinar cells utilise compound exocytosis to secrete zymogens. Large EVs form in pancreatic acinar cells as a result of aberrant compound endocytosis of secretory (zymogen) granules (Sherwood *et al.*, 2007; Voronina *et al.*, 2015). Comparing the size of EVs with that of secretory granules we can conclude that tens and even hundreds of secretory granules have to first form a post-exocytic Ω-shaped structure and subsequently this structure has to disconnect from the plasma membrane to form the large EVs (up to 12μm in diameter) observed in the acinar cells stimulated with TLC-S or supramaximal CCK. It is likely that trypsinogen activation observed in endocytic vacuoles occurs because not all zymogen is released from the post-exocytic structures through the fusion pore (which is known to be restricted and rather short-lived (Larina *et al.*, 2007)).

The EVs containing potentially damaging digestive enzymes, notably trypsin, should be harmless until the enzymes escape into the cytosol or extracellular solution. The rupture and fusion of the EVs, described in this study, delineate the escape routes that are likely to initiate autodigestion of the organ.

Rupture of a large EV is a catastrophic event for the cell, usually resulting in cell death within 30 minutes. The large size of EVs, formed by the inducers of acute pancreatitis, allowed us to definitively resolve ruptures of these organelles, but experiments aimed at imaging ruptures of the EVs were time consuming and the majority of confocal imaging sessions ended without resolving a single EV rupture. However, even the substantial experimental time necessary to resolve a single rupture (8 hours) is still shorter than the time required for inducing acute pancreatitis in the cerulein model (12 hours or 24 hours; reviewed (Lerch & Gorelick, 2013)). In our experiments on isolated acinar cells and cell clusters we found that approximately 40-50% of cells will have at least one large vacuole (of the size that we used to image ruptures, see Fig. 1C). Considering that the frequency of vacuolar ruptures is approximately one rupture per 8 hours, we can assume that approximately 20-30% of cells will experience a catastrophic rupture of an endocytic vacuole during the time necessary to induce acute pancreatitis in an animal model. Assuming similar rates of cellular vacuolisation and vacuolar rupture in the pancreata of mice undergoing induction of acute pancreatitis, one could infer that a significant proportion of the acinar cells of the glands (perhaps 20-30% of cells) may be damaged by the processes initiated by vacuolar rupture.

The experiments utilising a complimentary approach for detecting the escape of the content from the vacuoles into the cytosol (relying on the distribution of cytosolic fluorescence intensities, as shown on the Fig. 3) also suggests that a substantial proportion of cells experienced ruptures or leakage of endocytic vacuoles following supramaximal CCK stimulation. The increases of cytosolic fluorescence recorded in these experiments are usually very small and comparable to autofluorescence of the cells in the red part of the spectrum. This technical approach does not reveal the identity of the ruptured vacuoles (e.g. some of the vacuoles could be small and incapable of inducing cell death by releasing vacuolar content), but the high proportion of affected cells (approximately 30%) further highlights the importance of this phenomenon.

 The results of this study suggest that intravacuolar trypsinogen activation and cathepsin B activity are not essential for the rupture of EVs. So what is the mechanism rupturing these organelles? In this and a previous study (Sherwood *et al.*, 2007) we have observed long-distance movement of EVs and considerable changes of their shapes. A clear example of the change in the shape of a vacuole before rupture is shown in Fig. 7 (lower vacuole). These movements and shape changes probably reflect interactions of EVs vacuoles with cytoskeletal elements and/or cellular organelles in the very dynamic and crowded interior of acinar cells. We consider that forces produced by such interactions could be responsible for rupturing EVs. In this study we discovered and characterised F-actin coating of EVs. Such actination probably serves to protect EVs from rupture and consequently the loss of actin coat could make the large EVs fragile and unstable. The dynamics of F-actin on the endocytic vacuoles is now the subject of a separate investigation in our laboratory.

The observation time necessary to resolve a fusion event between an EV and the basolateral plasma membrane (15.3 hours of confocal imaging) is substantial but similar to the induction time in some models of acute pancreatitis (reviewed in (Lerch & Gorelick, 2013)). It is therefore likely that one or more fusion events will occur during the induction time. The fusion of trypsin-containing vacuoles with the basolateral membrane probably occurs because of the documented basolateral appearance of proteins involved in exocytosis in this cell type under pathological conditions (e.g. (Dolai *et al.*, 2012; Dolai *et al.*, 2017)). Formation of endocytic vacuoles depends on exocytosis of zymogen granules (Sherwood *et al.*, 2007). Actin plays an important role in exocytosis of zymogen granules in pancreatic acinar cells. Indeed stabilisation of actin by phalloidin or jasplakinolide inhibits this process (Muallem *et al.*, 1995; Valentijn *et al.*, 2000). It has been clearly documented that in pancreatic acinar cells zymogen granules are coated by actin (Valentijn *et al.*, 2000; Nemoto *et al.*, 2004; Jang *et al.*, 2012) but only after they fuse with the plasma membrane (Jang *et al.*, 2012). This suggests that normal exocytosis of secretory granules occurs when granules do not have an actin coat that could prevent the contact between the granule and the plasma membrane. It is likely that the same applies to exocytosis of EVs which are derived from the secretory granules. The actin coat should therefore prevent both rupture and fusion of EVs.

The fusion of EVs with the apical part of the plasma membrane presumably utilises the same mechanism as used by secretory granules (reviewed in (Thorn & Gaisano, 2012)). In this case trypsin and other digestive enzymes could be delivered directly into the lumen of pancreatic ducts. The degree of the damage to the ducts and the organ as a whole in this scenario is likely to depend on the efficiency of fluid and bicarbonate secretion (Pallagi *et al.*, 2011; Pallagi *et al.*, 2015). Notably trypsin reduces ductal bicarbonate secretion (Pallagi *et al.*, 2011). The effect of trypsin secreted from EVs could be further amplified by other cells of the acinus delivering zymogens by ‘normal’ release from secretory granules to the same duct and subsequent activation these zymogens by trypsin released from EVs.

The current study describes an important step in the pathway initiated by inducers of acute pancreatitis. It follows aberrant Ca2+-dependent compound endocytosis and formation of large EVs. It involves ruptures of EVs and their fusion with the plasma membrane; processes that mediate the release of trypsin into intracellular and extracellular milieu.

**Competing interests**: The authors declare that they have no conflicts of interest with the contents of this article.

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All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

**Authors' Translational Perspective**

Our study shows that the endocytic vacuoles, formed in pancreatic acinar cells in pathological settings, may rupture and precipitate the acinar cell death. Endocytic vacuoles contain zymogens, including trypsinogen that can undergo activation inside these organelles. Although this activation does not affect the rupture process itself, it may be critical for the downstream events and the cell fate. On the other hand, the aberrant endocytosis followed by the rupture of endocytic vacuoles delineates a potentially novel route for the delivery of membrane impermeant molecules to the cytosol of the acinar cell. It is conceivable that in the future this pathway could be utilised to deliver pharmacologically relevant compounds aiming to minimise or prevent acinar cell damage. Notably, such membrane-impermeant compounds will reach the cytosolic targets only in vulnerable acinar cells that have experienced the sequence of aberrant endocytosis and rupture of the endocytic vacuoles. Our study also highlights the possibility of the re-uptake of zymogens (and potentially active proteases) into endocytic vacuoles from the acinar lumen. This is a novel route for the delivery of damaging enzymes into endocytic structures and later to the cellular cytosol. The existence of this route further highlights the importance of an efficient acinar and ductal fluid secretion as an endogenous mechanism for the prevention of acute pancreatitis and emphasises the potential utility of treatment development aiming to normalise or stimulate fluid secretion.

**Figure Legends**

**Figure 1. Labelling, formation and rupture of endocytic vacuoles.**

**A**. The upper panels illustrate the ability of molecules with different molecular weight to enter and label endocytic vacuoles (EVs) in cells stimulated by physiological (left panel) and supramaximal (right panel) CCK concentrations. The EVs were revealed by accumulation of low molecular weight probe Lucifer Yellow (LY). In these experiments we also used fluorescently-labelled dextrans with different molecular weights: dextran Texas Red 3000 MW neutral (TRD 3kD; this smallest of the labelled dextrans is abbreviated to TRD in the rest of the manuscript, here we specified its molecular weight in abbreviation to distinguish it from the larger fluorescently-labelled dextrans), dextran tetramethylrhodamine 10000 MW neutral (TMRD 10kD), dextran Texas Red 40000 MW neutral (TRD 40kD) and dextran tetramethylrhodamine 70000 MW neutral (TMRD 70kD). The graphs show the ratios of the number of vacuoles containing labelled dextrans to the total number of vacuoles revealed by LY; \* indicates statistically significant difference from the total number of the vacuoles (P<0.05). The lower panels show the ratios of the number of EVs containing labelled bovine trypsinogen (CBQCA Atto-trypsinogen) to the total number of vacuoles (revealed by diS-Cy5) in cells stimulated with 10pM CCK and 10nM CCK. The proportion of vacuoles stained with CBQCA Atto-trypsinogen was significantly higher in cells stimulated with supramaximal CCK.

**B**. Time course of endocytic vacuole (EV) formation in unstimulated cells and cells stimulated with CCK or TLC-S; \* indicate statistically significant difference (P<0.05) from control (unstimulated cells). **C**. The distribution of EV diameters in control (unstimulated) cells and cells stimulated for 30 min with CCK or TLC-S. The mean diameter ± SEM was: 1.47 ± 0.04 μm (n= 190 vacuoles) in control conditions (unstimulated cells); 1.50 ± 0.03 μm (n= 384 vacuoles) for CCK 10pM; 1.68 ± 0.04 μm (n= 264 vacuoles) for CCK 500pM; 1.79 ± 0.04 μm (n= 403 vacuoles) for CCK 10nM; 1.62 ± 0.05 μm (n=297 vacuoles) for TLCS 500 μM. The vacuoles induced by supramaximal CCK concentrations and TLC-S were larger than the vacuoles induced by the physiological concentration of CCK (P<0.05). **D**. Rupture of an EV membrane and the loss of its content into cytosol in a cell stimulated by 10 nM CCK. The rupturing vacuole is indicated by the white arrow. Blue arrowheads indicate cell membrane blebs. The inset on the right shows the EV undergoing rupture in an expanded scale and the traces of fluorescence recorded from the EV and the cytosol (the regions of interest are shown by dashed lines), black arrows on the graph indicate the time points and fluorescence intensities corresponding to the inset images. Scale bar corresponds to 10 µm.

**Figure 2. Activation of trypsinogen and rupture of an endocytic vacuole.**

**A**. The cells were stimulated with 10 nM CCK. Endocytic vacuoles (EVs) were revealed by accumulated dextran Texas Red 3000 MW neutral (TRD). Left panels show the cluster of acinar cells (transmitted light and TRD fluorescence). The endocytic vacuole containing TRD is shown by a white arrow. Scale bars correspond to 10 µm. The boundary of the region displayed in the right gallery of images is shown by the white dashed line. Trypsinogen activation (i.e. increase of fluorescence as a result of BZiPAR cleavage) is shown in green. Note the trypsinogen activation in the endocytic vacuole that started after the vacuole was formed. The intravacuolar increase of BZiPAR - mediated fluorescence was usually transient; the decline of fluorescence started before the rupture of the vacuole (i.e. before the loss of TRD). A possible reason for this is that the final product of BZiPAR cleavage R110 is a membrane permeant probe and therefore poorly retained in cellular compartments. Changes of fluorescence recorded in the region of interest containing the vacuole are shown in the graph. At the end of the experiment we observed an increase of TRD fluorescence in the cytosol of the cell, signifying the loss of the plasma membrane integrity i.e. cell death. The right column of images illustrates the beginning of this process. **B.** Lower left image shows a doublet of pancreatic acinar cells in transmitted light. Scale bar corresponds to 10 µm. EVs were revealed by accumulated TRD. In this type of experiment benzamidine (1mM) was applied at least 30min before the start of experiment and maintained in the extracellular solution for the duration of the experiment. Cells were stimulated by 10 nM CCK. The rupturing EV is indicated by the white arrow. The insert on the lower left panel shows the EV undergoing rupture on an expanded scale. The traces of fluorescence shown on the right panel were recorded from the EV (red) and the cytosol (black); the specific regions of interest are shown by dashed lines on the lower left panel. The numbers on the traces (1-3) correspond to the numbers on the images and define the time points at which the images and corresponding fluorescence intensities were recorded.

**Figure 3. Visualising the appearance of membrane-impermeant fluorescence probes in the cellular cytosol.**

**A.** Cells were stimulated by 10nM CCK for 2h at 35oC in the presence of di-sulfo Cy5 (diS-Cy5) and dextran Texas Red 3000 MW neutral (TRD). Cells were then washed by perfusion with standard extracellular solution. Following the wash cells were immersed in the extracellular solution containing FITC-dextran 40000 MW (FITCD) and imaged. Upper left panel shows transmitted light image of the cells. Scale bar corresponds to 10 μm. Images of the fluorescence of the cells and extracellular milieu recorded using excitation/emission corresponding to the specified probes are shown on other panels of this part of the figure. Note the endocytic vacuole (EV) in the right cell and increased cytosolic fluorescence on diS-Cy5 and TRD images of the left cell. FITCD image indicates that the plasma membrane of the cell is intact. **B**. Representative fluorescence emission spectra recorded from cells stimulated as in A but in the presence of diS-Cy5 only. The fluorescence was excited by a 595nm laser line. The graphs show fluorescence spectra recorded from an endocytic vacuole (EV, green), cytosol of a cell that satisfies criteria for detecting the EV’s rupture / leakage (red, see Methods and part C), cytosol of a cell that did not satisfy criteria for detecting rupture / leakage (black, see Methods and part C). Blue trace in the insert is produced by subtracting black trace (mainly determined by cellular autofluorescence) from the cytosolic fluorescence of a cell that satisfies the criteria for detecting rupture/leakage. The residual trace shows a spectrum that is similar to the spectrum of diS-Cy5 recorded from an EV. The red and black traces were recorded from different cells and shown on the same graph for illustration purposes. **C**. Left panel shows a frequency histogram representing intensities of cytosolic fluorescence in cells that were immersed in the indicator-containing diS-Cy5 solution for 30 minutes but were not stimulated. Only a very few small vacuoles are expected to form during this period of time (see Fig.1B and C) and the distribution should therefore reflect cytosolic fluorescence in the cells that did not have ruptured EVs. The blue trace represents a single Gaussian approximation of the distribution. Right panel shows the frequency histogram of cells after two hours of incubation with diS-Cy5. CCK concentration was 10nM. First two Gaussian peaks of the approximation are shown by blue and magenta lines. Cells with cytosolic fluorescence above threshold (central value of the first peak plus 3 sigma) are classified as the cells that experienced rupture / leakage of endocytic vacuole(s). **D**. The method illustrated in parts A-C of this figure was used to evaluate the proportions of cells with ruptured vacuoles. CCK concentration was 10 nM (in specified experiments). Neither inhibition of serine protease with benzamidine (1mM) nor inhibition of cathepsin B with combination of CA074 (10μM) and CA074-Me (1 μM) (abbreviated as CA074/Me) produced a significant difference in the proportion of cells with increased cytosolic fluorescence from control. Inhibition of V-ATPase with 100nM of bafilomycin A1 (Baf on the figure) also did not produce a statistically significant change in the proportion of cells with increased cytosolic fluorescence. The number of experiments in each condition was: n=20 experiments for control (unstimulated cells) and CCK; n= 9 for CA074/Me and CA074/Me +CCK; n=8 for benzamidine and benzamidine + CCK; n=6 for bafilomycin A1 and bafilomycin A1+ CCK. Each of individual experiments involved acquisition and analysis of a fluorescence distribution similar to that shown on the right panel of part C.

**Figure 4. Cytosolic presence of membrane-impermeant fluorescence probe in the cell located in undissociated pancreatic fragment.**

Small (approximately 1mm) section of mouse pancreas was stimulated by 100nM CCK for 2 hours at 35oC in the presence of di-sulfo Cy5 (diS-Cy5, shown in magenta), washed and imaged in the presence of FITC-dextran 40000 MW (FITCD, shown in green). The lower gallery of images depicts the fragment containing two cells within the section, one with a large intact endocytic vacuole (EV, white arrow) and the adjacent cell with increased cytosolic fluorescence of diS-Cy5. The FITCD image indicates that the plasma membrane of this cell is intact, suggesting that the increase of the cytosolic fluorescence occurred as a result of an EV’s rupture. The figure is representative of 6 similar experiments.

**Figure 5. LifeAct staining reveals an association of F-actin with endocytic vacuoles.**

Pancreatic acinar cells were stimulated by CCK 10nM for 30 minutes. Scale bar corresponds to 10 µm.

**A**. Transmitted light images of pancreatic acinar cells are shown in the 1st column. The actin distribution was revealed by LifeAct expressed in the live acinar cells (2nd column) and correlated with the fluorescence of endocytosed diS-Cy5 (3rd column). Merged images are shown in 4th column. The 5th column shows individual vacuoles on an expanded scale. The upper row of images illustrates complete coats of F-actin on endocytic vacuoles (EVs). The central row shows one large vacuole with asymmetric complete F-actin coat and a number of smaller uncoated vacuoles with little or no associated actin. The bottom row of images shows EVs with incomplete F-actin coat and uncoated EVs with little or no associated F-actin. The traces on the panels show examples of fluorescence intensity profiles reflecting relative localisation of actin (LifeAct staining, black traces) and endocytosed diS-Cy5 (green traces). The fluorescence intensities were measured along the arrows indicated on the images.

**Figure 6**. **Interaction of F-actin with endocytic vacuoles and the effect of Jasplakinolide.**

 **A.** The actin distribution was revealed by SirActin (2nd column) and correlated with endocytosed probe (3rd column) in fixed pancreatic acinar cells. Merged images are shown in the 4th column. The 5th column shows individual vacuoles on an expanded scale. The upper row of images shows an EV coated with F-actin. The lower row of images shows an EV with no associated F-actin. Scale bars correspond to 10 µm

**B**. Illustrates apparent non-uniformities and fenestrations of the actin coat. The sequence of images is similar to that described in part A. Scale bars correspond to 10 µm. The fifth column illustrates actin coat and a merged image on an expanded scale. The yellow arrow shows an apparent fenestration of the actin coat whilst the white arrow indicates an increased density.

**C**. Treatment of the acinar cells with 1μM Jasplakinolide (Jasp) induced a significant (P= 0.03) reduction in cytosolic diS-Cy5 in CCK-stimulated cells (n=12 for all experimental conditions).

**Figure 7. Actin staining and rupture of endocytic vacuoles.**

Cells were stimulated by 10nM CCK. Endocytosed diS-Cy5 is shown by in green. The upper gallery of images shows the ruptures of two endocytic vacuoles. LifeAct staining is shown in white. Scale bar corresponds to 10 µm. The lower gallery of images depicts the fragment containing the two large vacuoles undergoing rupture (corresponding to the first 3 images of the upper gallery). Note the fluorescent probe escaping the upper vacuole via the fenestration in the actin coat (indicated by the yellow arrow). Merged images are shown in the bottom row. On the merged images actin is shown in magenta.

Both vacuoles undergo shape changes before the rupture but these changes are particularly prominent in the lower vacuole, which is not coated with actin.

**Figure 8. Exocytosis of endocytic vacuoles.**

**A**. Fusion of LY-filled EV (green; the EV undergoing fusion is highlighted by an arrow) with the basal plasma membrane of a CCK stimulated acinar cell. The extracellular medium contains TRD (red), and a fusion event is characterised by mixing of the probes (yellow). Following fusion, the EV loses LY and gains TRD. The region containing the fusing EV is shown in the bottom row of images. The graph shows the time-course of fluorescence of the two dyes recorded in the EV (highlighted by the dashed line). **B**. LY-filled EV (green) fuses with TRD-filled (red) post-exocytic structure in the apical region of the CCK stimulated cell. The region containing the fusing EV (white arrow) is displayed in the bottom row of images. Following fusion the composite structure contains both probes (yellow) (representative of n=7 observations). Scale bars correspond to 10 µm.

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