Ex vivo human pancreatic slice preparations offer a valuable model for studying pancreatic exocrine biology

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A genuine understanding of human exocrine pancreas biology and pathobiology has been hampered by a lack of suitable preparations and reliance on rodent models employing dispersed acini preparations. We have developed an organotypic slice preparation of the normal portions of human pancreas obtained from cancer resections. The preparation was assessed for physiologic and pathologic responses to the cholinergic agonist carbachol (Cch) and cholecystokinin (CCK-8), including for physiologic and pathologic responses to the cholinergic agonist carbachol (Cch) and cholecystokinin (CCK-8), including 1) amylase secretion, 2) exocytosis, 3) intracellular Ca2+ responses, 4) cytoplasmic autophagic vacuole formation, and 5) protease activation. Cch and CCK-8 both dose-dependently stimulated secretory responses from human pancreas slices similar to those previously observed in dispersed rodent acini. Confocal microscopy imaging showed that these responses were accounted for by efficient apical exocytosis at physiologic doses of both agonists and by apical blockade and redirection of exocytosis to the basolateral plasma membrane at supramaximal doses. The secretory responses and exocytotic events evoked by CCK-8 were mediated by CCK-A and not CCK-B receptors. Physiologic agonist doses evoked oscillatory Ca2+ increases across the acini. Supraphysiologic doses induced formation of cytoplasmic autophagic vacuoles and activation of proteases (trypsin, chymotrypsin). Maximal atropine pretreatment that completely blocked all the Cch-evoked responses did not affect any of the CCK-8-evoked responses, indicating that rather than acting on the nerves within the pancreas slice, CCK cellular actions directly affected human acinar cells. Human pancreas slices represent excellent preparations to examine pancreatic cell biology and pathobiology and could help screen for potential treatments for human pancreatitis.

Extensive studies on pancreatic acinar cell stimulus-secretion coupling have for more than three and a half decades employed dispersed acini preparations of rodent pancreas (1, 2). The ultimate intent of rodent studies has been to model the human pancreas to understand the regulation of digestive enzyme secretion and the cellular mechanism of human pancreatitis (2, 3). Few studies have directly been performed using the human pancreas. The group of Petersen and co-workers (4) has observed cellular responses from dispersed human acini preparation of normal pancreas portions obtained from patients undergoing pancreatic resection of non-obstructing pancreatic tumors. This group characterized the human pancreatic acinar Ca2+ signaling evoked by acetylcholine and also physiologic cholecystokinin octapeptide (CCK-8)‡ (4). These results were surprising and controversial (5) as previous studies of dispersed human pancreatic acini showed little to no secretory response to CCK-8 (6–8), which had led to the belief that CCK effects on human pancreas are attributed to vagal nerves within the pancreatic tissue (5). Although considerable work has been done on CCK stimulus-secretion coupling in the rodent exocrine pancreas (2, 3), CCK receptors were either not present or were present in small quantities in the human exocrine pancreas (7–9). As CCK receptors are of two subtypes, CCK-A and CCK-B, which CCK receptor subtype is in human pancreatic acinar cells has been a subject of continued debate. Using reverse transcriptase PCR, very low levels of both CCK-A and CCK-B receptors mRNA were present in the adult human pancreas (9). Using quantitative PCR, higher levels of CCK-A receptor mRNA were found in human pancreas (10). Which of these CCK receptors, CCK-A or CCK-B, are functional in human pancreatic acinar cells, has not been investigated.

Supramaximal cholinergic or CCK-8 stimulation of rodent pancreatic acini causes inhibition of amylase secretion from maximal levels resulting from blockade of normal regulated apical exocytosis and diversion of exocytosis to the basolateral plasma membrane; the latter would result in interstitial pancreatitis (11, 12). Blockade of basolateral exocytosis by genetic deletion of a putative exocytotic fusion protein prevented pan-

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‡ The abbreviations used are: CCK-8, cholecystokinin-octapeptide; Cch, carbachol; [Ca2+], intracellular calcium concentration; ROI, region of interest; TTX, tetrodotoxin; AMC, 7-amino-4-methylcoumarin; KRBH, Krebs-Ringer bicarbonate HEPES buffer.
creatitis induced by both supramaximal concentration of the cholinergic agonist carbachol (Cch) and a model of alcoholic pancreatitis (12). Pathologic basolateral exocytosis (11, 12) along with intracellular protease activation occurring initially within large cytoplasmic vacuoles (13–15) are now the established cellular mechanisms of pancreatitis (16). Although both of these events of acinar cell injury could be evoked by supramaximal CCK-8 or carbachol stimulation of rodent pancreas, it has yet to be proven whether in human pancreas these events are also the cellular mechanisms of human pancreatitis.

Recent reports showed that rodent pancreas can be prepared as thin pancreas slices (100–140 μm thick) cut by a microtome, where both exocrine and endocrine functions and cellular mechanisms remain intact (17, 18). This more genuinely physiologic preparation of intact pancreatic tissue has advantages over the dispersed tissue models, acini, or isolated islets, which require damaging enzymatic and mechanical dissociation. These dispersed pancreatic tissue preparations could perturb exocrine and endocrine cell biology that may confound the results derived from experiments employing these preparations (17, 18). Conceivably, these preparations could damage the small amount of CCK receptors present on the plasma membrane surface of the human pancreatic acinar cells.

In this work we have employed the slice preparation on normal human pancreas obtained after surgical resections of pancreatic cancer. We show the human exocrine pancreas displayed a typical concentration-dependent amylase secretion, acinar exocytosis, and intracellular calcium ([Ca^{2+}]) rise and supramaximal agonist stimulation-evoked cytoplasmic vacuole formation and protease activation. Of note, all these events were evoked by carbachol and also CCK-8 stimulation, the latter via pancreatic acinar CCK-A receptors and not mediated by neuronal input. The latter supports a direct CCK-8 signaling pathway for secretion and pancreatitis in the human pancreatic acinar cell.

Results

Carbachol and CCK-8 stimulate amylase secretion from human pancreas slices

We first examined cholinergic stimulation with carbachol, where physiologic concentrations (1–10 μmol/liter) stimulated amylase release and supraphysiologic concentrations caused concentration-dependent inhibition of secretion from maximal levels (Fig. 1A). This response from human pancreas slices is similar to what is evoked at the same concentrations from rodent dispersed acini (19). We also observed a similar CCK-8-stimulated release at physiologic concentrations of 1–10 pmol/liter with further increase at maximal CCK-8 (100 pmol/liter), and further supramaximal increases in concentrations also led to concentration-dependent reduction of secretion (Fig. 1B). This dose response from human pancreas slices is also similar to rodent dispersed acini studies (11). Nonetheless, this CCK-8 stimulated response could have been derived from vagal neural innervations, which would be intact within the fresh pancreas slice. We first determined the optimal dose of the cholinergic...
receptor antagonist, atropine that blocked maximal Cch (10 μmol/liter)-stimulated release to near basal levels, which is 50 μmol/liter atropine (Fig. 1C). 50 μmol/liter atropine had no effect on maximal CCK-8 (100 pmol/liter)-stimulated amylase release (Fig. 1D), indicating that CCK-8 acted directly on the acinar cells.

**Carbachol and CCK-8 evoke apical and basolateral exocytosis in human pancreas slices**

We previously showed that both carbachol and CCK-8 evoke apical exocytosis at physiologic concentrations and at supra-maximal concentrations cause apical blockade and redirection of exocytosis to the basolateral plasma membrane (11, 12, 19, 20). Maximal Cch (10 μmol/liter) likewise stimulated apical exocytosis in the human pancreas slices (Fig. 2A, representative of 6 experiments) shown as FM1–43 fluorescence occupying an increasing area in the apical pole of an acinus, with no fluorescence hotspots in the basolateral regions. In contrast, supramaximal Cch (2 mmol/liter) evoked exocytosis in several basolateral regions of the acinus (white arrows in Fig. 2B, representative of 5 experiments) with very little apical exocytosis, the latter indicating apical blockade. The fluorescence trace analyses of Fig. 2, A and B, are shown respectively in Fig. 2, C and D. On expanded views of a region with a large amount of basolateral exocytosis (series of bottom images in Fig. 2B), we observed an initial two exocytotic events progressing to four and then dispersion of fluorescence from the four (last image) into the interstitial space indicating emptying of zymogen granule contents.
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We next confirmed that the CCK-8-mediated apical exocytosis is a direct effect of CCK-8 on the human pancreatic acinar cells and not due to indirect effects of CCK-8 on releasing acetylcholine from vagal neurons or stellate cells (21) that are present within the intact pancreatic slice tissue (Fig. 3). Apical exocytosis by maximal Chch stimulation (Fig. 3A, top images) was completely blocked by 50 μmol/liter atropine (Fig. 3A, bottom images). In contrast, maximal CCK-8-evoked exocytosis (Fig. 3B, top images) was unaffected by 50 μmol/liter atropine (Fig. 3B, middle images). CCK-8-stimulated apical exocytosis was also unaffected by further addition of 100 nmol/liter tetrodo- 
xin (TTX) (4), which was used to poison the nerves and stellate cells (Fig. 3B, bottom images). The analyses for Fig. 3, A and B, are shown in Fig. 3, C and D, respectively.

**Carcbol and CCK-8 stimulate oscillatory \([Ca^{2+}]\), rises in acini of human pancreatic slices**

Murphy et al. (4) report that both Cch and CCK-8 at physiologic concentrations evoked \([Ca^{2+}]\), oscillations in dispersed acini of human pancreas. We show very similar results with Cch (5 μmol/liter, Fig. 4A) and CCK-8 (20 pmol/liter, Fig. 4C) stimulation of the human pancreas slices. Whereas Ch-ch-evoked \([Ca^{2+}]\), oscillations of the acini were completely blocked by atropine (50 μmole/liter) pretreatment (Fig. 4B), CCK-8-evoked \([Ca^{2+}]\), oscillations were unaffected either by atropine (50 μmol/liter) pretreatment (Fig. 4D) or atropine (50 μmol/ 
liter) plus tetrodotoxin (100 nmol/liter) pretreatment (Fig. 4E). This is depicted in the \([Ca^{2+}]\), fluorescence traces on the right of each set of images whereby the maximal \([Ca^{2+}]\), fluorescence peaks are correspondingly indicated by the numbers. These results indicate that \([Ca^{2+}]\), responses between adjacent acinar cells are relatively synchronized or coupled. Currently, we do not yet have the microscopy resolution capability to track with higher spatiotemporal resolution single acinar \([Ca^{2+}]\), kinetics of single cells within the slice.

**CCK-A receptors, but not CCK-B receptors, are present in acini of human pancreatic slices, which are responsive to CCK-8-stimulated secretion**

Imunofluorescence microscopy with specific antibodies (22–24) showed that CCK-A receptors (top images), but not CCK-B receptors (bottom images), are more abundant in human acini within the pancreas slice (Fig. 5A). Phalloidin- 
labeled F-actin (left panels) marked the acinus ductal lumen and also the basolateral plasma membrane, whereas ZO-1 (right panels) is concentrated in the acinar junctional complex that marked out the acini apical lumen. CCK-A recep- 
tors were notably present on the acinar basal plasma membrane with small amounts also on the lateral plasma membrane.

CCK-A receptor antagonist devazepide (also called L364, 718) dose-dependently (10⁻⁸–10⁻⁵ mmol/liter) (25) blocked maximal CCK-8-stimulated amylase secretion (Fig. 5B, left). In contrast, increasing concentrations of CCK-B receptor antago- 
nist L365,240 (10⁻⁹–10⁻⁶ mmol/liter) (26), had no effect on CCK-8-stimulated secretion (Fig. 5B, right). To confirm, maximal CCK-8-evoked apical exocytosis was also effectively blocked by maximal dose (10⁻⁵ mmol/liter) CCK-A receptor antagonist devazepide (Fig. 5C, upper images) but was not blocked by maximal dose (10⁻⁶ mmol/liter) CCK-B receptor antagonist L365,240 (Fig. 5C, bottom images). TTX was present in all these secretory and exocytotic studies to negate any con- 
tribution from neurons or stellate cells present in the intact pancreatic tissue slice.

**Supramaximal Cch and CCK-8 induce large cytoplasmic autophagic vacuole formation in acinar cells of human pancreas slices and consequent protease activation**

Accumulation of large cytoplasmic vacuoles in the acinar cells is an early feature of pancreatitis that can be induced by supramaximal Chch or CCK-8 in vivo or in vitro (13–15). It has been postulated that pancreatic enzymes (i.e. trypsinogen, chymotrypsinogen) are prematurely activated within these vacu- 
oles leading to pancreatitis (14, 15). The biogenesis of cytoplasmic vacuole formation and protease activation were recently elucidated to be at least in part due to impairment of autophagy (27). A marker of autophagic activity is LC3A to LC3B conversion and its accumulation within the large autophagic vacuoles (27). We next examined whether these events would take place in human pancreatic acinar cells within the pancreas slices.

Indeed, supramaximal Chch (2 mmol/liter, Fig. 6A) and CCK-8 (10 nmol/liter, Fig. 6B) induced large vacuole formation in the acini of the human pancreas slices that were detected by the LC3B antibody. Atropine pretreatment blocked the formation of these LC3B-marked vacuoles induced by 2 mmol/liter Chch (Fig. 6A) but not 10 nmol/liter CCK-8. Neither maximal Chch (10 μmol/liter, Fig. 6A) nor CCK-8 (100 pmol/liter, Fig. 6B) induced cytoplasmic vacuole formation. For quantitative analysis, we measured the size (Fig. 6C) and density (Fig. 6D) of the vacuoles, which confirmed the above findings.

To track in real time the formation of these autophagic vacu- 
ules, we transduced human pancreas slices with adenovirus-LC3B-GFP (Fig. 7). With supramaximal CCK-8 (10 nmol/liter stimulation, a time-dependent (over 20 min) increase in formation of discrete autophagic hotspots in the acinar cells (Fig. 7A) was observed. Here, the initial diffused LC3B-GFP fluorescence (box 1) progressively accumulated into discrete vacular structures (boxes 2 and 3). This increased formation of discrete autophagic hotspots was not observed in maximal CCK-8 (100 pmol/liter CCK-8)-stimulated human slices (Fig. 7B) or in unstimulated slices (data not shown). In fact, the initially weak and diffused fluorescence (box 1) became more dispersed (boxes 2 and 3) or the loss of fluorescence signal may be due to photobleaching by the repeated laser exposures.

Finally, we determined protease activation, specifically tryp- 
sin (Fig. 8A) and chymotrypsin (Fig. 8B) activity, in the human pancreas slices stimulated by Chch and CCK-8. Whereas maximal concentrations of both agonists did not induce activation of either proteases, supramaximal concentrations of both agonists induced trypsin and chymotrypsin activity. Atropine blocked the 2 mmol/liter Chch-induced protease activation but not 10 nmol/liter CCK-8-induced protease activation. After repeated attempts, we were unsuccessful in establishing the elegant method of imaging the cellular trypsin activity as reported by Lerch and co-workers (28) in the pancreatic slice.
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Discussion

This study demonstrates the human pancreas slice to be an excellent preparation to assess human exocrine physiology and pathophysiology. We showed that physiologic agonist (Cch and CCK-8) stimulation evoked oscillatory rises in cytosolic Ca2+ leading to apical exocytosis that efficiently released amylase. We also showed that supraphysiologic agonist stimulation caused apical blockade and redirected exocytosis to the basolateral plasma membrane, resulting in overall reduction of amylase secretion from maximal levels of release. All of these secretory events are consistent with the responses observed with dispersed rodent acini. This preparation has many advantages over dispersed preparations of acini. First, the pancreas slice retains normal morphology and secretory integrity even after several days in culture (17), which is not the case with dispersed acini. Mouse pancreatic slices could be cultured for 4–7 days and still retain the ability of stimulated amylase secretory responses (17), suggesting that human pancreas slices could be conducive to the virally induced gene (as was done here with Adenovirus-LC3B-GFP) and target protein up- and down-regulation, which requires several days. Second, it is a faster preparation and provides a larger amount of tissue that is the closest to the normal native state to study than dispersing human pancreatic acini into acini (4). Third, this preparation procedure is less damaging than dispersion of acini, which might alter acinar biology, such as CCK receptor coupling. In fact, we did not see significant damage to the acini in the pancreas slice. The mechanical slicing with the microtome is less damaging than mechanical and enzymatic dispersion into acini. Any release of pancreatic enzymes was washed out of the tissue and diluted by the large amount of buffer. The agarose gel is sufficiently porous to allow permeation of nutrients and oxygen from the buffer.

Dispersed pancreatic acini preparations are known to disrupt tight junctions (29), which would in turn disrupt or at least cause some rearrangement in the inositol trisphosphate (IP3) arrangements of inositol trisphosphate receptors in salivary gland acini versus pancreatic acini were reported to account for the different Ca2+ release responses (31). Therefore, the perturbation of tight junctions may in part explain the more variable responses observed in the study of dispersed human pancreatic acini (4). Further study in acinar Ca2+ release pathways should, therefore, be conducted on the human pancreatic slice. A caveat is that although we only obtained normal pancreas portions that are distal from the disease margins (i.e. cancer), there is a remote possibility that the disease portions of the pancreas might have unanticipated effects on normal acinar functions that might have affected our results.

Importantly, we were able to verify that CCK-8 stimulus-secretion coupling is present in human pancreatic acinar cells within a pancreas slice, similar to the results obtained with dispersed human pancreatic acini (4). In the pancreas slice, vagal neural tissues are certainly intact. However, we optimized the atropine concentration to completely block maximal Cch-stimulated responses, which was 50 times that used in the study by Murphy et al. (4). Treatment with 50 μmol/liter atropine prevents any possibility that CCK-8 could be acting through neural release of acetylcholine. In fact, 50 μmol/liter atropine had no effect on any of the CCK-8-stimulated responses, including amylase secretion, [Ca2+]i, responses, exocytosis, cytoplasmic vacuole formation, and protease activation. Another study using dispersed human pancreatic acini showed no response to CCK-8 (7), clearly different from our study and that of Murphy et al. (4). This discrepancy could be attributed to human pancreatic acinar CCK receptors being particularly sensitive to damage by collagenase, which is known to digest the acinar basement membrane (29). In fact, Murphy et al. (4) noted that when pancreatic specimens were obtained from clinically obstructed conditions, they did not observe the CCK-evoked responses. Here, we further defined the functionally important CCK receptor subtypes in human to be CCK-A. Interestingly, the CCK-A receptors were found not only on the basal plasma membrane but also on the lateral plasma membrane (Fig. 5A) closed to the tight junctions. We confirmed the CCK-A receptor function by blockade of CCK-stimulated secretion and apical exocytosis by a CCK-A receptor antagonist (devazepide) but not by the CCK-B receptor antagonist (L365,260).

Supramaximal Cch and CCK-8 induce a number of pathologic fusion events that contribute to cell injury. Basolateral exocytosis observed in the human pancreas slices were similarly observed in our previous studies employing dispersed acini from rats and mice (11, 12, 19, 20). Release and activation of proteases in the interstitial space would be expected to cause pancreatic tissue damage (32). Nonetheless, the dominant thinking is that supramaximal stimulation induces formation of cytoplasmic vacuoles where proteases are inadvertently and prematurely activated (13–15). These pathologic events leading to pancreatitis were shown to be due to impairment in the autophagic process (27). All of these events of cell injury were observed in the human pancreas slice.

In conclusion, this human pancreas slice preparation retains the intact architecture of the gland including the luminal domains (also basolateral domains and interstitial space), blood vessels, nerves, and surrounding tissues (stellate cells, islets, pancreatic ducts) that could also influence acinar function. The pancreatic slice retains the full polarity of acinar cells that can be profoundly affected by adjacent cues; both are lost in isolated cells and perturbed in dispersed acini preparations. Along with the intact CCK-A receptor signaling, the human pancreas slice preparation is ideal to assess other postulated mechanisms of cell injury, particularly those that mimic clinical pancreatitis.
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(33), and to use as a platform for testing potential treatments. The human pancreatic slice is, therefore, the best preparation to date and a new standard for translational medicine. Further development, such as long term culture (17) of human pancreatic slices, will enable gene manipulation by viral transduction, and also high spatiotemporal resolution imaging (4, 34, 35) will extend the usefulness of this preparation in understanding exocrine pancreatic function and pathobiology.

Materials and methods

Antibodies and reagents

Flu 4 acetoxyethyl ester and FM 1–43 dyes were from Molecular Probes (Eugene, OR). Substrates for trypsin (t-butoxy carbonyl (Boc)-Gln-Ala-Arg-7-amino-4-methyl coumarin (AMC) and chymotrypsin (succinyl (Suc)-Ala-Ala-Pro-Phe-AMC) were from Peptides International (Louisville, KY) and Calbiochem (EMD Biosciences, Billerica, MA), respectively. Tetrodotoxin is from Cedarlane Labs (Burlington, Ontario, Canada). Devazepide (CCK-A receptor antagonist) is from Sigma. L365,260 (CCK-B receptor antagonist) is from R&D Systems (Minneapolis, MN). CCK-A receptor antibody (catalog #EB06768) and CCK-B receptor antibody (catalog #EB06767) were from One World Lab (San Diego, CA). Alexa Fluor 594-conjugated mouse monoclonal ZO-1 antibody and Alexa Fluor 488 phalloidin were from Molecular Probes (Eugene, OR). LC-3B antibody was from Santa Cruz Biotechnology (Dallas, TX). LC3B-GFP adenovirus was a kind gift from Dr. Junichi Sadoshima (Rutgers New Jersey Medical School, NJ). Sulfated CCK-8 was from Research Plus (Barnegat, NJ). Carbachol chloride (Cch), sulfated atropine, and other chemicals unless specified were obtained from Sigma.

Human pancreas slice preparation and culture

Preoperative written consent was obtained from 18 patients operated for pancreatic tumors (8 adenocarcinomas) from July 2011 to January 2016, ages ranging from 42 to 73 years old (10 females, 8 males). After surgical resection, resected pancreas was assessed by the surgical pathology laboratory, and a normal portion distant from tumor margins immediately obtained and was assessed by the surgical pathology laboratory, and a normal portion distant from tumor margins immediately obtained and a normal portion distant from tumor margins immediately obtained and placed in cooled extracellular solution, and our laboratory called to retrieve the specimen. The slicing procedure is performed within 2 h of the call. All procedures involving human pancreas tissues were approved by the Research Ethics Boards of the University Health Network and University of Toronto.

Pancreas tissue slices were prepared in a manner similar to our previous reports with mouse pancreas but some variations tailored to the human pancreas (36, 37). The normal human pancreatic tissue was cut into smaller pieces then injected and embedded with 37 °C low melting 3.8% agarose gel (catalog #15517-022; Invitrogen) and cooled down by ice to solidify the gel. The gelled tissue blocks were glued onto the sample plate and cooled down by ice to solidify the embedded with 37 °C low melting 3.8% agarose gel (catalog #15517-022; Invitrogen) and cooled down by ice to solidify the embedded with 37 °C low melting 3.8% agarose gel (catalog #15517-022; Invitrogen) and cooled down by ice to solidify the.

Figure 4. Cch-evoked, but not CCK8-evoked, oscillatory [Ca2+]i rises in the acini of human pancreas slices are blocked by atropine. Trace analysis of [Ca2+]i fluorescence images were shown on the right as F/Fo, where F is fluorescence signal from the Ca2+ dye within the acini and Fo is baseline fluorescence signal. Maximal Cch (10 μmol/liter)-evoked [Ca2+]i oscillations (A, n = 21 acinar cells from 6 pancreas slices) were blocked by atropine (50 μmol/liter in B, n = 18 acinar cells from 5 pancreas slices). Maximal CCK-8 (100 pmol/liter)-evoked Ca2+ oscillations (C, n = 20 acinar cells from 6 pancreas slices) were not affected by atropine (D, n = 18 acinar cells from 5 pancreas slices) or by atropine + tetrodotoxin (E, n = 17 acinar cells from 5 pancreas slices). Scale bar = 2 μm.
Figure 5. CCK-A receptors, but not CCK-B receptors, are present in acini of human pancreatic slices, which are responsive to CCK-stimulated secretion. A, acini in the pancreas slice were double-labeled with antibodies to CCK-A receptor or CCK-B receptor along with Alexa Fluor 488 phalloidin to label F-actin (left images), which tracked the ductal lumen and basolateral plasma membrane, or Alexa Fluor 594-conjugated ZO-1 antibody (to label junctional complexes (right images), which tracked the apical lumen. ZO-1 and CCK-A/B receptor fluorescence images were superimposed on the differential interference contrast images. Scale bar/H11005 10/H9262 m. DIC, differential interference contrast. B, CCK-8-stimulated amylase secretion was blocked by CCK-A receptor antagonist (devazepide) in a dose-dependent manner but not by CCK-B receptor antagonist (L365,240). Each value, shown within a scattered plot, was from single or duplicate samples per experiment (left, 2 independent experiments, n = 3; right, 2 independent experiments, n = 3) with S.D. indicated by vertical lines within each plot. **, p < 0.01; ***, p < 0.001. NS, no significant difference determined by paired t test on two populations. C, CCK-A receptor antagonist devazepide blocked CCK-8-induced apical exocytosis in human acini (top images), whereas CCK-B receptor antagonist L365,240 did not block the apical exocytosis (bottom images). Analysis of fluorescence intensity traces of exocytosis at the apical pole is shown on the right (n = 5 acini for devazepide, n = 5 acini for L365,240). Scale bar = 10 μm. AU, arbitrary unit.
Figure 6. Supramaximal Cch but not supramaximal CCK-8-induced cytoplasmic vacuole formation in acini of human pancreas slices was blocked by atropine. Human pancreas slices were stimulated with indicated doses of Cch (A) or CCK-8 (B) for 40 min with or without pretreatment with atropine (50 μmol/liter, 30 min) then immunostained with LC3B antibody (red spots in 2 mmol/liter CCh and 10 nmol/liter CCK-8-stimulated cells). From the four independent experiments performed, we analyzed the diameter (in μm) (C) and density of vacuoles (number of vacuoles/pancreas slice area (10^7 × μm^2) (D) that formed in each condition (total number of vacuoles analyzed in each condition indicated). ***, p < 0.001. NS, no significant difference determined by paired t test on two populations. Scale bar = 20 μm.
FM1–43 fluorescence within the indicated regions of interest (ROIs) to monitor zymogen granule exocytosis occurring at different sites of the acinus within the slice. Quantitative analysis of ROIs fluorescent signals changing with time was normalized to the background noise.

**Ca²⁺ imaging**

100-μm-Thick pancreas slices were pretreated with sonicated Fluo 4 acetoxymethyl ester (5 μmol/liter) in extracellular solution for 30 min at room temperature, then washed for another 15–20 min in a buffer without the dye. Slices were mounted onto a perfusion chamber, which was then mounted onto the spinning disc confocal microscope. Ca²⁺ images were acquired with a 20× objective (0.7 NA) to capture a larger area using a 491-nm laser for 5 min at 1 frame/s and 200-ms exposure time. ROIs were drawn around single acinar cells. Fluorescent signals of the first 20 s of recording were averaged and set as the baseline (F₀). All changes in fluorescent signals with time (F) were normalized to the baseline and presented as F/F₀ (34).

**Confocal immunofluorescence microscopy**

80-μm-Thick pancreas slices were fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, and immuno-stained with LC-3B (1:200), CCK-A receptor (1:50), or CCK-B receptor antibody (1:50, 8 h). Images were captured with a 20× objective in the above spinning disc confocal system (0.75 NA), and analysis performed with the Velocity 3DM software.

**Adenovirus-LC3B-GFP imaging**

Human pancreas slices were infected with Ad-LC3B-GFP (10⁷ pfu) for 2.5 h, then placed on slice culture slits (EMD Biosciences) that were coated with rat tail collagen (5 mg/ml, Invitrogen) and placed in Waymouth’s medium (Invitrogen) for 12 h at 37 °C in a 5% CO₂ incubator. The culture slits were then mounted in a thermal chamber equilibrated at 37 °C. Images were acquired using the above confocal system with an oil immersion 63× objective (1.35 NA) and 491-nm laser at 500-ms exposure time for 25 min. Images were deconvolved using specific point spread function (confidence limit = 95%; iteration limit = 20); noise was removed.

**Measurement of trypsin and chymotrypsin activation**

Trypsin and chymotrypsin activity in pancreas slice homogenates were measured as we had described (34) with minor modifications. Briefly, after agonist stimulation, the slices were homogenized on ice and subjected to centrifugation at 1000 × g for 1 min. 50 μl of each postnuclear supernatant were added to wells of 96-well black μ-clear plates (Greiner Bio-One,


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Figure 8. Atropine impeded supramaximal Cch- but not CCK-8-evoked protease activity in human pancreatic slices. Effect of atropine (50 μM) on supramaximal Cch (2 mmol/liter) or CCK-8 (110 μM) protease activity. Each value was shown within a scattered plot; S.D. is indicated by vertical lines within each plot. NS, no significant difference determined by paired t test on two populations.

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Author contributions—All except M. S. C., E. W., and H. Y. G. participated in the acquisition of data, although T. L. and S. D. generated most of the data. M. S. C. and E. W. procured the human pancreatic tissues. T. L., S. D., P. T., and H. Y. G. drafted the manuscript; all reviewed the manuscript. H. Y. G. is the guarantor of this work and, as such, has full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Statistical analysis

Data are presented as scattered plots of raw data with S.D. or as means ± S.E. Statistical analysis of all data were carried out using SigmaStat v3.1 (Systat Software Inc., San Jose, CA). Differences between experimental groups were evaluated using the paired t test with p < 0.05 considered as significant. 15–30% of preparations were not viable but were experimented on because these specimens were precious. However, results from the majority of these bad tissue preparations were not interpretable and were not included in the analysis; those entire experiments were discarded. Tissue non-viability was due a number of reasons, including poor cutting and handling of specimens at the surgical pathology laboratory, delay in notification by the surgical path lab or late pick up of the specimens, and suboptimal sectioning during pancreatic slice preparations.
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