THE ROLE OF CYSTIC FIBROSIS TRANSMEMBRANE
CONDUCTANCE REGULATOR CL CHANNEL IN
PANCREATITIS

Ph.D. Thesis

Linda Judák

Szeged
2014
THE ROLE OF CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR CL CHANNEL IN PANCREATITIS

Ph.D. Thesis

Linda Judák

Supervisor at Pharmacology and Pharmacotherapy, University of Szeged: Dr. Viktória Venglovecz, Ph.D.
Supervisor at First Department of Medicine, University of Szeged: Péter Hegyi, M.D., Ph.D., DSc.

Pharmacology and Pharmacotherapy, First Department of Medicine
University of Szeged
Szeged, Hungary
2014
# TABLE OF CONTENTS

LIST OF
ABBREVIATIONS .......................................................................................................................... 2
ARTICLES CLOSELY RELATED TO THE SUBJECT OF THE THESIS AND CITED IN THE THESIS .......................................................................................................................... 3
SUMMARY ........................................................................................................................................ 5

## 1. INTRODUCTION .................................................................................................................. 7

## 2. MATERIALS AND METHODS ........................................................................................... 11

- 2.1. ETHICS .................................................................................................................................. 11
- 2.2. CAPAN-1 CELL CULTURE .................................................................................................. 11
- 2.3. ISOLATION OF PANCREATIC DUCTAL CELLS .................................................................. 11
- 2.4. WHOLE CELL CURRENT RECORDING ............................................................................. 12
- 2.5. MEASUREMENT OF INTRACELLULAR ATP ...................................................................... 12
- 2.6. CHEMICALS ....................................................................................................................... 13
- 2.7. STATISTICS ....................................................................................................................... 13

## 3. RESULTS .................................................................................................................................. 14

- 3.1. MEASUREMENT OF CFTR CONDUCTANCE IN GUINEA PIG PDECs ................................ 14
- 3.2. ETOH INCREASES THE BASAL BUT INHIBITS THE FORSKOLIN-STIMULATED CFTR CURRENTS ................................................................................................................................. 15
- 3.3. EFFECTS OF ETOH METABOLITES ON BASAL AND FORSKOLIN-STIMULATED CFTR CURRENTS ................................................................................................................................. 18
- 3.4. ETOH, POAEE AND POA CAUSE ATP DEPLETION IN PDECs, WHICH IS LINKED TO CFTR INHIBITION .............................................................................................................................. 21
- 3.5. INTRACELLULAR ATP SUPPLEMENTATION REVERSES THE INHIBITORY EFFECT OF ETOH, POAEE AND POA ON CFTR ACTIVITY IN PDECs ............................................................................. 23
- 3.6. LOW CONCENTRATION OF ETOH STIMULATES WHEREAS HIGH CONCENTRATION OF ETOH AND FATTY ACIDS INHIBIT FORSKOLIN-STIMULATED CFTR CURRENT IN HUMAN PDECs ................................................................................................................................. 24
- 3.7. ATP, SUPPLEMENTATION IN HUMAN PDECs .................................................................... 26
- 3.8. TRYPsin AND PAR-2-AP INHIBIT CFTR ............................................................................ 27

## 4. DISCUSSION .......................................................................................................................... 29

## 5. ACKNOWLEDGEMENTS ........................................................................................................ 32

## 6. REFERENCES ......................................................................................................................... 34

## 7. ANNEX .................................................................................................................................... 40
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac</td>
<td>acetaldehyde</td>
</tr>
<tr>
<td>AP</td>
<td>acute pancreatitis</td>
</tr>
<tr>
<td>ATP&lt;sub&gt;i&lt;/sub&gt;</td>
<td>intracellular ATP</td>
</tr>
<tr>
<td>BNPP</td>
<td>bis-(4-Nitrophenyl) phosphate</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt;</td>
<td>intracellular calcium concentration</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CFTR&lt;sub&gt;inh-172&lt;/sub&gt;</td>
<td>CFTR inhibitor-172</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DOG</td>
<td>2-Deoxyglucose</td>
</tr>
<tr>
<td>IAA</td>
<td>iodoacetamide</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>FAEE</td>
<td>fatty acid ethyl ester</td>
</tr>
<tr>
<td>MgGreen-AM</td>
<td>Magnesium Green-AM</td>
</tr>
<tr>
<td>PAR-2</td>
<td>proteinase-activated receptor-2</td>
</tr>
<tr>
<td>PAR-2-AP</td>
<td>PAR-2 activating peptide</td>
</tr>
<tr>
<td>PAR-2-ANT</td>
<td>PAR-2 antagonist</td>
</tr>
<tr>
<td>PDECs</td>
<td>pancreatic ductal epithelial cells</td>
</tr>
<tr>
<td>POA</td>
<td>palmitoleic acid</td>
</tr>
<tr>
<td>POAEE</td>
<td>palmitoleic acid ethyl ester</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>SBTI</td>
<td>soybean trypsin inhibitor</td>
</tr>
<tr>
<td>SLC26</td>
<td>solute carrier family 26</td>
</tr>
</tbody>
</table>
Articles closely related to the subject of the thesis and cited in the thesis


*IF: 4.463 (2012)*


*IF: 11.675*

Articles not related to the subject of the thesis


*IF: 3.431 (2012)*


*IF: 1.858*
Number of full publications: 4 (1 first author)
Cumulative impact factor: 21,427
SUMMARY

Background. Acute pancreatitis (AP) is a potentially fatal disease characterized by local complications and/or systemic organ failure. Recent investigations suggest that pancreatic ductal epithelial cells (PDECs) help to protect the pancreas from noxious agents, such as alcohol or premature trypsinogen activation in the early phase of the disease. Since the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel plays a major role in PDEC physiology our aims in this study were to investigate the direct effects of (i) ethanol (EtOH) and its metabolites and (ii) trypsin on CFTR activity.

Methods. Human ductal adenocarcinoma cell line (Capan-1) and guinea pig PDECs were used. We investigated the effects of EtOH (1, 10 and 100 mM), palmitoleic acid (POA; 10, 100 and 200 µM), palmitoleic acid ethyl ester (POAEE; 10, 100 and 200 µM) and trypsin (10 µM) on CFTR Cl⁻ currents using the whole cell configuration of the patch clamp technique. Changes in intracellular ATP (ATPᵢ) were estimated by the Mg²⁺-sensitive fluorescent dye, Magnesium Green-AM (MgGreen-AM).

Results and Conclusions. In the first part of this thesis we tested the hypothesis that EtOH exerts a direct effect on CFTR to impair ductal function. Administrations of low concentration of EtOH (1 mM) had no significant effect on isolated PDECs either on basal or forskolin-stimulated CFTR currents. However, at higher concentrations such as 10 or 100 mM EtOH increased the basal and decreased the forskolin-stimulated CFTR currents. Using equivalent concentration of mannitol, we showed that the stimulatory effect of EtOH is a non-specific, osmotic effect; whereas the inhibitory effect of EtOH is independent of its osmotic effect. The unsaturated fatty acid, POA and its esterized form POAEE, reversibly and time-dependently inhibited CFTR currents at higher concentrations (100 and 200 µM). Both EtOH, POA and POAEE significantly decreased the level of ATPᵢ in the ductal cells, whereas administration of ATPᵢ back to the cell via the patch pipette almost completely abolished the inhibitory effect of EtOH and its metabolites on CFTR. In summary, our results show that high concentration of EtOH, POA and POAEE strongly inhibit CFTR Cl⁻ channel in the ductal cells through the depletion of ATPᵢ. We speculate that restoration of the intracellular energetic pool by ATP supplementation may represent a novel therapeutic tool for the treatment of alcohol-induced pancreatitis.

In the second part of the thesis we showed, that both trypsin and proteinase-activated receptor-2 (PAR-2) activating peptide (PAR-2-AP) irreversibly decreased forskolin-stimulated CFTR Cl⁻ current in PDECs. Pretreatment with either soybean trypsin
inhibitor (SBTI) or PAR-2 antagonist (PAR-2-ANT) completely prevented the inhibitory effect of trypsin. In summary, these data suggests, that trypsin inhibits CFTR Cl− currents by activation of PAR-2, which might have an important role in the pathogenesis of chronic pancreatitis.
1. INTRODUCTION

The exocrine pancreas consist of two main cell types: the acinar and the ductal cells. The digestive enzymes are synthetized and secreted by the acinar cells, whereas the ductal cells secrete a bicarbonate-rich isotonic solution which is essential for the transport of digestive enzymes into the duodenum, and insures an optimal pH environment for the action of digestive enzymes.\(^1\) Ductal HCO\(_3^-\) secretion is not only regulated by gastrointestinal hormones and cholinergic nerves but is influenced by luminal factors, intraductal pressure, Ca\(^{2+}\) concentration, or pathological activation of proteases. The human pancreatic ductal system secretes about 2 litres of pancreatic juice per day, which containing as much as 140 mmol/l HCO\(_3^-\) at maximal stimulation.\(^3\) Previous studies indicated that reduced or deficient fluid and HCO\(_3^-\) secretion leads to the destruction of the pancreas, and causes different pancreatic diseases, such as cystic fibrosis.\(^4,5\)

PDECs possess several transporters and ion channels, which play a crucial role in HCO\(_3^-\) secretion (Fig. 1).

![Cellular mechanism of pancreatic ductal HCO\(_3^-\) secretion](image)

**Fig. 1. Cellular mechanism of pancreatic ductal HCO\(_3^-\) secretion.** Intracellular HCO\(_3^-\) is derived from CO\(_2\) through the action of carbonic anhydrase. Redundant H\(^+\) than leaves the cell through the Na\(^+\)/H\(^+\) exchanger and the proton pump. The alternative pathway involves the direct uptake of HCO\(_3^-\) from the blood by the Na\(^+\)/HCO\(_3^-\) cotransporter. HCO\(_3^-\) than leaves the cells through the Slc26 Cl\(^-\)/HCO\(_3^-\) exchanger and the CFTR Cl\(^-\) channel at the apical membrane. CA: carbonic anhydrase, CFTR: cystic fibrosis transmembrane conductance regulator, Slc26: solute carrier family 26,CACC: calcium-activated chloride channel.
It is now generally accepted that accumulation of HCO$_3^-$ across the basolateral membrane is achieved by passive diffusion of CO$_2$, its hydration and dissociation to H$^+$ and HCO$_3^-$ ions. Other mechanism for HCO$_3^-$ uptake is based on the aligned function of the Na$^+/HCO_3^-$ cotransporter, the Na$^+/H^+$ exchanger and the H$^+$-ATPase.\textsuperscript{1,6} In contrast, HCO$_3^-$ secretion at the apical membrane is driven by the CFTR Cl$^-$ channel, the Cl$^-/HCO_3^-$ exchanger and calcium–activated chloride channel (Fig. 1).\textsuperscript{2,7,8}

The CFTR protein is a cAMP-regulated Cl$^-$ channel with multiple functions in epithelial cells (Fig. 2).\textsuperscript{9} In the exocrine pancreas the CFTR plays a key role in the apical Cl$^-$, HCO$_3^-$ and water transport in PDECs. The severe loss of functions, caused by mutations of the CFTR gene, leads to pathological lesions of the pancreas. The characteristic behavior of CFTR is determined by the function of the different domains from which CFTR is assembled. These include the two membrane-spanning domains (MSDs) that are each composed of six transmembrane segments, the two nucleotide-binding domains (NBDs) that each contain motifs which interact with ATP, and the unique R (regulatory) domain that contains multiple consensus phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC) moreover many charged amino acids.

![Fig. 2. Model showing proposed domain structure of cystic fibrosis transmembrane conductance regulator (CFTR). MSD: membrane-spanning domain; NBD: nucleotide-binding domain; R: regulatory domain; PKA: cAMP-dependent protein kinase.](image-url)
The MSDs assemble to form the Cl⁻ selective pore while the NBDs and R domain control CFTR channel gating. The classical view is that CFTR mainly secretes Cl⁻ and having a relatively low permeability to HCO₃⁻. However, under stimulated secretion the channel permeability changes and become more permeable for HCO₃⁻ than Cl⁻. It is well documented that insufficient function of CFTR increases the risk of AP.

AP is a sudden and severe disease characterized by local complications and/or systemic organ failure. Heavy alcohol consumption is one of the leading causes of AP, however, the cellular mechanism by which EtOH induces pancreatitis is not well understood. EtOH can be metabolized by two different pathways: the oxidative and the nonoxidative pathways. The oxidative pathway involves alcohol dehydrogenase, which enzyme catalyses EtOH conversion to acetaldehyde (Ac). Ac than further oxidized by Ac dehydrogenase to acetate. This conversion happens dominantly in the liver. In contrast, the non-oxidative metabolism of EtOH involves the esterification of EtOH with fatty acids (FAs) and the production of fatty acid ethyl esters (FAEEs). The formation of FAEEs from EtOH and FAs is catalysed by the enzyme, FAEE synthase, whereas FAEE hydrolase is responsible for the reverse reaction. The effects of EtOH or its metabolites on the pancreas were mainly investigated on acinar and stellate cells whereas much less studies have been done on PDECs. Yamamoto et al. showed that low concentrations (0.3–30mM) of EtOH augmented ductal fluid secretion stimulated with physiological or pharmacological concentrations of secretin, whereas a high concentration of EtOH (100 mM) completely abolished this effect. However, no data is available regarding the effect of EtOH or its metabolites on the activity of CFTR Cl⁻ channel.

The possible role of CFTR in the pathomechanism of alcoholic pancreatitis has been first raised due to Henry Sarles. He investigated 205 patients with different form of pancreatitis. Almost all of his patients were alcoholics. In 100 patients, he observed the presence of protein plugs in the intra/interlobular pancreatic ducts and these patients secreted significantly higher Cl⁻ and Na⁺ compare to controls. These results clearly suggest that the CFTR Cl⁻ channel did not work properly in these patients. Therefore, our main aim in this study was to characterize the direct effects of EtOH, FA and FAEE on PDECs, especially on CFTR channel activity.
It is generally accepted that inappropriate or premature activation of digestive enzymes leads to autodigestive injury and therefore is a key event in the development of chronic or acute pancreatitis. Under physiological conditions, trypsinogen is synthesized by the acinar cells as inactive precursor, transferred to the duodenum via the pancreatic ducts, and then activated by enteropeptidases in the small intestine. PAR-2 is a cell surface receptor for trypsin-like proteases. Several studies indicated that trypsin stimulates enzyme secretion from acinar cells via PAR-2. In contrast, the effect of trypsin on PDECs is contradictory, highly depends on the species and the localization of PAR-2. Trypsin stimulates HCO$_3^-$ secretion in Capan-1, human pancreatic ductal cell line, whereas inhibit HCO$_3^-$ efflux from bovine PDECs. Moreover, activation of basolateral PAR-2 stimulates, whereas apical PAR-2 inhibits ductal HCO$_3^-$ secretion. In contrast, no data is available regarding the effect of trypsin on CFTR, however CFTR Cl$^-$ channel plays an essential role in ductal HCO$_3^-$ secretion. Therefore, in this study our aim was to characterize the effect of trypsin and the PAR-2-AP on CFTR activity.
2. MATERIALS AND METHODS

2.1. Ethics

All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (U.S.A. NIH publication No85-23, revised 1985). Animal experiments were approved by the Regional Ethical Board at the University of Szeged, Hungary.

2.2. Capan-1 cell culture

Capan-1 cells were grown in Roswell Park Memorial Institute (RPMI)-1640 Medium supplemented with 15% fetal bovine serum, 1% penicillin–streptomycin. Cells from passage numbers 20–60 were used in this study. For electrophysiological measurements, 5×10^5 cells were plated onto 24 mm diameter glass coverslips and used for experiments after 3 to 4 days of culture (~60–80% confluency).

2.3. Isolation of pancreatic ductal cells

Four- to eight-week-old guinea pigs were humanly killed by cervical dislocation and then the pancreas was removed. Intra/interlobular ducts were isolated by enzymatic digestion and microdissection from the pancreas and then cultured overnight as previously described. To obtain single pancreatic ductal cells, cultured ducts were incubated for 50 min at 37°C in 50 U/ml elastase dissolved in storage solution (Dulbecco's modified Eagle's medium containing 3% (w/v) bovine serum albumin (pH 7.4 with NaOH)). The ducts were then washed in a Ca^{2+}/Mg^{2+}-free Kreb's–Ringer HEPES buffer (pH 7.4) for 10 min further in order to stop elastase activity. After the incubation, ducts were teased apart using stainless steel needles.

In the patch clamp experiments, one drop of cell suspension was placed within a transparent recording chamber, and single ductal cells were allowed to settle and attach to the bottom of the chamber for at least 30 min before perfusion was initiated. In the microfluorometric measurements, one drop of cell suspension was transferred to a poly-L-lysine-coated cover slip (24 mm) forming the base of a perfusion chamber. Cells were then washed continuously with solutions at a rate of 1.5 or 5–6ml/min in the patch-clamp and microfluorometric measurements, respectively. Individual ductal cells were used within 3–4 h after the isolation.
2.4. Whole cell current recording

Patch-clamp micropipettes were fabricated from borosilicate glass capillaries (Clark, Reading, UK) using a P-97 Flaming/Brown micropipette puller (Sutter Co., Novato, CA, USA) and had resistances between 2.5 and 4 MΩ. Whole cell currents were recorded with Axopatch-1D patch clamp amplifier (Molecular Devices-Axon Instruments, Union City, CA, USA) at 37 °C.

The standard extracellular solution contained (in mM) 145 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES and 5 glucose (pH 7.4 with NaOH). The osmolarity of the external solution was 300 mOsm/l. The standard pipette solution contained (in mM) 120 CsCl, 2 MgCl₂, 10 HEPES, 5 ethylene glycol-bis(β-aminoethyl ether)-N,N,N8,N8-tetraacetic acid (EGTA) and 1 Na₂ATP (pH 7.2 with CsCl). The osmolarity of the pipette solution was 240 mOsm/l. In some experiments, the ATP concentration was increased to 2 or 5 mM.

After establishing a high (1–10 GOhm) resistance seal by gentle suction, the cell membrane was disrupted by further suction or by applying a 1.5 V electrical pulse for 1–5ms. The series resistance was typically 4-8 MOhm before compensation (50-80 %, depending on the voltage protocol). Membrane currents were digitized using a 333-kHz analog-to-digital converter (Digidata 1200, Molecular Devices-Axon Instruments, Union City, CA, USA) after low-pass filtering at 1 kHz under software control (PCLAMP 6.0, Molecular Devices-Axon Instruments, Union City, CA, USA). To obtain current/voltage (I/V) relationships, the membrane potential (V_m) was held at 0 mV and then clamped to ±100 mV in 20 mV increments for 500 ms, with an 800 ms interval between each pulse. Currents were measured over a 4 ms period starting 495 ms into the voltage pulse. Mean current amplitudes were calculated at ±60 mV and normalized to cell capacitance (pF).

2.5. Measurement of intracellular ATP

Changes in intracellular ATP (ATPᵢ) were estimated using the Mg²⁺-sensitive fluorescent dye, Magnesium Green-AM (MgGreen-AM). Most of the Mg²⁺ ions in the cells are bound to ATP. Since ATP has a higher affinity for Mg²⁺ than ADP, upon ATP hydrolysis the concentration of cytosolic free Mg²⁺ increases. Upon binding Mg²⁺, MgGreen exhibits an increase in fluorescence emission intensity which allows the indirect measurement of ATPᵢ. Cells were incubated with 5 µM MgGreen-AM in the presence of 0.05 % pluronic F-127 in standard HEPES solution (in mM: 130 NaCl, 5 KCl, 1 CaCl₂, 1
MgCl₂, 10 D-glucose and 10 Na-HEPES; pH was set to 7.4 with HCl) for 30 min at room temperature.

Five to ten cells (region of interest, ROIs) were then excited with light at a wavelength of 490 nm, with emitted light monitored at 535 nm. The fluorescence emissions were captured by a CCD camera and digitized by an Xcellence imaging system (Olympus, Budapest, Hungary). One ATP measurement was obtained per second. Fluorescence signals were normalised to initial fluorescence intensity (F/F₀) and expressed as relative fluorescence.

2.6. Chemicals

Chromatographically pure collagenase was purchased from Worthington (Lakewood, NJ, USA). Mg-Green was from Invitrogen (Eugene, OR, USA). Forskolin was obtained from Tocris (Bristol, UK) and made up as a 50 mM stock solution in dimethyl sulphoxide (DMSO). Palmitoleic acid ethyl ester (POAEE) was purchased from Cayman Chemical (Estonia, Tallinn). PAR-2 antagonist (PAR-2-ANT, H-Phe-Ser-Leu-Leu-Arg-Try-NH₂) and PAR-2 activating peptide (PAR-2-AP, H-Ser-Leu-Ile-Gly-Arg-Leu-amin trifluoroacetate salt) was from Peptides International (Louisville, Kentucky, USA). All other chemicals were purchased from Sigma-Aldrich (Budapest, Hungary) and were of the highest grade available. Palmitoleic acid (POA) and POAEE were made up as a 10 mM stock solution in DMSO, and on the day of the experiment, an appropriate quantity of the stock POA was dissolved in Krebs or HEPES. Emulsification of POA was achieved by sonication.

2.7. Statistics

Data are expressed as means ± SEM. Significant difference between groups was determined by Student's t-test or by analysis of variance. Probability values ≤ 0.05 were accepted as significantly different.
3. RESULTS

3.1. Measurement of CFTR conductance in guinea pig PDECs

Our experimental conditions were designed to selectively study CFTR channel activity in PDECs as described previously. In order to block potassium conductances, we used a CsCl-rich pipette solution, whereas Ca$^{2+}$-activated Cl$^{-}$ currents were inhibited by using 5 mM intracellular EGTA to lower Ca$^{2+}$ levels to ~10 nM. After establishing a whole cell recording, a small resting current was observed (23±2.5 pA/pF at ± 60 mV; Fig. 3A—i), with a mean reversal potential of −5.6 mV, which is very close to the Cl$^{-}$ equilibrium potential (~ −5.7 mV; Fig. 3B).

Fig. 3. CFTR Cl$^{-}$ currents in single native guinea pig PDECs. Whole cell currents were obtained by applying 500 ms duration voltage-clamp steps ranging between −100 and +100 mV in 20 mV steps from a holding potential of 0 mV in a normal bath solution with a CsCl-rich pipette solution. (A) Representative whole cell currents recorded before stimulation (i), during stimulation with forskolin (5 µM) (ii) and after washout of forskolin from the bath (iii). (B) Current/voltage (I/V) relationships were obtained by measuring currents over a 4 ms period starting 495 ms into the voltage pulse. The currents were measured under basal conditions (diamonds), during exposure to forskolin (squares) and after washout of forskolin from the bath (triangles). (C, D) Summary of current densities (measured at +60 mV) in basal (C) and in forskolin-stimulated (D) cells in the presence of CFTR inhibitor, CFTRinh-172 (10 µM). The current densities have been normalised to the cell input capacitance. Data are presented as means ± SEM. *p <0.05 vs forskolin, n =7–10.

14
Administration of 5 µM forskolin (cAMP agonist) to the bathing solution increased whole cell currents by approximately tenfold in 70% of cells (Fig. 3A—ii). The forskolin-activated currents were voltage-independent with a linear I/V relationship (Fig. 3B) and decreased after removal of forskolin from the external solution (Fig. 3A—ii). To confirm that the basal and stimulated currents were both mediated by CFTR, we tested CFTRinh-172, a potent inhibitor of the channel. Administration of 10 µM CFTRinh-172 reversibly and significantly blocked the basal currents by 58.2 ± 5.7% (at + 60 mV; Fig. 3C) and forskolin-stimulated currents by 66.6 ± 6.4% (at + 60 mV; Fig. 3D), providing further evidence that under our experimental conditions the whole cell currents were due to CFTR.

3.2. EtOH increases the basal but inhibits the forskolin-stimulated CFTR currents

We examined the effect of 1–100 mM EtOH on both basal and forskolin-stimulated whole cell currents. Exposing duct cells to 1 mM EtOH had no significant effect on basal or forskolin-activated CFTR currents, even after 15 min of administration (Fig. 4C, F). Increasing the EtOH concentration to 10 mM significantly increased the basal current by 32.6 ± 10.2% after 15 min of administration (at + 60 mV; Fig. 4A–C), whereas 100 mM EtOH induced a robust increase within 5 min (110.1 ± 7.2% at + 60 mV; Fig. 4A–C). In contrast, both 10 and 100 mM EtOH blocked the forskolin-stimulated whole cell currents (31.4 ± 9.6 and 66.5 ± 5.4%, respectively, after 15 mins at + 60 mV; Fig. 4D–F). Both the stimulatory and inhibitory effects of EtOH were reversible on washout. In addition, block by EtOH was not voltage dependent (Fig. 4B, E).
Fig. 4. Effect of EtOH on basal and forskolin-stimulated CFTR currents in single native guinea pig PDECs. Whole cell currents were recorded as described in Fig. 3. **Basal conditions (A–C):** (A) representative whole cell currents recorded under basal conditions (i), after 15 min from administration of EtOH (100 mM) (ii) and after washout of EtOH from the bath (iii). (B) Current–voltage (I/V) relationships were obtained as described in Fig. 3. The currents were measured under basal conditions (diamonds), during 15 min of exposure to 100mM EtOH (squares) and after washout of EtOH from the bath (triangles). (C) Summary of current densities (measured at +60 mV) in control, in the presence of EtOH (1, 10 and 100 mM) and after washout of EtOH from the bath. The current densities have been normalised to the cell input capacitance. Data are presented as means ± SEM. *p<0.05 vs control, n=10–13. **Forskolin-stimulated cells (D–F):** (D) representative whole cell currents recorded before stimulation (i), during stimulation with forskolin (5 µM)
(ii), in the presence of 100 mM EtOH (15 min) (iii), after removal of EtOH from the bath (iv) and after washout of forskolin from the bath (v). (E) Current/voltage (I/V) relationships were obtained as described in Fig. 3. The currents were measured under basal conditions (diamonds), during stimulation with forskolin in the absence (squares) and presence (triangles) of EtOH (100 mM) and after washout of forskolin from the bath (circles). (F) Summary of current densities (measured at + 60 mV) in control, in forskolin-stimulated cells, in the presence of EtOH (1, 10 and 100 mM, respectively) and after washout of forskolin and EtOH from the bath. The current densities have been normalised to the cell input capacitance. Data are presented as means ± SEM. *p <0.05 vs forskolin, n =10–13.

The rate of the mannitol-induced increase in whole cell currents was similar to the rate of EtOH-induced increase, indicating that the stimulatory effect of a high concentration of EtOH on the basal current is likely to be via an osmotic effect. In contrast to EtOH, mannitol did not decrease but significantly increased the forskolin-stimulated currents by 49.7±10.2 % (after 15 mins at + 60 mV; Fig. 5B), suggesting that the inhibitory effect of EtOH is independent of its osmotic effect.

Fig. 5. Effect of mannitol on basal and forskolin-stimulated CFTR currents in single native guinea pig PDECs. Summary of current densities (measured at + 60 mV) in basal (A) and forskolin-stimulated cells (B) in the presence of 177 mM mannitol. The current densities have been normalised to the cell input capacitance. Data are presented as means ± SEM. *p <0.05 vs control, **p <0.05 vs forskolin, n =6–7
3.3. Effects of EtOH metabolites on basal and forskolin-stimulated CFTR currents

Ac is the end product of oxidative EtOH degradation, whereas the non-oxidative degradation of EtOH produces FAEEs. Using the same conditions as the EtOH experiments, neither 1 nor 5 mM Ac had any effect on basal or forskolin-stimulated CFTR currents (data not shown, n = 5).
Fig. 6. Effect of POAEE on basal and forskolin-stimulated CFTR currents in single native guinea pig PDECs.
Whole cell currents were recorded as described in Fig. 3. Basal conditions (A–C): (A) representative whole cell currents recorded under basal conditions (i), after 5 min of administration of POAEE (200 µM) (ii) after 10 min of administration of POAEE (200 µM) (iii) after 15 min of administration of POAEE (200 µM) (iv) and after washout of POAEE from the bath (v). (B) Current–voltage (I/V) relationships were obtained as described in Fig. 3. The currents were measured under basal conditions (diamonds), during 15 min of exposure to 200 µM POAEE (squares) and after washout of POAEE from the bath (triangles). (C) Summary of current densities (measured at + 60 mV) in control, in the presence of POAEE (10, 100 and 200 µM, respectively) and after washout of POAEE from the bath. The current densities have been normalised to the cell input capacitance. Data are presented as means ± SEM. *p <0.05 vs control, n =10–13. Forskolin-stimulated cells (D–F): (D) representative whole cell currents recorded before stimulation (i), during stimulation with forskolin (5 µM) (ii), in the presence of 200 µM POAEE (15 min) (iii), after removal of POAEE from the bath (iv) and after washout of forskolin from the bath (v). (E) Current/voltage (I/V) relationships were obtained as described in Fig. 3. The currents were measured under basal conditions (diamonds), during stimulation with forskolin in the absence (squares) and presence (triangles) of POAEE (200 µM) and after washout of forskolin from the bath (circles). (F) Summary of current densities (measured at + 60 mV) in control, in forskolin-stimulated cells, in the presence of POAEE (10, 100 and 200 µM, respectively) and after washout of forskolin and POAEE from the bath. The current densities have been normalised to the cell input capacitance. Data are presented as means ± SEM. *p <0.05 vs forskolin, n =8-10.

POAEE, an unsaturated non-oxidative metabolite of EtOH, also had no effect on the basal currents over the concentration range of 10–200 µM (Fig. 6A–C). However, in marked contrast, 200 µM POAEE was able to block the forskolin-stimulated CFTR currents by 56.1± 12.3 % after 15 min of exposure (at + 60 mV; Fig. 6D–F).

The unsaturated FA, POA, at low concentrations (10 µM) did not affect the basal or forskolin-stimulated CFTR currents (Fig. 7C, F). However, at higher concentrations (100 and 200 µM), POA induced a dose-dependent and significant decrease in both the basal currents (63.6±5.2 and 68.4±5.5 % at+60 mV, respectively; Fig. 7A–C) and forskolin-stimulated CFTR currents (72.1±4.2 and 70.1±6.7 % at + 60 mV, respectively; Fig. 7D–F). The inhibitory effect of POAEE and POA (100 and 200 µM) reached a maximum after 5 and 15 min of administration, respectively, and were always reversible on washout.

The formation of FAEEs from EtOH and FAs is catalysed by the enzyme FAEE synthase, whereas FAEE hydrolase is responsible for the reverse reaction. The highest activities of FAEE synthases/hydrolases are found in the liver and pancreas. A number of FAEE synthases have been identified, and among them pancreatic cholesterol ester synthase and pancreatic carboxylester synthase have been shown to possess some FAEE-synthesizing activity in the pancreas. Although several studies have demonstrated that FAEEs are involved in organ injury, we wanted to test whether the inhibitory effect of POAEE depended on its hydrolysis. For this, we treated the cells with 200 µM bis-(4-nitrophenyl) phosphate (BNPP), an inhibitor of FAEE hydrolase. Fifteen minutes of
treatment with this agent did not change the basal or forskolin-stimulated whole cell currents, indicating that BNPP alone has no effect on CFTR activity. In contrast, BNPP treatment almost completely abolished the inhibitory effect of POAEE on forskolin-stimulated currents (187.3±12.9 to 141.6±4.7 pA/pF; 15 min of administration of POAEE at +60 mV), strongly suggesting that formation of POA is a crucial step in the inhibitory effect of POAEE/EtOH.

Fig. 7. Effect of POA on basal and forskolin-stimulated CFTR currents in single native guinea pig PDECs. Whole cell currents were recorded as described in Fig. 3. Basal conditions (A–C): (A) Representative whole cell currents recorded under basal conditions (i), after 5 min of administration of POA (200 µM) (ii) after 10 min of administration of POA (200 µM) (iii) after 15 min of administration of POA (200 µM) (iv) after 15 min of administration of POA (200 µM) (v) after washout. (B) Current density (µA/pF) as a function of voltage (mV) for control (■) and after 5 min (□), 10 min (△), 15 min (∇) of administration of POA (200 µM). (C) Current density (µA/pF) as a function of time (min) for control (■), 5 min (□), 10 min (△), 15 min (∇) of administration of POA (200 µM). (D) Representative whole cell currents recorded after 5 min of administration of forskolin (200 µM) (i), after 5 min of administration of forskolin (200 µM) and POA (200 µM) (ii), after 15 min of administration of forskolin (200 µM) and POA (200 µM) (iii) after 15 min of administration of forskolin (200 µM) and POA (200 µM) (iv) after washout. (E) Current density (µA/pF) as a function of voltage (mV) for control (■) and after 5 min (□), 10 min (△), 15 min (∇) of administration of forskolin (200 µM) and POA (200 µM). (F) Current density (µA/pF) as a function of time (min) for control (■), 5 min (□), 10 min (△), 15 min (∇) of administration of forskolin (200 µM) and POA (200 µM).
POA (200 µM) (iii) after 15 min of administration of POA (200 µM) (iv) and after washout of POA from the bath (v). (B) Current–voltage (I/V) relationships were obtained as described in Fig. 3. The currents were measured under basal conditions (diamonds), during 15 min of exposure to 200 µM POA (squares) and after washout of POA from the bath (triangles). (C) Summary of current densities (measured at +60 mV) in control, in the presence of POA (10, 100 and 200 µM, respectively) and after washout of POA from the bath. The current densities have been normalised to the cell input capacitance. Data are presented as means ± SEM. *p <0.05 vs control, n =10–13. Forskolin-stimulated cells (D–F): (D) representative whole cell currents recorded before stimulation (i), during stimulation with forskolin (5 µM) (ii), in the presence of 200 µM POA (15 min) (iii), after removal of POA from the bath (iv) and after washout of forskolin from the bath (v). (E) Current/voltage (I/V) relationships were obtained as described in Fig. 3. The currents were measured under basal conditions (diamonds), during stimulation with forskolin in the absence (squares) and presence (triangles) of POA (200 µM) and after washout of forskolin from the bath (circles). (F) Summary of current densities (measured at +60 mV) in control, in forskolin-stimulated cells, in the presence of POA (10, 100 and 200 µM, respectively) and after washout of forskolin and POA from the bath. The current densities have been normalised to the cell input capacitance. Data are presented as means ± SEM. *p <0.05 vs forskolin, n =8-10.

3.4. EtOH, POAEE and POA cause ATP

We next investigated the intracellular mechanism by which EtOH, and its non-oxidative metabolites, exerts its inhibitory effect on CFTR. Previous studies have shown that EtOH and monounsaturated FAs, such as POA, promote opening of the permeability transition pore, which leads to mitochondrial depolarization and finally cell death.\cite{35, 36} Since no data are available regarding the effect of FAs on mitochondrial function in PDECs, we investigated the effect of EtOH, and its non-oxidative metabolites, on mitochondrial injury using microfluorometric technique. PDECs were loaded with the Mg\textsuperscript{2+}-sensitive fluorescent dye, MgGreen-AM, which allows an indirect measurement of ATP\textsubscript{i}.\cite{29} Administration of 100 mM EtOH caused a small ATP\textsubscript{i} depletion in 75% of cells (Fig. 8A–C), whereas POA or POAEE (200 µM) induced a marked increase in MgGreen fluorescence intensity, which reached a plateau about 5 min after the administration of the metabolites (Fig. 8A–C). We have previously shown that inhibitors of both oxidative and glycolytic metabolism, carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 100 µM) and 2-deoxyglucose/iodoacetamide (DOG/IAA, 10 mM), respectively, caused a marked and irreversible depletion of ATP\textsubscript{i} in guinea pig pancreatic ductal cells.\cite{37}
Fig. 8. EtOH, POA and POAEE cause ATP<sub>i</sub> depletion in single native guinea pig PDECs and reduce the activity of CFTR. (A) Representative curves of the MgGreen fluorescence experiments. A decrease in [ATP]<sub>i</sub> is shown by an increase in fluorescence intensity. High concentration of EtOH (100 mM), POAEE (200 µM) and POA (200 µM) induced significant ATP<sub>i</sub> depletion after 15 min of administration. (B) Summary data for the maximal fluorescence intensity changes. Data are shown as means ± SEM from 25–35 ROIs in three to five experiments for all groups. *p<0.05 vs forskolin, **p <0.05 vs control. (C) Light (1) and fluorescent images (2 and 3) of isolated pancreatic ductal cells showing the effects of 100 mM EtOH, 200 µM POAEE or 200 µM POA on [ATP]<sub>i</sub>. Pictures were taken before (1 and 2) and after (3) exposure of the cells to EtOH, POAEE or POA. (D) Summary of current densities (measured at +60 mV) for basal and forskolin-stimulated (E) cells in the presence of a mixture of 100 µM CCCP and 10 mM DOG/IAA. The current densities have been normalised to the cell input capacitance. Data are presented as means ± SEM. *p<0.05 vs control and forskolin, n =3. N.D. not detected

In order to decide whether the inhibitory effect of EtOH, POA and POAEE on CFTR was linked to the depletion of ATP<sub>i</sub>, we tested the effect of CCCP/DOG/IAA administration on CFTR currents and found that this combination of inhibitors blocked both the basal (Fig. 8D) and forskolin-stimulated currents (Fig. 8E) in a similar manner to EtOH, POA and POAEE. After the removal of CCCP/DOG/IAA from the external solution, the currents did not return to the initial levels, indicating that CCCP/DOG/IAA irreversibly inhibit CFTR, probably due to the maintained depletion of ATP<sub>i</sub>.
3.5. Intracellular ATP supplementation reverses the inhibitory effect of EtOH, POAEE and POA on CFTR activity in PDECs

To investigate further the apparent relationship between the inhibitory effect of EtOH, POA and POAEE on CFTR channel activity and depletion of ATP$_i$, we tested whether raising ATP$_i$ levels in PDECs would result in a better tolerance to these agents. Increasing the ATP$_i$ concentration in the pipette solution from 1 mM to either 2 or 5 mM did not produce a detectable alteration in the size, or properties, of the basal or forskolin-stimulated CFTR currents (data not shown), suggesting that ATP was not limiting for CFTR activity under all conditions. Next, we studied the effect of 100 mM EtOH, 200 µM POAEE or 200 µM POA on CFTR currents in the presence of 2 mM ATP in the pipette. Supplementation of the pipette solution with 2 mM ATP did not influence the effects of EtOH or its non-oxidative metabolites on CFTR currents (data not shown). In contrast, increasing the ATP concentration to 5 mM completely prevented the inhibitory effect of EtOH (Fig. 9A) and POAEE (Fig. 9B) on forskolin-stimulated currents, as well as the effect of POA on the basal (Fig. 9C) and forskolin-stimulated CFTR currents (Fig. 9D). These results indicate that inhibition of CFTR by EtOH//POAEE/POA is likely due to a reduction of ATP$_i$ and/or to a change in ATP$_i$ interaction with CFTR.
Intracellular ATP supplementation reversed the inhibitory effect of EtOH, POAEE and POA on CFTR activity in guinea pig PDECs. Summary of current densities (measured at + 60 mV) in forskolin-stimulated cells in the presence of 100 mM EtOH (A), 200 µM POAEE (B) and 200 µM POA (C) and under basal conditions in the presence of 200 µM POA (D). Current densities have been normalised to the cell input capacitance. Data are presented as means ± SEM. *p <0.05 vs. forskolin, **p <0.05 vs. control, n =5–10.

3.6. Low concentration of EtOH stimulates whereas high concentration os EtOH and fatty acids inhibit forskolin-stimulated CFTR current in human PDECs

To extend these studies to human PDECs, we used a human ductal adenocarcinoma cell line, Capan-1, which has been shown to express CFTR. We used the same concentrations of EtOH, POA and POAEE than on guinea pig PDEC and we found similar results. Consequently, patch-clamp experiments revealed that 10 mM EtOH increased, whereas, 100 mM EtOH or 200 µM POA significantly decreased forskolin-stimulated CFTR Cl⁻ currents (Fig.10). In both cases, inhibition was voltage-independent and irreversible (unpublished data).
Fig. 10. Effect of EtOH and its metabolites on forskolin-stimulated CFTR currents in CAPAN-1 cell line.

(A) Representative fast whole cell CFTR Cl− current recordings from Capan-1 cells. (i) Unstimulated currents, currents after 10 min stimulation with 10µM forskolin (Forsk), and (iii) stimulated currents following 10 min exposure to 10 or 100mM ethanol and 200µM POA (iv) I/V relationships. Diamonds represent unstimulated currents, squares represent forskolin-stimulated currents, and triangles represent forskolin stimulated currents in the presence of the tested agents. 

(B) Summary of the current density (pA/pF) data measured at Erev ±60 mV. The current densities have been normalised to the cell input capacitance. Exposing the Capan-1 cells to 10mM ethanol stimulated, 100mM ethanol or 200µM POA blocked the forskolin-stimulated CFTR Cl− currents. n: 5-6 for all groups. a: p<0.05 vs basal current; b: p<0.05 vs forskolin-stimulated current.
3.7. ATP$_i$ supplementation in human PDECs

Next we investigated the effect of ATP$_i$ supplementation on the inhibitory effect of POA. Similar to guinea pig PDECs, 200 µM POA decreased the forskolin-stimulated CFTR currents by 69.7±3.2 % (from 86.7±7.1 to 27.7±6.8 pA/pF at + 60 mV; Fig. 11A, B). Administration of 2 mM intracellular ATP did not affect the inhibitory effect of POA; however, in the presence of 5 mM intracellular ATP, this inhibition was significantly reduced to 36.1±3.2 % (Fig. 11A, B).

![Figure 11](image_url)

Fig. 11. Effect of intracellular ATP supplementation in Capan-1 cells exposed to POA. (A) Representative whole cell currents recorded under basal conditions (i), after administration of forskolin (5 µM) (ii), after 15 min of administration of POA (200 µM) (iii), in the presence of 1.0 mM ATP (top set of traces) or 5.0 mM ATP (bottom set of current traces). Current–voltage (I/V) relationships were obtained as described in Fig. 2. The currents were measured under basal conditions (diamonds), during stimulation with forskolin (squares) and in the presence of 200 µM POA (triangles). (B) Summary of current densities measured at + 60 mV. Current densities have been normalised to the cell input capacitance. Data are presented as means ± SEM. a p<0.05 vs forskolin, b p< 0.05 vs 1 mM ATP (200 µM POA), n =8–12
3.8. Trypsin and PAR-2-AP inhibit CFTR

Exposure of PDECs to 10 µM trypsin did not affect the basal CFTR currents; however, administration of either 10 µM PAR-2-AP (Fig. 12A (iii)) or 10 µM trypsin (Fig. 12B (iii)) inhibited forskolin-stimulated CFTR currents by 51.7% ± 10.5% and 57.4% ± 4.0%, respectively. In both cases, the inhibition was voltage independent and irreversible. Pretreatment with either soybean trypsin inhibitor (SBTI; 10 µM; Fig. 10C (iii)) or PAR-2 antagonist (PAR-2-ANT; 10 µM; Fig. 12D (iii)) completely prevented the inhibitory effect of trypsin on the forskolin-stimulated CFTR currents. Fig. 12E is a summary of these data, which suggest that trypsin inhibits CFTR currents by activation of PAR-2.
Figure 12. Effects of trypsin and PAR-2-AP on CFTR Cl- currents of guinea pig pancreatic duct cells.
Representative fast whole cell current recordings from PDECs. (A–D) (i) Unstimulated currents, (ii) currents after stimulation with 5 µM forskolin, and (iii) currents following 3 minute exposure to (A) 10 µM PAR-2-AP, (B) 10 µM trypsin, (C) 10 µM trypsin/5 µM SBTI, and (D) 10 µM trypsin/10 µM PAR-2-ANT. (iv) I/V relationships. The currents were measured under basal condition (diamonds), during stimulation with forskolin (squares), and triangles represent forskolin-stimulated currents in the presence of the tested agents (see previous text). (E) Summary of the current density (pA/pF) data obtained from A–D measured at E_rev ± 60 mV. Exposing PDECs to either PAR-2-AP or trypsin blocked the forskolin-stimulated CFTR Cl- currents, while administration of SBTI or PAR-2-ANT prevented the inhibitory effect of trypsin. n = 6 for all groups. *: p<0.05 vs the unstimulated cells, **: p<0.05 vs forskolin. FORSK, forskolin; TRYP, trypsin, PAR-AP, PAR-2 activating peptide; PAR2-ANT, PAR-2 antagonist; SBTI: soybean trypsin inhibitor.
4. DISCUSSION

AP is a sudden inflammation of the pancreas. The severity of the disease can vary from a mild to a fulminant disorder with high morbidity and mortality. Most cases of AP are closely linked to heavy alcohol consumption, therefore a number of studies have investigated the effects of EtOH on the pancreas. Most of these studies are focused on the effect of EtOH on acinar and stellate cells, however much less research have been done on ductal cells.

One of the major functional cell types of the exocrine pancreas is the ductal cell. In these cells, the CFTR Cl⁻ channel operate paralell with the Cl/HCO₃⁻ exchangers on the apical membrane to provide a daily supply of ~2.5 L of a HCO₃⁻ rich fluid secretion, which is essential for normal exocrine gland function. Mutations in the cftr gene are often associated with pancreatitis. Several loss of function CFTR mutations have been shown to impair ductal fluid and HCO₃⁻ secretion and lead to the development of acute or chronic pancreatitis or are responsible for certain cases of idiopathic pancreatitis. In addition, the frequency of CFTR mutations in patients with alcoholic pancreatitis is twice as high compared to the general population. In contrast, the functional insufficiency of normal CFTR and its role in alcoholic pancreatitis is less characterized. In the present study, we show that EtOH, its non-oxidative metabolite POAEE and the FA, POA strongly inhibit CFTR Cl⁻ channel activity in guinea pig PDECs when measured using the whole cell configuration of the patch clamp technique. We demonstrated that 1 mM EtOH (blood alcohol level reached during moderate social drinking) had no effect on basal or forskolin-stimulated CFTR channel activity. However, at higher concentrations such as 10 or 100 mM (which is equivalent to a lethal blood alcohol level in humans), EtOH increased the basal and decreased the forskolin-stimulated CFTR currents. The present observation that low concentrations of EtOH (1 mM) had no effect whereas high concentrations (10 and 100 mM) inhibit secretagogue-stimulated CFTR activity is in agreement with previous observations that alcohol exerts dose-dependent effects on the pancreas. Using an equivalent concentration of mannitol, we show that the stimulatory effect of EtOH is likely to be due to an osmotic effect, whereas the inhibitory effect is independent of any the change in osmolarity.
Several experimental studies have shown a key role of EtOH metabolites in the development of pancreatitis. Therefore, in this study we also investigated the effect of the oxidative and non-oxidative EtOH metabolites on the activity of CFTR. Previous studies have demonstrated that Ac, an oxidative metabolite of EtOH, strongly inhibits NF-κB binding activity and cholecystokinin-induced secretion in isolated rat pancreatic acinar cells and, at higher concentrations, causes morphological alterations in the rat pancreas. In contrast, our study showed that Ac had no effect on CFTR activity or on ATP; furthermore, it did not cause any morphological changes in ductal cells even at high concentrations (5 mM), indicating that PDECs are probably not involved in the toxic effect of Ac in the pancreas. On the contrary to oxidative metabolism, the pancreas has a very high capacity to metabolise EtOH through a non-oxidative pathway, and consequently the concentration of these metabolites is higher in the pancreas than in any other organ. Previous studies have shown that at relatively low concentrations FAEEs are able to induce pancreatic injury, whereas at higher concentrations they cause toxic calcium signalling in acinar cells and disrupt the mitochondrial oxidative chain, which leads to ATP depletion and finally cell death. In addition, the pivotal role of FAEEs in the pathophysiology of alcoholic pancreatitis is also supposed by the fact that high concentrations of FAEEs have been detected in the pancreas at autopsy after acute alcohol intoxication and in chronic alcoholics. Our studies showed for the first time that the unsaturated FA, POA and its esterized form, POAEE reversibly and time-dependently inhibit CFTR activity in PDECs.

In the next step we wanted to identify the intracellular mechanism by which EtOH, POA and POAEE exert their inhibitory effect on CFTR. The CFTR Cl⁻ channel is a member of ATP-binding cassette (ABC) transporter superfamily, which also binds and hydrolyses ATP in order to drive conformational rearrangements of its transmembrane domains. Thus, a supply of ATP is essential for CFTR function, and in the absence of ATP the activity of CFTR dramatically decreases. Since FAs and FAEEs have previously been shown to cause ATP depletion in rat pancreatic acinar cells, we investigated if ATP depletion could underlie the effects of EtOH, POAEE and POA on CFTR activity in PDECs. Our results show that all three compounds decrease the level of ATP; in the ductal cells (Fig. 9). POA caused a significantly greater decrease in ATP; than POAEE. In addition, longer administration of POA did not cause a further increase in ATP; (as based on Mg-Green fluorescence intensity), indicating that 200 µM POA likely induced nearly complete ATP; depletion in PDECs. Consistent with this, inhibition of glycolytic and mitochondrial ATP production reduced CFTR currents to a similar degree to that
caused by EtOH, POA and POAEE, which strongly suggests that depletion of ATP_i is a key step in the inhibitory effect of these agents on CFTR. Based on these results, we tested whether administration of ATP_i back to the cell via the patch pipette is able to prevent the inhibitory effect of EtOH, POA and POAA on CFTR. Addition of 5 mM intracellular ATP almost completely abolished the inhibitory effect of these agents on CFTR activity (Fig. 8). Moreover similar results were found in Capan-1 cells, where supplementation of ATP_i was also able to decrease the inhibitory effect of POA on CFTR. 

In the second part of this thesis we investigated the effect of trypsin on CFTR activity. It is generally believed that the earliest events in acute pancreatitis is the zymogen activation which is mediated by lysosomal hydrolases such as cathepsin B and produces active trypsin. Active trypsin than activates other zymogens resulting in cell injury. Since acinar cells synthesize, store and secrete the inactive proforms of digestive enzymes and this cell type accounts for 80.3% of the total pancreas volume, most of the research on the effect of trypsin have been done on acinar cells. In contrast, much less studies have been done on ductal cells. PAR-2 is a specific receptor of trypsin and it has been shown that activation of this receptor stimulates enzyme secretion from acinar cells. In the ductal cells, activation of PAR-2 is able to modulate the rate of HCO_3^- secretion. These studies mainly focused on the Cl/HCO_3^- exchangers whereas no data is available regarding the effect of trypsin on the CFTR Cl^- channel. Therefore, our next challenge was to identify whether trypsin can reduce ductal bicarbonate secretion via PAR-2 dependent inhibition of the CFTR Cl^- channel in guinea pig PDECs using the whole cell configuration of the patch clamp technique. We showed, that both trypsin and PAR-2-AP irreversibly decreased forskolin-stimulated CFTR currents in PDECs and this inhibitory effect could be completely prevented by SBTI or PAR-2-ANT. 

Taken together we showed for the first time that high concentrations of EtOH, POAEE, POA and physiological concentration of trypsin and PAR-2-AP all strongly inhibit epithelial CFTR channel activity. Since the CFTR Cl^- channel plays an essential role in ductal electrolyte and fluid secretion, we hypothesise that functional inhibition of this channel may be involved in the pathogenesis of AP. In addition, we found that the inhibitory effect of EtOH, POAEE and POA is mediated through the depletion of ATP_i. Since the deleterious effects of EtOH and its metabolites could be prevented by maintaining cytosolic ATP levels we speculate that restoration of the intracellular energetic pool by ATP supplementation may represent a novel therapeutic target in the treatment of the disease.
5. ACKNOWLEDGEMENTS

There is a multitude of people who aided my efforts to finish my thesis. First of all, I would like to thank Prof. János Lonovics and Prof. Tibor Wittmann, past and present Head of First Department of Medicine, University of Szeged, for providing me with the possibility to do the PhD work in their department.

I would like to thank my supervisors for guiding me through this wonderful scientific adventure. I am clearly indebted to Dr. Viktória Venglovecz, who tirelessly instructed me to learn the elaborate patch-clamp method and Prof. Péter Hegyi, who introduced me into the scientific research of the pancreas. I deeply appreciate the valuable knowledge learnt from them.

I am also grateful to Prof. András Varró, the Head of Department of Pharmacology and Pharmacotherapy, who provided us the opportunity to work in his department.

I am really thankful to Prof. Barry E. Argent and Dr. Mike A. Gray our collaborators from the University of Newcastle, UK for their support and help in our projects.

I would also like to thank my colleagues at the First Department of Medicine and at the Department of Pharmacology and Pharmacotherapy for their help.

This work would not have been possible to accomplish without the assistance of Edit Magyarné Pálfí, Zoltánné Fuksz, Béláné Horesnyi, Miklósné Árva, Judit Lakatosné Ficzere, Rea Fritz and Zoltán Kocsispéter.

This work was supported by by Hungarian National Development Agency grants (TÁMOP-4.2.2.A-11/1/KONV-2012-0035, TÁMOP-4.2.2-A-11/1/KONV-2012-0052, TÁMOP-4.2.2.A-11/1/KONV-2012-0073, TÁMOP-4.2.4.A/2-11-1-2012-0001), the Hungarian Scientific Research Fund (OTKA NF105758, NF100677, K109756), the Hungarian Academy of Sciences (BO 00174/10/5 and BO/00531/11/5), and a European Pancreatic Club fellowship.
Last, but not least, I would like to thank to my family for their unflagging love and support throughout my life; this dissertation would have been impossible to accomplish without their help. I dedicate this thesis to them.
6. REFERENCES


36. Lamarche F, Carcenac C, Gonthier B, Cottet-Rousselle C, Chauvin C, Barret L, 
Leverve X, Savasta M, Fontaine E. Mitochondrial Permeability Transition Pore 
Inhibitors Prevent Ethanol-Induced Neuronal Death in Mice. Chem Res Toxicol 
2013.

37. Maleth J, Venglovecz V, Razga Z, Tiszlavicz L, Rakonczay Z, Jr., Hegyi P. Non- 
conjugated chenodeoxycholate induces severe mitochondrial damage and inhibits 

38. Pandol SJ, Saluja AK, Imrie CW, Banks PA. Acute pancreatitis: bench to the 

39. Pandol SJ, Periskic S, Gukovsky I, Zaninovic V, Jung Y, Zong Y, Solomon TE, 
Gukovskaya AS, Tsukamoto H. Ethanol diet increases the sensitivity of rats to 
pancreatitis induced by cholecystokinin octapeptide. Gastroenterology 

40. Gukovskaya AS, Mouria M, Gukovsky I, Reyes CN, Kasho VN, Faller LD, Pandol 
SJ. Ethanol metabolism and transcription factor activation in pancreatic acinar cells 

Ethanol differentially regulates NF-kappaB activation in pancreatic acinar cells 
through calcium and protein kinase C pathways. Am J Physiol Gastrointest Liver 

Chronic alcohol consumption accelerates fibrosis in response to cerulein-induced 

43. Criddle DN, Raraty MG, Neoptolemos JP, Tepikin AV, Petersen OH, Sutton R. 
Ethanol toxicity in pancreatic acinar cells: mediation by nonoxidative fatty acid 

44. Apte MV, Wilson JS. Stellate cell activation in alcoholic pancreatitis. Pancreas 


7. ANNEX
A kisztás fibrózis transzmembrán konduktancia regulátor Cl⁻ csatorna szerepe a pankreatitisz kialakulásában

Tézis kivonat

I. BEVEZETÉS

A pankreássz egyik leggyakrabban előforduló megbetegedése az akut pankreatitisz (AP). Az AP a hasnyálmirigy hirtelen fellépő gyulladása, amelynek kialakulásában az emésztőenzimek idő előtti aktiválódása játszik alapvető szerepet. Mivel a hasnyálmirigy állományának nagy részét az acinus sejtek teszik ki, illetve az AP során fellépő szöveti károsodás elsősorban az acinus sejteket érinti, az AP-el kapcsolatos vizsgálatok középpontjában az acinus sejtek tanulmányozása áll. Az 1980-as évektől fordult a figyelem a duktális epitél sejtek (PDEC) felé, amikor is egy sejtizolálási metodika segítségével lehetőség nyílt az intakt duk tuszok vizsgálatára. A PDEC legfőbb élettani szerepe, hogy egy HCO₃⁻-ban gazdag, izotónikus folyadéket szekretál, amely kimossa a duktális fából az emésztőenzimeket, valamint megfelelő pH-t biztosít a duodénumban az enzimek működéséhez. Az utóbbi évek kutatásai igazolták, hogy a duktális sejtek bikarbonát szekréciójában fontos szerepet játszhatnak a betegség kialakulásában és lefolyásában, ezért a duktális sejtek funkcionális elváltozásainak felderítése alapvető fontosságú lehet az akut pankreatitisz patogenezínek pontos megértéséhez, illetve terápiájának fejlődéséhez.

A humán pankreássz duktális epitél sejtek igen nagy, 140 mM koncentrációban képesek HCO₃⁻ szekrécióra, melyben számos transzporter, ioncsatorna játszik szerepet. Ezen ion csatornák közül kiemelkedő az apikális membránon található cisztás fibrózis transzmembrán konduktancia regulátor (CFTR) Cl⁻ csatorna szerepe. A CFTR egy cAMP által szabályozott ioncsatorna, amely elsősorban a Cl⁻ transzportjában vesz részt. Működése a Cl⁻/HCO₃⁻ kicserélővel szoros összhangban történik, biztosítva ezáltal a normál duktális folyadékszekréciót, amely a pankreássz integritásának megőrzéséhez elengedhetetlen. A CFTR alapvető szerepe, hogy Cl⁻-ot transzportál a duktális lumenbe és ezáltal biztosítja a Cl⁻ forrást a Cl⁻/HCO₃⁻ kicserélő működéséhez. Az utóbbi évek kutatásai rámutattak arra, hogy stimulált szekréció során a csatorna permeabilitása megváltozik és a Cl⁻ transzportja mellett a HCO₃⁻-ra is permeábilissá válik, azonban a mechanizmus nem teljesen tisztázott.
Köztudott, hogy a pankreatitisz kialakulásában az egyik fő etiológiai tényező a túlzott alkoholfogyasztás, míg a betegség pathomechanizmusában az idő előtt aktiválódott tripszin játszik alapvető szerepet.

Az etanol és a tripszin hatását a duktális sejtekben elsősorban a HCO$_3^-$ szekrécióra vizsgálták. Yamamoto és mtsai. kimutatták, hogy kis dózisú etanol hatására fokozódik a duktális sejtek szekretin-indukálta HCO$_3^-$ szekréciója, míg a nagy koncentrációban (100 mM) adott etanol esetén a duktális szekréció csökken. A tripszin esetében elég ellentmondásosak az eredmények. A tripszin hatása a HCO$_3^-$ szekrécióra nagymértékben függ a fajtól és a proteáz-aktiválta receptor -2 (PAR-2) lokalizációjától. Humán pankreász duktális sejtvonalban a tripszin fokozza, míg szarvasmarhából izolált PDEC-n gátolja a HCO$_3^-$ szekréciót. Továbbá a bazolaterális oldalon kifejeződő PAR-2 fokozza, míg az apikális oldalon kifejeződő PAR-2 gátolja a duktális HCO$_3^-$ szekréciót. Arra vonatkozóan azonban, hogyan befolyásolja az etanol vagy a tripszin a duktális CFTR csatorna működését nem lelhető fel adat az irodalomban.

II. CÉLKITŰZÉSEK

Kísérleteink során célul tüztük ki (i) az etanol (EtOH) és bomlástermékeinek vizsgálatát a CFTR csatorna aktivitására tengerimalac PDEC-ben és humán pankreász sejtvonalon, valamint a (ii) tripszin hatásának vizsgálatát a CFTR Cl$^-$ csatorna aktivitására tengerimalac PDEC-ben.

III. ANYAGOK ÉS MÓDSZEREK

**Intakt pankreász duktuszok izolálása**

Az intra/interlobuláris duktuszokat 150-250 gramm tömegű tengerimalacok hasnyálmirigyeiből izoláltuk. A hasnyálmirigyet kollagenáz enzimmel 30 perccig emészettük 37 °C-on, majd a duktuszokat sztereomikroszkóp alatt mikrodisszekciós technikával izoláltuk. Az izolált duktuszokat 37 °C-on 5% CO$_2$-t tartalmazó környezetben inkubáltuk 24 órán keresztül. Ezt követően az egyedi sejteket a duktuszokból 50 perces elasztázos emésztes és mikrodisszekciós technika kombinációjával nyertük.

**CAPAN-1 sejt vonal**

A Capan-1 sejteket Roswell Park Memorial Institute (RPMI)-1640 Medium oldatban tenyészettük kiegészítve 15 % magzati marha szérum, valamint 1 % penicillin–streptomycin
kombinációjával. A mérések során 20–60 passzázst elért sejteket használtunk. Az elektrofiziológiai vizsgálatokhoz 5x10³ sejtet szélesztettünk 24 mm átmérőjű üveg lemezekre. A kísérleteket 3-5 nap elteltével végeztük, amikor a sejtek transzepitéliális ellenállása (TER) szignifikáns mértékben megemelkedett és a konfluencia elérte a ~60–80 % -ot.

**Intracelluláris ATP (ATPᵢ) mérés**

Az ATPᵢ szint méréséhez Xcellence (Olympus, Hungary) képalkotó rendszert használtunk. A sejteket 5 µmol/L Magnesium-Green-AM festékkel 30 percig töltöttük szobahőmérsékleten. Tekintettel arra, hogy az ATP-nek az ADP-hez viszonyítva 10x erősebb a magnézium ionokat kötő képessége, a sejten belüli Mg²⁺ ionok nagy része Mg²⁺-ATP formájúban van jelen. Ennek következtében a Mg²⁺-Green által kibocsátott fluoreszcens jel arányos lesz az ADP/ATP hányadossal, ezáltal jól követhető az ATPᵢ szint változása. Ha a fluoreszcens intenzitás emelkedik, akkor az ATPᵢ szint csökken. A sejteket 476 nm-en világítottuk meg és a fluoreszcens intenzitást 535 nm-en gyűjtöttük.

**Teljes sejtes patch-clamp mérés**

A CFTR csatorna aktivitását patch-clamp technika teljes-sejt (whole-cell) konfigurációjával vizsgáltuk. A kísérleteket szobahőmérsékleten végeztük. Méréseinkhez Axopatch-1D erősítőt használtunk, feszültség-zár üzemmódban. Az ingerlő feszültség impulzusokat és az adatgyűjtést számítógép-vezérelt AxonDigidata 1200 A/D konverter és pCLAMP 6 szoftver segítségével végeztük. Méréseink során szükség esetén soros ellenállás kompenzációt alkalmaztunk( 50-80%), a soros ellenálláson eső feszültségből adódó hiba korrekciójának érdekében. A pipettákat boroszilikát (Clark) üvegkapillárisokból húztuk, majd hővel políroztuk a hegyüket, így a pipetták ellenállása 2.5-4 MOhm volt. Az oldatok cseréjét gravitáció által hajtott perfüziós rendszer segítségével valósítottuk meg, folyamatos elszívás mellett. A külső oldat összetétele mM-ban: 145 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES and 5 glucose (pH 7.4 ). A pipetta oldat összetétele mM-ban: 120 CsCl, 2 MgCl₂, 10 HEPES, 5 ethyleneglycol-bis(b-aminoethylether)-N,N,N8,N8-tetraacetic acid (EGTA) and 1 Na₂ATP (pH 7.2). A külső oldat őzomalaritása 300 mOsm/l volt, míg a pipetta oldaté 240 mOsm/l. A kísérleteink során az ATP koncentrációja 2-5 mM –ig növeltük.
IV. EREDMÉNYEK

Az etanol valamint bomlástermékeinek dózis- és időfüggő hatásának vizsgálata a CFTR Cl⁻ csatorna aktivitására

1. Az EtOH (100 mM), a palmitoleinsav etil-észter (POAEE; 200 µM) valamint a zsírsav, palmitoleinsav (POA; 100 és 200 µM) reverzibilisen gátolja a forskolin-stimulált CFTR csatornát a tengerimalac hasnyálmirigy duktális sejtekben, valamint humán pankreáisz sejtvonalon.

2. A POAEE gátló hatása a zsírsav- etil-észter hidroláz gátló, BNPP segítségével kivédhető volt.

3. Az EtOH, POAEE és POA hatására a sejten belüli ATP szint jelentős lecsökkent.

4. Az intracelluláris ATP pótlásával kivédhető volt az EtOH, POAEE valamint a POA CFTR csatornára gyakorolt gátló hatása.

A tripszin hatásának vizsgálata a CFTR Cl⁻ csatorna aktivitására

5. A tripszin (10 µM) gátolja a CFTR csatorna aktivitását, mely gátló hatás a PAR-2 receptoron keresztül valósul meg.

V. KÖVETKEZTETÉSEK

A CFTR Cl⁻ csatorna a hasnyálmirigy duktális sejtek luminális membránján elhelyezkedő anion csatorna, amely alapvető szerepet játszik a normál, duktális folyadékszekréció fenntartásában és ez által a hasnyálmirigy integritásának megőrzésében. Munkánk során elsőként mutattuk ki, hogy az EtOH, nem-oxidatív bomlásterméke, a POAEE, a zsírsav, POA valamint a tripszin gátolja a CFTR Cl⁻ csatorna aktivitását. Kísérleteink során kimutattuk, hogy a POAEE gátló hatásában a zsírsav keletkezése alapvető fontosságú. Továbbá azt találtuk, hogy az EtOH, POAEE és POA hatására markáns ATPi csökkenés következik be, mely alapvető szerepet játszik a CFTR csatornára kifejtett gátló hatásukban. Az ATPi visszaadásával mind az EtOH, POAEE és POA gátló hatása kivédhetővé vált.

Eredményeink azt mutatják, hogy a pankreatitisz kialakulásában szerepet játszó faktorok, úgy mint az EtOH vagy az idő előtti tripszin aktiválódás erőteljesen gátolják a CFTR Cl⁻ csatorna működését, amely alapvető szerepet játszhat az AP pathomechanizmusában. Továbbá eredményeink azt sugallják, hogy a sejten belüli ATP szint emelése egy új, terápiás lehetőséget jelenthet a betegség kezelésében.
Articles closely related to the subject of the thesis and cited in the thesis
I.
Ethanol and its non-oxidative metabolites profoundly inhibit CFTR function in pancreatic epithelial cells which is prevented by ATP supplementation

L. Judák · P. Hegyi · Z. Rakonczay Jr. · J. Maléth · M. A. Gray · V. Venglovecz

Received: 7 May 2013 / Revised: 26 July 2013 / Accepted: 30 July 2013 © Springer-Verlag Berlin Heidelberg 2013

Abstract Excessive alcohol consumption is a major cause of acute pancreatitis, but the mechanism involved is not well understood. Recent investigations suggest that pancreatic ductal epithelial cells (PDECs) help defend the pancreas from noxious agents such as alcohol. Because the cystic fibrosis transmembrane conductance regulator (CFTR) Cl\(^-\) channel plays a major role in PDEC physiology and mutated CFTR is often associated with pancreatitis, we tested the hypothesis that ethanol affects CFTR to impair ductal function. Electrophysiological studies on native PDECs showed that ethanol (10 and 100 mM) increased basal, but reversibly blocked, forskolin-stimulated CFTR currents. The inhibitory effect of ethanol was mimicked by its non-oxidative metabolites, palmitoleic acid ethyl ester (POAEE) and palmitoleic acid (POA), but not by the oxidative metabolite, acetaldehyde. Ethanol, POAEE and POA markedly reduced intracellular ATP (ATP\(_i\)) which was linked to CFTR inhibition since the inhibitory effects were almost completely abolished if ATP\(_i\) depletion was prevented. We propose that ethanol causes functional damage of CFTR through an ATP\(_i\)-dependent mechanism, which compromises ductal fluid secretion and likely contributes to the pathogenesis of acute pancreatitis. We suggest that the maintenance of ATP\(_i\) may represent a therapeutic option in the treatment of the disease.

Keywords Acute pancreatitis · Ethanol · CFTR · Fatty acids · ATP

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac</td>
<td>Acetaldehyde</td>
</tr>
<tr>
<td>AP</td>
<td>Acute pancreatitis</td>
</tr>
<tr>
<td>ATP(_i)</td>
<td>Intracellular ATP</td>
</tr>
<tr>
<td>BNPP</td>
<td>bis-(4-Nitrophenyl) phosphate</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide m-chlorophenyl hydrzone</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOG</td>
<td>2-Deoxyglucose</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FAEE</td>
<td>Fatty acid ethyl ester</td>
</tr>
<tr>
<td>MgGreen-AM</td>
<td>Magnesium Green-AM</td>
</tr>
<tr>
<td>PDECs</td>
<td>Pancreatic ductal epithelial cells</td>
</tr>
<tr>
<td>POA</td>
<td>Palmitoleic acid</td>
</tr>
<tr>
<td>POAEE</td>
<td>Palmitoleic acid ethyl ester</td>
</tr>
</tbody>
</table>

Introduction

Acute pancreatitis (AP) is a potentially fatal disease characterized by local complications (pancreatic necrosis, abscess and pseudocyst) and/or systemic organ failure. The overall mortality rate of AP is between 2.1 and 7.8 % worldwide [66]. This unacceptably high mortality rate is primarily due to the lack of specific clinical treatments. It is well known that heavy alcohol consumption is one of the leading causes of acute pancreatitis [49, 68]. The cellular mechanism by which ethanol induces pancreatitis is not completely understood. Most of...
the research into the pathogenesis of alcoholic pancreatitis has focused on the functional effects of ethanol on pancreatic acinar and stellate cells. Studies on acinar cells have shown that ethanol exerts its toxic effect by sensitizing acini to cholecystokinin-induced zymogen [34] and NF-κB activation [23] in vitro or to various forms of pancreatitis in vivo [52, 57]. In addition, Criddle et al. [15] have shown that at relatively high concentrations, ethanol induces a small increase in intracellular calcium concentration of mouse acini. Moreover, they found that the end products of non-oxidative ethanol degradation, fatty acid ethyl esters (FAEEs), were more toxic to the cells than ethanol itself because these FAEEs led to sustained intracellular calcium elevation and ATP depletion in acinar cells [15, 16]. The toxic effects of FAEEs on pancreatic acini were also confirmed in vivo [79]. Ethanol infusion in the presence of inhibitors of oxidative ethanol metabolism worsened the ethanol-induced pancreatic injury in the rat [79]. The effect of ethanol on pancreatic stellate cells has been mainly characterized by Apte et al. [2, 4, 45]. They showed that both ethanol and acetaldehyde (Ac) activate stellate cells through the switching on of mitogen-activated protein kinases. Activation of stellate cells then leads to pancreatic fibrosis, one of the characteristic features of alcoholic pancreatitis [2, 4, 45].

In contrast to acinar and stellate cells, the effect of ethanol, or its non-oxidative metabolites, on pancreatic ductal epithelial cells (PDECs) is much less characterized. PDECs secrete a bicarbonate-rich fluid which is crucially important in the maintenance of normal pancreatic function [6]. This isotonic, alkaline fluid neutralises protons secreted by acinar cells [27], curtails premature trypsinogen activation [51] and washes out digestive enzymes from the pancreas [6]. Impaired or decreased fluid secretion can lead to pancreatic injury or even pancreatitis [19, 20, 64]. Yamamoto et al. [81] showed that low concentrations (0.3–30 mM) of ethanol augmented ductal fluid secretion stimulated with physiological or pharmacological concentrations of secretin, whereas a high concentration of ethanol (100 mM) completely abolished this effect [81]. However, the cellular mechanisms underlying these responses to alcohol are unknown.

One of the key players in ductal fluid secretion is the cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channel which works closely with SLC26 anion exchangers to regulate normal ductal HCO3− and fluid secretion [53, 59, 71, 78]. It is well documented that mutations in the cfr gene are often associated with pancreatitis [19]; however, little data are available regarding the effect of ethanol, or its metabolites, on CFTR function. Recently, Raju et al. demonstrated that 24 h of preincubation of airway epithelial cells with 100 mM ethanol inhibited CFTR-mediated chloride secretion by directly affecting cellular cAMP levels [58].

In this article, we show that ethanol, its non-oxidative metabolites, palmitoleic acid ethyl ester (POAEE) and the fatty acid (FA) palmitoleic acid (POA), strongly inhibit CFTR Cl− channel activity via a reduction in intracellular ATP (ATPi) levels. Importantly, these deleterious effects on CFTR activity were prevented by increasing cytosolic ATP. We speculate that supplementation of ATPi in pancreatic ductal cells may represent a novel therapeutic tool in the treatment of acute alcohol-induced pancreatitis.

Materials and methods

Capan-1 cell culture

Capan-1 cells were grown in Roswell Park Memorial Institute (RPMI)-1640 Medium supplemented with 15 % fetal bovine serum, 1 % penicillin-streptomycin. Cells from passage numbers 20–60 were used in this study. For electrophysiological measurements, 5×105 cells were plated onto 24-mm-diameter glass coverslips and used for experiments after 3 to 4 days of culture (~60–80 % confluency).

Isolation of pancreatic ductal cells

Four- to eight-week-old guinea pigs were killed by cervical dislocation in accordance with protocols approved by the local ethical board of the University of Szeged. Intra/interlobular ducts were isolated by enzymatic digestion and microdissection from the pancreas and then cultured overnight as previously described [5]. To obtain single pancreatic ductal cells, cultured ducts were incubated for 50 min at 37 °C in 50 U/ml elastase dissolved in storage solution (Dulbecco's modified Eagle's medium containing 3 % (w/v) bovine serum albumin (pH 7.4 with NaOH)). The ducts were then washed in a Ca2+/Mg2+-free Kreb's HEPES buffer (pH 7.4) for 10 min further in order to stop elastase activity. After the incubation, ducts were teased apart using stainless steel needles. In the patch clamp experiments, one drop of cell suspension was placed within a transparent recording chamber, and single ductal cells were allowed to settle and attach to the bottom of the chamber for at least 30 min before perfusion was initiated. In the microfluorometric measurements, one drop of cell suspension was transferred to a poly-l-lysine-coated cover slip (24 mm) forming the base of a perfusion chamber. Cells were then washed continuously with solutions at a rate of 1.5 or 5–6 ml/min in the patch clamp and microfluorometric measurements, respectively. Individual ductal cells were used within 3–4 h after the isolation.

Whole cell current recording

Patch-clamp micropipettes were pulled from borosilicate glass capillaries using a P-97 Flaming/Brown micropipette puller (Sutter Co. Novato, CA, USA) and had resistances between 2.5 and 4 MΩ. Whole cell currents were recorded with
Axopatch-1D patch clamp amplifier (Molecular Devices-Axon Instruments, Union City, CA, USA) at 37 °C. The standard extracellular solution contained (in mM) 145 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES and 5 glucose (pH 7.4 with NaOH). The osmolarity of the external solution was 300 mOsm/l. The standard pipette solution contained (in mM) 120 CsCl, 2 MgCl₂, 10 HEPES, 5 ethylene glycol-bis(b-aminoethyl ether)-N,N,N′,N′,N⁵,N⁶-tetraacetic acid (EGTA) and 1 Na₂ATP (pH 7.2 with CsCl). The osmolarity of the pipette solution was 240 mOsm/l. In some experiments, the ATP concentration was increased to 2 or 5 mM. After establishing a high (1–10 GOhm) resistance seal by gentle suction, the cell membrane was disrupted by further suction or by applying a 1.5-V electrical pulse for 1–5 ms. Membrane currents were digitized using a 333-kHz analog-to-digital converter (Digidata 1200, Molecular Devices-Axon Instruments, Union City, CA, USA) after low-pass filtering at 1 kHz under software control (PCLAMP 6.0, Molecular Devices-Axon Instruments, Union City, CA, USA). To obtain current/voltage (I/V) relationships, the membrane potential (V_m) was held at 0 mV and then clamped to ±100 mV in 20-mV increments for 500 ms, with an 800-ms interval between each pulse. Currents were measured over a 4-ms period starting 495 ms into the voltage pulse. Mean current amplitudes were calculated at ±60 mV and normalised to cell capacitance (pF).

Measurement of intracellular ATP

Changes in intracellular ATP (ATP_i) were estimated using the Mg²⁺-sensitive fluorescent dye, Magnesium Green-AM (MgGreen-AM). Most of the Mg²⁺ ions in the cells are bound to ATP. Since ATP has a higher affinity for Mg²⁺ than ADP, upon ATP hydrolysis the concentration of cytosolic free Mg²⁺ increases [47]. Upon binding Mg²⁺, MgGreen exhibits an increase in fluorescence emission intensity which allows the indirect measurement of ATP_i. Cells were incubated with 5 μM MgGreen-AM in the presence of 0.05 % pluronic F-127 in standard HEPES solution (in mM: 130 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 D-glucose and 10 Na-HEPES; pH was set to 7.4 with HCl) for 30 min at room temperature. Five to ten cells (region of interest, ROIs) were then excited with light at a wavelength of 490 nm, with emitted light monitored at 535 nm. The fluorescence emissions were captured by a CCD camera and digitized by an Xcellence imaging system (Olympus, Budapest, Hungary). One ATP_i measurement was obtained per second. Fluorescence signals were normalised to initial fluorescence intensity (F/F₀) and expressed as relative fluorescence.

Chemicals

Chromatographically pure collagenase was purchased from Worthington (Lakewood, NJ, USA). Mg-Green was from Invitrogen (Eugene, OR, USA). Forskolin was obtained from Tocris (Bristol, UK) and made up as a 50-mM stock solution in dimethyl sulfoxide (DMSO). Palmitoleic acid ethyl ester was purchased from Cayman Chemical (Estonia, Tallinn). All other chemicals were purchased from Sigma (Budapest, Hungary) and were of the highest grade available. Palmitoleic acid and POAE were made up as a 10-mM stock solution in DMSO, and on the day of the experiment, an appropriate quantity of the stock POA was dissolved in Krebs or HEPES. Emulsification of POA was achieved by sonication.

Statistics

Data are expressed as means±SEM. Significant difference between groups was determined by Student's t-test or by analysis of variance. P values≤0.05 were accepted as significantly different.

Results

Measurement of CFTR conductance in guinea pig PDECs

Our experimental conditions were designed to selectively study CFTR channel activity in PDECs as described previously [50]. In order to block potassium conductances, we used a CsCl-rich pipette solution, whereas Ca²⁺-activated Cl⁻ currents were inhibited by using 5 mM intracellular EGTA to lower Ca²⁺ levels to ~10 nM. After establishing a whole cell recording, a small resting current was observed (23±2.5 pA/pF at ±60 mV; Fig. 1a—i), with a mean reversal potential of −5.6 mV, which is very close to the Cl⁻ equilibrium potential (~−5.7 mV; Fig. 1b). Administration of 5 μM forskolin (a cAMP agonist) to the bathing solution increased whole cell currents by approximately tenfold in 70 % of cells (Fig. 1a—ii). The forskolin-activated currents were voltage-independent with a linear I/V relationship (Fig. 1b) and decreased after removal of forskolin from the external solution (Fig. 1a—ii). To confirm that the basal and stimulated currents were both mediated by CFTR, we tested CFTRinh-172, a potent inhibitor of the channel [40, 46]. Administration of 10 μM CFTRinh-172 reversibly and significantly blocked the basal currents by 58.2±5.7 % (at ±60 mV; Fig. 1c) and forskolin-stimulated currents by 66.6±6.4 % (at ±60 mV; Fig. 1d), providing further evidence that under our experimental conditions the whole cell currents were due to CFTR.

Ethanol increases the basal but inhibits the forskolin-stimulated CFTR currents

We examined the effect of 1–100 mM ethanol on both basal and forskolin-stimulated whole cell currents. Exposing duct cells to 1 mM ethanol had no significant effect on basal or...
forskolin-activated CFTR currents, even after 15 min of administration. Increasing the ethanol concentration to 10 mM significantly increased the basal current by 32.6±10.2 % after 15 min of administration (at +60 mV; Fig. 2a–c), whereas 100 mM ethanol induced a robust increase within 5 min (110.1±7.2 % at +60 mV; Fig. 2a–c). In contrast, both 10 and 100 mM ethanol blocked the forskolin-stimulated whole cell currents (31.4±9.6 and 66.5±5.4 %, respectively, after 15 mins at +60 mV; Fig. 2d–f). Both the stimulatory and inhibitory effects of ethanol were reversible on washout. In addition, block by ethanol was not voltage dependent (Fig. 2d, e).

We next investigated the mechanism by which ethanol exerts its effects on CFTR. Since the plasma membrane is permeable to ethanol, it can diffuse into the cell and bind to water molecules which will disrupt the osmotic environment and lead to cellular dehydration. Under these conditions, the flow of ions and water could change. In order to decide whether ethanol has an indirect osmotic effect on CFTR or whether it can directly affect the channel or its regulation, we exposed cells to mannitol, a cell-impermeable molecule, in order to osmotically shrink the cells. An osmotically equivalent concentration of mannitol (177 mM) increased the basal CFTR currents by fourfold (from 24.4±3.5 to 97.5±22 pA/pF at +60 mV; Fig. 3a). The rate of the mannitol-induced increase in whole cell currents was similar to the rate of ethanol-induced increase, indicating that the stimulatory effect of a high concentration of ethanol on the basal current is likely to be via an osmotic effect. In contrast to ethanol, mannitol did not decrease but significantly increased the forskolin-stimulated currents by 49.7±10.2 % (after 15 mins at +60 mV; Fig. 3b), suggesting that the inhibitory effect of ethanol is independent of its osmotic effect.

Effects of ethanol metabolites on basal and forskolin-stimulated CFTR currents

Acetaldehyde (Ac) is the end product of oxidative ethanol degradation, whereas the non-oxidative degradation of ethanol produces fatty acid ethyl esters (FAEEs). Using the same conditions as the ethanol experiments, neither 1 nor 5 mM Ac had any effect on basal or forskolin-stimulated CFTR currents (data not shown, n = 5). POAEE, an unsaturated non-oxidative metabolite of ethanol, also had no effect on the basal currents over the concentration range of 10–200 μM (Fig. 4a–c). However, in marked contrast, 200 μM POAEE was able to block the forskolin-stimulated CFTR currents by 56.1±12.3 % after 15 mins of exposure (at +60 mV; Fig. 4d–f). The unsaturated FA, POA, at low concentrations (10 μM) did not affect the basal or forskolin-stimulated CFTR currents (Fig. 5c, f). However, at higher concentrations (100 and 200 μM), POA induced a dose-dependent and significant
Fig. 2 Effect of ethanol on basal and forskolin-stimulated CFTR currents in single native guinea pig PDECs. Whole cell currents were recorded as described in Fig. 1. Basal conditions (a–c): a representative whole cell currents recorded under basal conditions (i), after 15 min from administration of ethanol (100 mM) (ii) and after washout of ethanol from the bath (iii). b Current–voltage (I/V) relationships were obtained as described in Fig. 1. The currents were measured under basal conditions (diamonds), during 15 min of exposure to 100 mM ethanol (squares) and after washout of ethanol from the bath (triangles). c Summary of current densities (measured at +60 mV) in control, in the presence of ethanol (1, 10 and 100 mM) and after washout of ethanol from the bath. The current densities have been normalised to the cell input capacitance. Data are presented as means±SEM. *p<0.05 vs control, n=10–13. Forskolin-stimulated cells (d–f): d representative whole cell currents recorded before stimulation (i), during stimulation with forskolin (5 μM) (ii), in the presence of 100 mM ethanol (15 min) (iii), after removal of ethanol from the bath (iv) and after washout of forskolin from the bath (v). e Current/voltage (I/V) relationships were obtained as described in Fig. 1. The currents were measured under basal conditions (diamonds), during stimulation with forskolin in the absence (squares) and presence (triangles) of ethanol (100 mM) and after washout of forskolin from the bath (circles). f Summary of current densities (measured at +60 mV) in control, in forskolin-stimulated cells, in the presence of ethanol (1, 10 and 100 mM, respectively) and after washout of forskolin and ethanol from the bath. The current densities have been normalised to the cell input capacitance. Data are presented as means±SEM. *p<0.05 vs forskolin, n=10–13. EtOH ethanol
decrease in both the basal currents (63.6±5.2 and 68.4±5.5 % at+60 mV, respectively; Fig.5a–c) and forskolin-stimulated CFTR currents (187.3±12.9 to 141.6±4.7 pA/pF; 15 min of administration of POAEE at+60 mV), strongly suggesting that formation of POA is a crucial step in the inhibitory effect of POAEE/ethanol.

Ethanol, POAEE and POA cause ATPi depletion in PDECs, which is linked to CFTR inhibition

We next investigated the intracellular mechanism by which ethanol, and its non-oxidative metabolites, exerts its inhibitory effect on CFTR. Previous studies have shown that ethanol and monounsaturated FAs, such as POA, promote opening of the permeability transition pore, which leads to mitochondrial depolarization and finally cell death [22, 36]. Since no data are available regarding the effect of FAs on mitochondrial function in PDECs, we investigated the effect of ethanol, and its non-oxidative metabolites, on mitochondrial injury using a microfluorometric technique. PDECs were loaded with the Mg\(^{2+}\)-sensitive fluorescent dye, MgGreen-AM, which allows an indirect measurement of ATPi [7]. Administration of 100 mM ethanol caused a small ATPi depletion in 75 % of cells (Fig. 6a–c), whereas POA or POAEE (200 μM) induced a marked increase in MgGreen fluorescence intensity, which
reached a plateau about 5 min after the administration of the metabolites (Fig. 6a–c).

We have previously shown that inhibitors of both oxidative and glycolytic metabolism, carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 100 µM) and 2-deoxyglucose/iodoacetamide (DOG/IAA, 10 mM), respectively, caused a marked and irreversible depletion of ATP_i in guinea pig pancreatic ductal cells [42]. In order to decide whether the inhibitory effect of ethanol, POA and POAEE on CFTR was linked to the depletion of ATP_i, we tested the effect of CCCP/DOG/IAA administration on CFTR currents and found that this combination of inhibitors blocked both the basal (Fig. 6d) and forskolin-stimulated currents (Fig. 6e) in a similar manner to ethanol, POA and POAEE. After the removal of CCCP/DOG/IAA from the external solution, the currents did not return to the initial levels, indicating that CCCP/DOG/IAA...
Fig. 5 Effect of POA on basal and forskolin-stimulated CFTR currents in single native guinea pig PDECs. Whole cell currents were recorded as described in Fig. 1. Basal conditions (a–c): a representative whole cell currents recorded under basal conditions (i), after 5 min of administration of POA (200 μM) (ii), after 10 min of administration of POA (200 μM) (iii), after 15 min of administration of POA (200 μM) (iv) and after washout of POA from the bath (v). b Current–voltage (I/V) relationships were obtained as described in Fig. 1. The currents were measured under basal conditions (diamonds), during 15 min of exposure to 200 μM POA (squares) and after washout of POA from the bath (triangles). c Summary of current densities (measured at +60 mV) in control, in the presence of POA (10, 100 and 200 μM, respectively) and after washout of POA from the bath. The current densities have been normalised to the cell input capacitance. Data are presented as means±SEM. *p <0.05 vs control, n = 8–10. Forskolin-stimulated cells (d–f): d representative whole cell currents recorded before stimulation (i), during stimulation with forskolin (5 μM) (ii), in the presence of 200 μM POA (15 min) (iii), after removal of POA from the bath (iv) and after washout of forskolin from the bath (v). e Current–voltage (I/V) relationships were obtained as described in Fig. 1. The currents were measured under basal conditions (diamonds), during stimulation with forskolin in the absence (squares) and presence (triangles) of 200 μM POA (15 min) and after washout of forskolin from the bath (circles). f Summary of current densities (measured at +60 mV) in control, in forskolin-stimulated cells, in the presence of POA (10, 100 and 200 μM, respectively) and after washout of forskolin and POA from the bath. The current densities have been normalised to the cell input capacitance. Data are presented as means±SEM. *p <0.05 vs forskolin, n = 8–10.
irreversibly inhibit CFTR, probably due to the maintained depletion of ATP$_i$. Intracellular ATP supplementation reverses the inhibitory effect of ethanol, POAEE and POA on CFTR activity in PDECs

To investigate further the apparent relationship between the inhibitory effect of ethanol, POA and POAEE on CFTR channel activity and depletion of ATP$_i$, we tested whether raising ATP$_i$ levels in PDECs would result in a better tolerance to these agents. Increasing the ATP$_i$ concentration in the pipette solution from 1 mM to either 2 or 5 mM did not produce a detectable alteration in the size, or properties, of the basal or forskolin-stimulated CFTR currents (data not shown), suggesting that ATP was not limiting for CFTR activity under all conditions. Next, we studied the effect of 100 mM ethanol, 200 μM POAEE or 200 μM POA on CFTR currents in the presence of 2 mM ATP in the pipette. Supplementation of the pipette solution with 2 mM ATP did not influence the effects of ethanol or its non-oxidative metabolites on CFTR currents (data not shown). In contrast, increasing the ATP concentration to 5 mM completely prevented the inhibitory effect of ethanol (Fig. 7a) and POAEE (Fig. 7b) on forskolin-stimulated (e) cells in the presence of a mixture of 100 μM CCCP and 10 mM DOG/IAA. The current densities have been normalised to the cell input capacitance. Data are presented as means±SEM. *p <0.05 vs control and forskolin, n=3. N.D. not detected

Intracellular ATP supplementation reverses the inhibitory effect of ethanol, POAEE and POA on CFTR activity in PDECs

To extend these studies to human PDECs, we used a human ductal adenocarcinoma cell line, Capan-1, which has been shown to express CFTR [11], and studied the effect of ATP$_i$ supplementation on the inhibitory effect of POA. Similar to guinea pig PDECs, 200 μM POA decreased the forskolin-stimulated CFTR currents by 69.7±3.2 % (from 86.7±7.1 to 27.7±6.8 pA/pF at +60 mV; Fig. 8a, b). Administration of 2 mM intracellular ATP did not affect the inhibitory effect of POA; however, in the presence of 5 mM intracellular ATP, this inhibition was significantly reduced to 36.1±3.2 % (Fig. 8a, b).
Discussion

Long-term use of alcohol is associated with several gastrointestinal [62, 73] and neurological [48] disorders and is one of the leading causes of certain types of cancer [65]. Therefore, a number of studies have investigated the effects of ethanol on various tissues. In hepatocytes, ethanol (100 mM) decreases cell viability and induces lipid peroxidation [82], whereas chronic ethanol feeding damages the hepatic mitochondria in rats [10]. In the heart, chronic alcohol intake elevates blood pressure and leads to alcohol-induced heart muscle disease [39]. It has also been shown that a very high concentration of ethanol (1.7 M) inhibits intracellular protease activities in human brain, liver and muscle in vitro [44]. In addition, neuroradiological studies have demonstrated that the brain undergoes loss of both white and gray matter in chronic alcoholics [9].

The effect of ethanol on the pancreas is also well characterized since excessive alcohol consumption is one of the leading causes of both chronic and acute pancreatitis [21]. One of the major functional cell types of the exocrine pancreas is the ductal cell. In these cells, the CFTR Cl⁻ channel works closely with apical Cl⁻/HCO₃⁻ exchangers to provide a daily supply of ~2.5 L of a HCO₃⁻-rich fluid secretion, which is essential for normal exocrine gland function [70, 71, 78]. Mutations in the cftr gene are often associated with pancreatitis. Several loss of function CFTR mutations have been shown to impair ductal fluid and HCO₃⁻ secretion and lead to the development of acute [12, 35, 56] or chronic pancreatitis [12, 31, 56, 60, 72] or are responsible for certain cases of idiopathic pancreatitis [13, 14]. In addition, the frequency of CFTR mutations in patients with alcoholic pancreatitis is twice as high compared to the general population [67].

In contrast, the functional insufficiency of normal CFTR and its role in alcoholic pancreatitis is less characterized. In the present study, we show that ethanol, its non-oxidative metabolite POAEE and the FA POA strongly inhibit CFTR Cl⁻ channel activity in guinea pig PDECs when measured using the whole cell configuration of the patch clamp technique. We demonstrate that 1 mM ethanol (a blood alcohol level reached during moderate social drinking) had no effect on basal or forskolin-stimulated CFTR channel activity. However, at 200 μM POA (c) and under basal conditions in the presence of 200 μM POA (d). Current densities have been normalised to the cell input capacitance. Data are presented as means±SEM. *p<0.05 vs. forskolin, **p<0.05 vs. control, n=5–10
higher concentrations such as 10 or 100 mM (which is equivalent to a lethal blood alcohol level in humans [69]), ethanol increased the basal and decreased the forskolin-stimulated CFTR currents. The present observation that low concentrations (1 mM) had no effect whereas high concentrations (10 and 100 mM) inhibit secretagogue-stimulated CFTR activity is in agreement with previous observations that alcohol exerts dose-related effects on the pancreas [18]. Using an equivalent concentration of mannitol, we show that the stimulatory effect of ethanol is likely to be due to an osmotic effect, whereas the inhibitory effect is independent of any change in osmolarity.

Several experimental studies have shown a key role of ethanol metabolites in the development of pancreatitis [80]. The oxidative metabolism of ethanol is catalysed by alcohol dehydrogenase and predominantly occurs in hepatocytes. However, it has been postulated that pancreatic acinar cells are also able to oxidatively metabolise a significant amount of ethanol [23, 25]. It has been shown that Ac inhibits NF-κB binding activity [23] and cholecystokinin-induced secretion [63] in isolated rat pancreatic acinar cells and, at higher concentrations, causes morphological alterations in the rat pancreas [41]. In our study, Ac had no effect on CFTR activity or on ATP; furthermore, it did not cause any morphological changes in ductal cells even at high concentrations (5 mM), indicating that PDECs are probably not involved in the toxic effect of Ac in the pancreas.

In contrast to oxidative metabolism, the pancreas has a very high capacity to metabolise ethanol through a non-oxidative pathway [26], and consequently the concentration of these metabolites is higher in the pancreas than in any other organ [38]. In addition, FAEE synthase activity [3], the key enzyme which catalyses FAEE production, is significantly higher in the pancreas than in the liver [80]. The effects of non-oxidative ethanol metabolites on the pancreas have been widely investigated. At low concentrations, FAEEs (50 μM) are able to induce pancreatic injury [24], whereas at higher concentrations (200 μM) they cause toxic calcium signalling in acinar
cells and disrupt the mitochondrial oxidative chain, which leads to ATP\(_i\) depletion and finally cell death [15, 16]. In addition, the pivotal role of FAEEs in the pathophysiology of alcoholic pancreatitis is also supposed by the fact that high concentrations of FAEEs have been detected in the pancreas at autopsy after acute alcohol intoxication and in chronic alcoholics [38]. Here we show for the first time that the unsaturated FA POA and its esterized form POAEE, which strongly suggests that inhibition of glycolytic and mitochondrial ATP production and a sustained rise in cytosolic Ca\(^{2+}\).

Taken together, we show for the first time that high concentrations of ethanol, POAEE and POA all strongly inhibit epithelial CFTR channel activity through depletion of ATP\(_i\). Importantly, these deleterious effects of ethanol and its metabolites could be prevented by maintaining cytosolic ATP levels. Since the CFTR Cl\(^{-}\) channel plays an essential role in ductal electrolyte and fluid secretion, we hypothesise that functional inhibition of this channel may be involved in the pathogenesis of alcoholic pancreatitis. Because restoration of the intracellular energetic pool by ATP supplementation maintains CFTR function in the presence of these metabolites, it may represent a potential novel therapeutic option in the treatment of pancreatitis.

Acknowledgments Our research is supported by Hungarian National Development Agency grants (TÁMOP-4.2.2.A-11/1/KONV-2012-0035, TÁMOP-4.2.2.A-11/1/KONV-2012-0052, TÁMOP-4.2.2.A-11/1/KONV-2012-0073, TÁMOP-4.2.4.A/2-11-1-2012-0001), the Hungarian Scientific Research Fund (OTKA NF105758, NF100677, K109756), the Hungarian Academy of Sciences (BO 00174/10/5 and BO/00531/11/5), and a European Pancreatic Club fellowship to Linda Judák.

Conflict of interest The authors hereby declare that there is no conflict of interest to disclose.

References


II.
Trypsin Reduces Pancreatic Ductal Bicarbonate Secretion by Inhibiting CFTR Cl⁻ Channels and Luminal Anion Exchangers

PETRA PALLAGI,* VIKTÓRIA VENGOVECZ,‡ ZOLTÁN RAKONCZAY Jr.*, KATALIN BORKA,* ANNA KOROMPAY,§ BÉLA ÖZSVÁRI,* LINDA JUDÁK,* MIKLÓS SAHIN–TÓTH,† ANDREA GEISZ,*† ANDREAS SCHNÜR,† JÓZSEF MALETH,* TAMÁS TAKÁCS,* MIKE A. GRAY,¶ BARRY E. ARGENT,¶ JULIA MAYERLE,# MARKUS M. LERCH,‡ TIBOR WITTMANN,* and PÉTER HEGYI*†‡§

†First Department of Medicine and ‡Department of Pharmacology, University of Szeged, Szeged, Hungary; §Second Department of Pathology, Semmelweis University, Budapest, Hungary; †Department of Molecular and Cell Biology, Henry M. Goldman School of Dental Medicine, Boston University, Boston, Massachusetts; ‡Institute for Cell & Molecular Biosciences, Newcastle University, Newcastle upon Tyne, England; and *Department of Medicine A, Greifswald University Hospital, Greifswald, Germany

BACKGROUND & AIMS: The effects of trypsin on pancreatic ductal epithelial cells (PDECs) vary among species and depend on the localization of proteinase-activated receptor 2 (PAR-2). We compared PAR-2 localization in human and guinea-pig PDECs, and used isolated guinea pig ducts to study the effects of trypsin and a PAR-2 agonist on bicarbonate secretion. METHODS: PAR-2 localization was analyzed by immunohistochemistry in guinea pig and human pancreatic tissue samples (from 15 patients with chronic pancreatitis and 15 without pancreatic disease). Functionally, guinea pig PDECs were studied by microperfusion of isolated ducts, measurements of intracellular pH and intracellular Ca²⁺ concentration, and patch clamp analysis. The effect of pH on trypsinogen autoactivation was assessed using recombinant human cationic trypsinogen. RESULTS: PAR-2 localized to the apical membrane of human and guinea pig PDECs. Trypsin increased intracellular Ca²⁺ concentration and intracellular pH and inhibited secretion of bicarbonate by the luminal anion exchanger and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel. Autoactivation of human cationic trypsinogen accelerated when the pH was reduced from 8.5 to 6.0. PAR-2 expression was strongly down-regulated, at transcriptional and protein levels, in the ducts of patients with chronic pancreatitis, consistent with increased activity of intraductal trypsin. Importantly, in PAR-2 knockout mice, the effects of trypsin were markedly reduced. CONCLUSIONS: Trypsin reduces pancreatic ductal bicarbonate secretion via PAR-2-dependent inhibition of the apical anion exchanger and the CFTR Cl⁻ channel. This could contribute to the development of chronic pancreatitis by decreasing luminal pH and promoting premature activation of trypsinogen in the pancreatic ducts.

Keywords: Acinar Cells; Ductal Epithelium; Animal Model; Pancreatic Enzymes.

Trypsinogen is the most abundant digestive protease in the pancreas. Under physiologic conditions, trypsinogen is synthesized and secreted by acinar cells, transferred to the duodenum via the pancreatic ducts, and then activated by enteropeptidase in the small intestine. There is substantial evidence that early intra-acinar or luminal activation of trypsinogen to trypsin is a key and common event in the development of acute and chronic pancreatitis. Importantly, almost all forms of acute pancreatitis are due to autodigestion of the gland by pancreatic enzymes.

Several studies have shown that trypsin stimulates enzyme secretion from acinar cells via proteinase-activated receptor 2 (PAR-2), whereas the effect of trypsin on pancreatic ductal epithelial cells (PDECs) is somewhat controversial. Trypsin activates ion channels in dog PDECs and stimulates bicarbonate secretion in the CAPAN-1 human pancreatic adenocarcinoma cell line, whereas it dose-dependently inhibits bicarbonate efflux from bovine PDECs. The effect of trypsin differs not only among species, but also with respect to the localization of PAR-2. When PAR-2 is localized to the basolateral membrane and activated by trypsin, the result is stimulation of bicarbonate secretion. In contrast, when the receptor is localized to the luminal membrane, the effect is inhibition. Interestingly, there are no data concerning the effects of trypsin on guinea pig PDECs which, in terms of bicarbonate secretion, are an excellent model of human PDECs.

The human pancreatic ductal epithelium secretes an alkaline fluid that may contain up to 140 mmol/L NaHCO₃. The first step in HCO₃⁻ secretion is the accumulation of HCO₃⁻ inside the cell, which is driven by basolateral Na⁺/HCO₃⁻ cotransporters, Na⁺/H⁺ exchangers, and H⁺-adenosine triphosphatases. Only 2 transporters have been identified on the apical membrane of

Abbreviations used in this paper: BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; CFTR, cystic fibrosis transmembrane conductance regulator Cl⁻ channel; CFTRinh-172, CFTR inhibitor-172; [Ca²⁺]; intracellular Ca²⁺ concentration; H₂DIDS, dicydro-4,4'-disothio-dianostilbene-2,2'-dissulfonic acid; PAR-2, proteinase-activated receptor-2; PAR-2-AP, PAR-2 activating peptide; PAR-2-ANT, PAR-2 antagonist; PDEC, pancreatic ductal epithelial cell; pH, intracellular pH; pHᵥ, luminal pH; SBTI, soybean trypsin inhibitor; SLC26, solute carrier family 26.

© 2012 by the AGA Institute
0016-5085/536.00
doi:10.1053/j.gastro.2011.08.039
cells in the proximal ducts that are the major sites of HCO$_3^-$ secretion: cystic fibrosis transmembrane conductance regulator (CFTR) and the solute carrier family 26 (SLC26) anion exchangers.\textsuperscript{12,13} How these transporters act in concert to produce a high HCO$_3^-$ secretion is controversial.\textsuperscript{14} Most likely, HCO$_3^-$ is secreted through the anion exchanger until the luminal concentration reaches about 70 mmol/L, after which the additional HCO$_3^-$ required to raise the luminal concentration to 140 mmol/L is transported via CFTR.\textsuperscript{15,16}

The role of PAR-2 in experimental acute pancreatitis is also controversial and highly dependent on the model of pancreatitis studied. PAR-2 was found to be protective in secretagogue-induced pancreatitis in mice\textsuperscript{7,17–19} and rats.\textsuperscript{20} However, PAR-2 is clearly harmful when pancreatitis is evoked by the clinically more relevant luminal administration of bile salts in mice.\textsuperscript{17}

In this study, we show for the first time that (1) PAR-2 is localized to the apical membrane of the human proximal PDECs, (2) the localization of PAR-2 in the guinea pig pancreas is identical to that in the human gland, (3) trypsin markedly reduces bicarbonate efflux through a (H$_2$DIDS)-sensitive apical SLC26 anion exchanger and (4) a decrease in pH within the ductal lumen will strongly accelerate the autoactivation of trypsinogen, and (5) trypsin down-regulates PAR-2 expression at both transcriptional and protein levels in PDECs of patients with chronic pancreatitis.

### Materials and Methods

A brief outline of the materials and methods is given in the following text. For further details, please see Supplementary Materials and Methods.

### Solutions

The compositions of the solutions used for microfluorimetry are shown in Table 1.

### Isolation of Pancreatic Ducts and Individual Ductal Cells

Small intralobular proximal ducts and individual ductal cells were isolated from guinea pigs or PAR-2 wild-type (PAR-2$^{-/-}$) and knockout (PAR-2$^{-/-}$) mice with a C57BL6 background by microdissection as described previously.\textsuperscript{21}

#### Measurement of Intracellular pH and Ca$^{2+}$ Concentration

Intracellular pH (pH$_i$) and calcium concentration ([Ca$^{2+}$]) were estimated by microfluorimetry using the pH- and Ca$^{2+}$-sensitive fluorescent dyes 2,7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxy methyl ester (BCECF-AM) and 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxyl)-ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acetoxy methyl ester (FURA 2-AM), respectively.

#### Microperfusion of Intact Pancreatic Ducts

The luminal perfusion of the cultured ducts was performed as described previously.\textsuperscript{22}

#### Electrophysiology

CFTR Cl$^{-}$ channel activity was investigated by whole cell patch clamp recordings on guinea pig single pancreatic ductal cells.

#### Measuring Autoactivation of Trypsinogen

Autoactivation of human cationic trypsinogen was determined in vitro at pH values ranging from 6.0 to 8.5. Experimental details are described in Supplementary Materials and Methods.

#### Immunohistochemistry

Five guinea pig, 2 PAR-2$^{-/-}$, 2 PAR-2$^{-/-}$, and 30 human pancreata were studied to analyze the expression pattern of PAR-2 protein. Relative optical densitometry was used to quantify the protein changes in the histologic sections. Patients’ data and the full methods are described in Supplementary Supplementary Materials and Methods.

#### Real-Time Reverse-Transcription Polymerase Chain Reaction

RNA was isolated from 30 human pancreata. Following reverse transcription, messenger RNA (mRNA) expression of PAR-2 and β-actin was determined by real-time polymerase chain reaction analysis.

### Results

#### Expression of PAR-2 in Guinea Pig and Human Pancreata

PAR-2 was highly expressed in the luminal membrane of small intralobular and interlobular ducts (Figure 1A [i]; cuboidal epithelial cells forming the proximal pancreatic ducts) but was almost undetectable in the larger interlobular ducts (Figure 1A [ii]; columnar epithelial cells forming the distal pancreatic ducts). The localization of PAR-2 in the human pancreas was identical to that in the guinea pig gland (Figure 1A [iv–vi]). Measurements of relative optical density confirmed the significant differences between the expression of PAR-2 in small intralobular and interlobular ducts and the larger interlobular ducts in both species (Figure 1C).

---

**Table 1.** Composition of Solutions for Microfluorimetry Studies

<table>
<thead>
<tr>
<th></th>
<th>Standard Cl/H$_2$O$_2^-$</th>
<th>Standard H$_2$O$_2^-$</th>
<th>Cl$^{-}$/free H$_2$O$_2^-$</th>
<th>Ca$^{2+}$/free H$_2$O$_2^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>130</td>
<td>115</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>1</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Sodium HEPES</td>
<td>10</td>
<td></td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>25</td>
<td>25</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Sodium gluconate</td>
<td>115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium gluconate</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium gluconate</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium sulfate</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*NOTE.* Values are concentrations in mmol/L.
Luminal Administration of PAR-2-AP and Trypsin Induces Dose-Dependent \([Ca^{2+}]_i\) Signals

Because PAR-2 expression was detected only on the luminal membrane of intralobular duct cells, we used the microperfusion technique to see whether these receptors can be activated by PAR-2 agonists. First, the experiments were performed at pH 7.4 to understand the effects of trypsin and PAR-2 under quasi-physiologic conditions (Figure 2). The fluorescent images in Figure 2A clearly show that luminal administration of PAR-2 activating peptide (PAR-2-AP) increased \([Ca^{2+}]_i\) in perfused pancreatic ducts. The \([Ca^{2+}]_i\) response was dose dependent and consisted of a peak in \([Ca^{2+}]_i\) that decayed in the continued presence of the agonist, possibly reflecting PAR-2 inactivation or depletion of intracellular \(Ca^{2+}\) stores (Figure 2B). Pretreatment of PDECs with 10 \(\mu M\) PAR-2 antagonist (PAR-2-ANT) for 10 minutes completely blocked the effects of 10 \(\mu M\) PAR-2-AP on \([Ca^{2+}]_i\) (Figure 2A and C). Removal of extracellular \(Ca^{2+}\) had no effect on the increase in \([Ca^{2+}]_i\), evoked by luminal administration of 10 \(\mu M\) PAR-2-AP; however, preloading ducts with the calcium chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'–tetraacetic acid (BAPTA-AM) at 40 \(\mu M\) totally blocked the response (Figure 2A and C).

Trypsin also induced a dose-dependent elevation in \([Ca^{2+}]_i\), similar to that evoked by PAR-2-AP (Figure 2E and F). Addition of 5 \(\mu M\) soybean trypsin inhibitor (SBTI), 10 \(\mu M\) PAR-2-ANT, and 40 \(\mu M\) BAPTA-AM totally blocked the increase in \([Ca^{2+}]_i\) (Figure 2D and F). These data show that trypsin activates PAR-2 on the luminal membrane of the duct cell, which leads to release of \(Ca^{2+}\) from intracellular stores and an elevation of \([Ca^{2+}]_i\).

Because the pH of pancreatic juice can vary between approximately 6.8 and 8.0,23,24 we also evaluated the effects of trypsin and PAR-2-AP on \([Ca^{2+}]_i\), at these pH values (Supplementary Figures 1 and 2, respectively). The
elevations of [Ca\(^{2+}\)]\(_i\) at pH 6.8 and 8.0 were generally very similar to the changes observed at pH 7.4. However, the increases in [Ca\(^{2+}\)]\(_i\) evoked by 1 \(\mu\)mol/L PAR-2-AP and 0.1 \(\mu\)mol/L trypsin were significantly lower at pH 6.8 compared with either pH 7.4 or 8.0 (Supplementary Figure 3).

**Luminal Exposure to PAR-2-AP and Trypsin Evokes Intracellular Alkalosis in PDECs**

Figure 3 shows pH recordings from microperfused pancreatic ducts. Luminal application of the CFTR inhibitor (CFTRinh) 172 (10 \(\mu\)mol/L) and the anion exchanger inhibitor H2DIDS (500 \(\mu\)mol/L) induced intracellular alkalization in PDECs (Figure 3A [i]). These data indicate that when bicarbonate efflux across the luminal membrane of PDECs (ie, bicarbonate secretion) is blocked, elevation of duct cell pH occurs, presumably because the basolateral transporters continue to move bicarbonate ions into the duct cell. Note also that the increase in pH evoked by the inhibitors is not sustained and begins to reverse before the inhibitors are withdrawn (Figure 3A [ii]), which might be explained by the regulation of pH by basolateral acid/base transporters.

Both luminal PAR-2-AP and trypsin induced a dose-dependent elevation of pH, (Figure 3A [ii and iii]), suggesting that activation of PAR-2 inhibits bicarbonate efflux across the apical membrane of the duct cell. Preincubation of PDECs with either 10 \(\mu\)mol/L PAR-2-ANT or 5 \(\mu\)mol/L SBTI or 40 \(\mu\)mol/L BAPTA-AM for 30 minutes totally blocked the effect of trypsin on pH, (Figure 3A [iv]). The inhibitory effect of the calcium chelator BAPTA-AM suggests that the actions of trypsin and PAR-2-AP on pH are mediated by the increase in [Ca\(^{2+}\)] that they evoke (Figure 2). Therefore, in this case, the transient nature of the pH response may reflect the transient effect that PAR-2 activators have on [Ca\(^{2+}\)]. (Figure 2B and E), as well as pH regulation by basolateral acid/base transporters.

Next we tested the effects of trypsin on pH, in Cl\(^{−}\)-free conditions and during pharmacologic inhibition of the
Figure 3. Effects of PAR-2-AP and trypsin on pHₐ in microperfused guinea pig pancreatic ducts. (A) Representative pHₐ traces showing the effects of luminal administration of different agents in microperfused pancreatic ducts. (i) A total of 10 μmol/L CFTRinh-172 and/or 500 μmol/L H₂DIDS caused alkalization of pHₐ. (ii) PAR-2-AP and (iii) trypsin induced a dose-dependent pHₐ elevation. (iv) Preincubation of ductal cells with 10 μmol/L PAR-2-ANT or 5 μmol/L SBTI or 40 μmol/L BAPTA-AM totally blocked the alkalization caused by 10 μmol/L trypsin. (v) Removal of luminal Cl⁻ or (vi) administration of H₂DIDS (500 μmol/L) decreased, but did not totally abolish, the effects of 10 μmol/L trypsin on pHₐ. (vii) Pretreatment with 10 μmol/L CFTRinh-172 also decreased the effects of trypsin (10 μmol/L) on pHₐ. (viii) Simultaneous administration of H₂DIDS and CFTRinh-172 strongly inhibited the effect of 10 μmol/L trypsin. (B and C) Summary of the effects of PAR-2-AP and trypsin on changes in pHₐ. ΔpHₐ was calculated from the experiments shown in A. (B) Effects of Cl⁻-free conditions. ΔpHₐ was calculated from the experiments shown in A. (C) Effects of Cl⁻-free conditions, H₂DIDS, CFTRinh-172, and a combination of the inhibitors all induced an intracellular alkalosis. Trypsin further increased the alkalization of pHₐ although the effect was markedly reduced when both H₂DIDS and CFTRinh-172 were present. n = 4–5 for all groups. (B) *P < .05 vs 0.1 μmol/L trypsin; **P < .001 vs 10 μmol/L trypsin. (C) *P < .05 vs 0.1 μmol/L PAR-2-AP; **P < .001 vs 10 μmol/L PAR-2-AP. (D) *P < .05 vs the respective filled column.
luminal anion exchangers and/or CFTR (Figure 3A [v–viii]). Luminal Cl−-free conditions increased the pHi of PDECs, presumably by driving HCO3− influx on the apical anion exchangers (Figure 3A [v]). Note that luminal administration of trypsin further elevated pHi in Cl−-free conditions (Figure 3A [v]) and also in the presence of H2DIDS (Figure 3A [vi]) and CFTRinh-172 (Figure 3A [vii]). However, pretreatment of ducts with a combination of H2DIDS and CFTRinh-172 markedly reduced the effect of trypsin on pHi (Figure 3A [viii]).

Figure 3B–D is a summary of the pHi experiments. Trypsin (Figure 3B) and PAR-2-AP (Figure 3C) both induced statistically significant, dose-dependent increases in pHi, and these effects were blocked by PAR-2-ANT, SBTI, and BAPTA-AM. Exposure of the ducts to luminal Cl−-free conditions, H2DIDS, CFTRinh-172, or a combination of the inhibitors also induced an intracellular alkalosis (Figure 3D). Also shown in Figure 3D is the additional, statistically significant increase in pHi caused by trypsin in ducts exposed to Cl−-free conditions and the individual inhibitors. However, when ducts were exposed to both CFTRinh-172 and H2DIDS simultaneously, the effect of trypsin on pHi was markedly reduced, although it remained statistically significant (Figure 3D). We interpret these results as indicating that trypsin inhibits both Cl−-dependent (ie, anion exchanger mediated; revealed when CFTR is blocked by CFTRinh-172) and Cl−-independent (ie, CFTR mediated; revealed in Cl−-free conditions and when the luminal exchangers are blocked by H2DIDS) bicarbonate secretory mechanisms in PDECs. Reduced bicarbonate secretion will lead to a decrease in intraductal pH.

**Trypsin and PAR-2-AP Inhibit CFTR**

Exposure of guinea pig PDECs to 5 μmol/L forskolin, which elevates intracellular adenosine 3′,5′-cyclic monophosphate levels, increased basal whole cell currents (Figure 4A–D [i]) from 8.9 ± 2.3 to 91.2 ± 13.5 pA/pF (Figure 4A–D [ii]) at +60 mV in 78% of cells (38/49). The forskolin-activated currents were time- and voltage-independent, with a near linear I/V relationship and a reversal potential of −5.15 ± 1.12 mV (Figure 4A–D [iv]). These biophysical characteristics indicate that the currents are carried by CFTR.

Exposure of PDECs to 10 μmol/L trypsin did not affect the basal currents; however, administration of either 10 μmol/L PAR-2-AP (Figure 4A [iii]) or 10 μmol/L trypsin (Figure 4B [iii]) inhibited forskolin-stimulated CFTR currents by 51.7% ± 10.5% and 57.4% ± 4.0%, respectively. In both cases, the inhibition was voltage independent and irreversible. Pretreatment with either SBTI (10 μmol/L; Figure 4C [iii]) or PAR-2-ANT (10 μmol/L; Figure 4D [iii]) completely prevented the inhibitory effect of trypsin on the forskolin-stimulated CFTR currents. Figure 4E is a summary of these data, which suggest that trypsin inhibits CFTR Cl− currents by activation of PAR-2.

**Autoactivation of Trypsinogen Is pH Dependent**

Trypsinogen can undergo autocatalytic activation during which trace amounts of trypsin are generated, which, in turn, can further activate trypsinogen in a self-amplifying reaction. Human trypsinogens are particularly prone to autoactivation, and mutations that facilitate autoactivation are associated with hereditary pancreatitis. To assess the effect of a decrease in intraductal pH (caused by reduced bicarbonate secretion) on trypsinogen activation, we measured autoactivation of human cationic trypsinogen in vitro at pH values ranging from 6.0 to 8.5 using a mixture of various buffers. As shown in Figure 5A, the rate at which cationic trypsinogen autoactivates was markedly increased as the pH was reduced from 8.5 to 7.0 when the buffer solution contained 1 mmol/L CaCl2 and no NaCl. However, a further reduction in pH, from 7.0 to 6.0, had little effect (Figure 5A [i]).

To rule out that the differences observed in autoactivation were due to the different ionic strengths of the buffers used, we repeated the experiments in the presence of a higher concentration of sodium (100 mmol/L NaCl, Figure 5A [ii]) or lower concentration of calcium (0.1 mmol/L CaCl2, Figure 5A [iii]). Although the overall autoactivation rates were much slower in the presence of NaCl, the pH profile of autoactivation was essentially identical to that observed in the absence of added salt (Figure 5A [ii]). Also, pH-dependent changes in the autoactivation of trypsinogen were still detectable when the experiments were performed using a low calcium buffer (Figure 5A [iii]).

**PAR-2 Is Down-regulated in Patients With Chronic Pancreatitis**

It has been documented that there is activated trypsin in the pancreatic ductal lumen in chronic pancreatitis in humans. If trypsin activity is elevated in the duct lumen, PAR-2 down-regulation should occur, which could be due to either (1) changes in PAR-2 mRNA transcription and/or (2) receptor internalization and translocation to the cytoplasm. Our data show a marked reduction in membranous PAR-2 protein level but no significant changes in cytoplasmic PAR-2 protein in chronic pancreatitis (Figure 5B [i–iv] and C). Furthermore, PAR-2 mRNA expression was markedly reduced in chronic pancreatitis (Figure 5D), suggesting that reduced PAR-2 mRNA transcription may cause PAR-2 down-regulation in chronic pancreatitis.

**Luminal Exposure to R122H Mutant Cationic Trypsin Induces Elevation of [Ca2+]i, and Evokes Alkalosis in PDECs**

It has been shown that mutations in cationic trypsinogen increase the risk of chronic pancreatitis, most likely because of the enhanced autoactivation exhibited by the mutant trypsinogens. Here we tested whether the commonest mutation in cationic trypsin, R122H, affected the ability of the protease to interact with PAR-2.
A cationic trypsin causes comparable changes in $pHi$ and $[Ca^{2+}]_{i}$ to 0.4 mol/L wild-type bovine trypsin, suggesting that a trypsin-mediated inhibition of bicarbonate secretion could play a role in the pathogenesis of hereditary as well as chronic pancreatitis.

Activation of PAR-2 Is Diminished in PAR-2−/− Mice

Finally, we investigated the effects of both PAR-2-AP and trypsin on PDECs isolated from PAR-2+/+ and PAR-2−/− mice (Figure 6C–E). First we confirmed using
immunohistochemistry that PAR-2\(^{+/+}\) mice do, whereas PAR-2\(^{-/-}\) mice do not, express PAR-2 in their PDECs (Figure 6C [i and iii]). Accordingly, our functional data clearly show that the pH, and \([Ca^{2+}]\), responses to luminal administration of either trypsin or PAR-2-AP were markedly diminished in PAR-2\(^{-/-}\) PDECs (Figure 6D and E).

**Discussion**

The human pancreatic ductal epithelium secretes 1 to 2 L of alkaline fluid every 24 hours that may contain up to 140 mmol/L NaHCO\(_3\).\(^{12,13}\) The physiologic function of this alkaline secretion is to wash digestive enzymes down the ductal tree and into the duodenum and to neutralize acidic chyme entering the duodenum from the stomach. There are important lines of evidence supporting the idea that pancreatic ducts play a role in the pathogenesis of pancreatitis: (1) ductal fluid and bicarbonate secretion are compromised in acute and chronic pancreatitis,\(^{30,31}\) (2) one of the main end points of chronic pancreatitis is the destruction of the ductal system,\(^{32,33}\) (3) mutations in CFTR may increase the risk of pancreatitis,\(^{30,31,34–36}\) and (4) etiologic factors for pancreatitis, such as bile acids or ethanol in high concentration, inhibit pancreatic ductal bicarbonate secretion.\(^{37–39}\) Despite the previously men-

---

**Figure 5.** The effects of pH on trypsinogen activation and analyses of PAR-2 expression in human pancreatic samples. The autoactivation of human cationic trypsinogen was determined in vitro at pH values ranging from 6.0 to 8.5. (A) (i) Trypsinogen at 2 \(\mu\)mol/L concentration was incubated with 40 nmol/L trypsin at 37°C in 0.1 mol/L Tris + 2-(N-morpholino)ethanesulfonic acid2-(N-morpholino)ethanesulfonic acid buffer mixture containing 1 mmol/L CaCl\(_2\). (ii) The same protocol was used in high (100 mmol/L) NaCl buffer solution. Autoactivation of cationic trypsinogen significantly increased as the pH was reduced from 8.5 to 6.0. (iii) The same protocol was used in low (0.1 mmol/L) Ca\(^{2+}\)-buffered solution buffer solution. (B) (i–iv) PAR-2 expression. (i) Representative section of normal human pancreas. (ii) No primary antiserum. (iii) Representative section of human pancreas from a patient with chronic pancreatitis (CP). (iv) No primary antiserum. (C) Representative section of human pancreas from a patient with chronic pancreatitis (CP). (iv) No primary antiserum. (C) Relative optical density, n = 15. *P < .05 vs CP.

**Figure 6.** Real-time reverse-transcription polymerase chain reaction analysis of PAR-2 mRNA expression of human pancreas. Data are given in 2^\(\Delta\)dCT. n = 15. *P < .05 vs CP.
tioned data, the role of PDECs in the development of pancreatitis has received relatively little attention.40

There are important species differences regarding the localization of PAR-2 in pancreatic ducts and in the effect of its activation on bicarbonate secretion. For example, CAPAN-1 cells10 and dog PDECs9 express PAR-2 only on the basolateral membrane, whereas bovine PDECs express PAR-2 on the luminal membrane.11 Therefore, one of our
The first aim was to determine which animal model best mimics human PAR-2 expression and thus would be the best for studying the effects of trypsin on PDEC function. Our results showed that in the human pancreas PAR-2 is localized to the luminal membrane of small proximal pancreatic ducts, which are probably the major site of bicarbonate and fluid secretion. Because CAPAN-1 cells and dog PDECs express PAR-2 only on the basolateral membrane, they do not mimic the human situation. Rats or mice are also not good models for the human gland because they secrete only 70 to 80 mmol/L bicarbonate.41,42 However, the guinea pig pancreas secretes ~140 mmol/L bicarbonate, as does the human gland, and the regulation of bicarbonate secretion is similar in both species.41,42 Because PAR-2 expression in the guinea pig pancreas was localized to the luminal membrane of duct cells, we performed our experiments on isolated guinea pig ducts.

First we characterized the effects of PAR-2 activation by trypsin and PAR-2-AP on PDECs. Previously, it has been shown that activation of the G protein–coupled PAR-2 by proteases requires proteolytic cleavage of the receptor, which is followed by an elevation of [Ca2+]i.43–45 As expected, luminal trypsin and PAR2-AP caused a dose-dependent elevation of [Ca2+]i in guinea pig ducts. Importantly, the trypsin inhibitor SBTI, PAR-2-ANT, and the intracellular calcium chelator BAPTA-AM all completely blocked the elevation of [Ca2+]i, whereas removal of extracellular Ca2+ had no effect. Acidosis (pH 6.8) also slightly reduced the changes in [Ca2+]i evoked by trypsin, most probably due to reduced cleavage activity of trypsin at an acidic pH. Next we characterized the effects of PAR-2 activation on pHi. Luminal application of trypsin and PAR-2-AP both caused a dose-dependent intracellular alkalization in PDECs. This alkalosis is most likely explained either by a reduction in the rate of bicarbonate efflux (ie, secretion) across the apical membrane of PDECs or by an increase in the rate of bicarbonate influx at the basolateral side of the cell. We favor the former explanation because luminal application of the anion exchange inhibitor H2DIDS or the CFTR inhibitor CFTRinh-172 produced a similar intracellular alkalization.22,46 Thus, PAR-2 activation inhibits bicarbonate secretion in PDECs by inhibiting SLC26 anion exchangers and CFTR Cl− channels. Our conclusion from these pHi and patch clamp data is that PAR-2 activation inhibits both the SLC26 anion exchanger (probably SLC26A6 [PAT-1])47 because SLC26A3 [DRA] is only weakly inhibited by disulfonic stilbenes48,49 and CFTR Cl− channels expressed on the apical membrane of the duct cell.

The pH of pancreatic juice (and therefore the luminal pH [pHl] in the duct) can vary between approximately 6.8 and 8.0. It has recently been shown that protons coreleased during exocytosis cause significant acidosis (up to 1 pH unit) in the lumen of the acini.23 However, Ishiguro et al49 have clearly shown that the pHl in pancreatic ducts is dependent on the level of bicarbonate secretion. pHl can be elevated from 7.2 to 8.5 by stimulation with secretin or forskolin, and this effect was strictly dependent on the presence of bicarbonate.24,49,50 Also, inhibition of ductal bicarbonate secretion with H2DIDS can decrease the pHl to less than 8.0.49 In view of these results, we tested whether trypsinogen autoactivation was affected by pH over the range of 6.0 to 8.5. Autoactivation of trypsinogen was relatively slow at pH 8.5, but decreasing the pH from 8.5 to 7 progressively stimulated autoactivation. These results suggest that under physiologic conditions bicarbonate secretion by PDECs is not only important for elevating the pH in the duodenum, but also for keeping pancreatic enzymes in an inactive state in the ductal system of the gland.

Receptor down-regulation is a phenomenon that occurs in the continued presence of an agonist and leads to a reduction in the sensitivity of the cell to the agonist. Potentially, there are 2 mechanisms that could underlie receptor down-regulation of PAR-2: (1) after proteolytic activation, the PAR-2 is internalized by a clathrin-mediated mechanism and then targeted to lysosomes45 and (2) if trypsin is present for a longer time in the lumen, PAR-2 may be down-regulated at the transcriptional level. In this study, we provide evidence that the second mechanism, transcriptional down-regulation, explains the reduced expression of PAR-2 seen in chronic pancreatitis.

Conflicting data can be found in the literature concerning the role of PAR-2 in acute pancreatitis. Singh et al57 showed that in secretagogue-induced experimental pancreatitis, PAR-2 deletion is associated with a more severe
pancreatitis. Although Laukkarinen et al\textsuperscript{17} confirmed these results in cerulein-induced pancreatitis, they also clearly showed that in taurocholate-induced pancreatitis, PAR-2 deletion markedly reduced the severity of the disease. There is no evidence to suggest that clinical pancreatitis is evoked by supramaximal secretagogue stimulation; however, the taurocholate-induced pancreatitis model may mimic the clinical situation. Therefore, Laukkarinen et al\textsuperscript{17} speculated that PAR-2 activation promotes the worsening of clinical pancreatitis and our data are consistent with that hypothesis.

Besides the clear pathophysiologic role of the trypsin/PAR-2 interaction in chronic pancreatitis, there is still a debate as to why PAR-2 are localized to the luminal membrane of PDEC in small ducts close to the acinar cells. What could the physiologic role of this PAR-2 be? A number of agents have been shown to have dual effects on PDECs at different concentrations. For example, bile acids in low concentrations stimulate but in high concentrations inhibit bicarbonate secretion.\textsuperscript{39} The same applies to ethanol.\textsuperscript{38} Under physiologic conditions, trypsin inhibitors are coreleased from acinar cells with trypsinogen and should block the activity of any trypsin that is generated spontaneously. Therefore, only very small amounts of active trypsin, if any, will be present in the duct lumen under normal conditions. However, there remains a possibility that very small amounts of active trypsin (ie, concentrations less than 0.1 \(\mu\)mol/L that would not cause an elevation of \([Ca^{2+}]_{i}\), or change in pH) could bind to PAR-2 on the luminal membrane of the ducts and augment other stimulatory mechanisms so as to enhance flushing of digestive enzymes down the ductal tree.

In conclusion, we suggest for the first time that one of the physiologic roles of bicarbonate secretion by PDECs is to curtail trypsinogen autoactivation within the pancreatic ductal system. However, if trypsin is present in the duct lumen (as may occur during the early stages of pancreatitis due to leakage from acinar cells), PAR-2 on the duct cell will be activated, leading to \(Ca^{2+}\) release from intracellular stores and an increase in cytosolic \(Ca^{2+}\) concentration. This causes inhibition of the luminal anion exchangers and CFTR Cl\textsuperscript{−} channels, reducing bicarbonate secretion by the duct cell. The decrease in bicarbonate secretion will increase the transit time of zymogens down the duct tree and decrease \(pH_{z}\), both of which will promote the autoactivation of trypsinogen. The trypsin so formed will further inhibit bicarbonate transport, leading to a vicious cycle generating further decreases in \(pH_{z}\) and enhanced trypsinogen activation, which will favor development of the pancreatitis (Supplementary Figure 4).

Finally, the R122H mutant cationic trypsin also elevated \([Ca^{2+}]_{i}\), and \(pH_{z}\) in duct cells, suggesting that this mechanism may be particularly important in hereditary pancreatitis in which the mutant trypsinogens more readily autoactivate.\textsuperscript{39}

### Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: [10.1053/j.gastro.2011.08.039].

### References


Supplementary Materials and Methods

Ethics

All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (USA NIH publication No 85-23, revised 1985). Animal experiments were approved by the Regional Ethical Board at the University of Szeged, Hungary.

Solutions and Chemicals

HEPES-buffered solutions were gassed with 100% O₂, and their pH was set to 7.4 with HCl at 37°C. HCO₃⁻-buffered solutions were gassed with 95% O₂/5% CO₂ to set pH to 7.4 at 37°C. For patch clamp studies, the standard extracellular solution contained in (mmol/L): 145 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 5 glucose (pH 7.4 adjusted with NaOH). The osmolarity of the extracellular solution was 300 mOsm/L. The standard pipette solution for the patch clamp experiments contained in (mmol/L): 120 CsCl, 2 MgCl₂, 0.2 ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA), 10 HEPES, and 1 Na₂ATP (pH 7.2 adjusted with NaOH). Chromatographically pure collagenase was purchased from Worthington (Lakewood, NJ). 2,7-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM), 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-ethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM), 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxo)ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acetoxymethyl ester (FURA 2-AM), dihydro-4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (H₂DIDS), and 1,2-bis(o-aminophenoxy)ethane- N,N,N′,N′-tetraacetic acid (BAPTA-AM) were from Invitrogen (Carlsbad, CA). PAR-2-ANT (H-Phe-Ser-Leu-Arg-Tyr-NH₂) and PAR-2-AP (H-Ser-Leu-Ile-Gly-Arg-Leu-amid trifluoroacetate salt) were from Teptides International (Louisville, KY). For skolin were from Tocris (Ellisville, MO). Rabbit PAR-2 polyclonal antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). All other chemicals were obtained from Sigma-Aldrich (Budapest, Hungary).

Isolation of Pancreatic Ducts and Individual Ductal Cells

Male guinea pigs weighing between 150 and 250 g or mice (PAR-2+/+ and PAR-2−/−) weighing between 18 and 21 g were humanely killed by cervical dislocation, the pancreas was removed, and small intralobular proximal ducts were isolated by microdissection as described previously. 1 PAR-2−/− mice (B6.Cg-F2rl1tm1Mslb/J) were previously generated by Schmidlin et al 1 and a kind gift from Ashok Saluja. 2 Isolated ducts were then cultured overnight in a 37°C incubator gassed with 5% CO₂/95% air. 3 To obtain single pancreatic ductal cells, cultured ducts were incubated for 50 minutes at 37°C in 50 U/mL elastase dissolved in storage solution (Dulbecco’s modified Eagle medium containing 3% [wt/vol] bovine serum albumin [pH 7.4 with NaOH]). Then the ducts were transferred to a Ca²⁺/Mg²⁺-free HEPES-buffered solution and incubated for a further 10 minutes at 37°C. After the incubation, the ducts were transferred to a coverslip and teased apart using stainless steel needles. The individual ductal cells were used for experiments within 3 to 4 hours after isolation.

Measurement of pH, and Ca²⁺ Concentration

Ducts were bathed in standard HEPES solution and loaded with BCECF-AM (2 μmol/L) or FURA 2-AM (5 μmol/L) for 30 to 60 minutes at room temperature.

Ducts were then transferred to a perfusion chamber mounted on an IX71 inverted microscope (Olympus, Budapest, Hungary) and perfused continuously with solutions at 37°C both from the luminal and basolateral side at a rate of 10 to 30 µL/min and 4 to 5 mL/min, respectively. Four to 5 small areas (region of interests) of 5 to 10 cells in each intact duct were excited with light at a given wavelength. Excitation of BCECF was at 495 and 440 nm, with emitted light monitored at 535 nm. Excitation of FURA-2 was at 380 and 340 nm, with emitted light monitored at 510 nm. The fluorescence emissions were captured by a charge-coupled device camera and digitized by a Cell imaging system (Olympus, Budapest, Hungary). Ratio images were collected at 1-second intervals. In situ calibration of pH, measured with BCECF was performed using the high K⁺-nigericin technique. 4,5

Electrophysiology

Guinea pig PDECs were isolated by an enzymatic microdissection procedure as described previously. Using a glass pipette, a few drops of cell suspension were placed within a perfusion chamber mounted on the stage of an inverted microscope (TMS; Nikon, Tokyo, Japan). The ductal cells were allowed to settle and attach to the bottom of the chamber for at least 30 minutes before the perfusion was started.

Patch clamp micropipettes were fabricated from boro-silicate glass capillaries (Clark, Reading, England) by using a P-97 Flaming/Brown micropipette puller (Sutter Co, Novato, CA). These pipettes had resistances between 1.5 and 2.5 MΩ. Membrane currents were recorded with an Axopatch 1D amplifier (Axon Instruments, Union City, CA) using the whole cell configuration of the patch clamp technique at 37°C. After establishing a high-resistance seal (1–10 GΩ) by gentle suction, the cell membrane beneath the tip of the pipette was disrupted by suction or by application of short electrical pulses. The series resistance was typically 4 to 8 MΩ before compensation (50–80%, depending on the voltage protocol). Current-voltage (I/V) relationships were obtained by holding Vₘ at 0 mV and clamping to ±100 mV in 20-mV increments. Membrane currents were digitized by using a 333-kHz analog-to-digital converter (Digidata 1200; Axon Instruments) under software control (pClamp 6;
Axon Instruments). Analyses were performed by using pClamp 6 software after low-pass filtering at 1 kHz.

Expression and Purification of Human Trypsinogens
Wild-type and R122H mutant human cationic trypsinogen was expressed in *Escherichia coli* and purified by ecotin-affinity chromatography as reported previously.6

Measuring Autoactivation of Trypsinogen
Autoactivation of trypsinogen was measured at 2 μmol/L concentration at 37°C in a polybuffer system (American Bioanalytical Inc, Natick, MA) containing 100 mmol/L 2-(N-morpholino)ethanesulfonic acid, 100 mmol/L HEPES, and 100 mmol/L Tris in 100 μL final volume. The pH of the Polybuffer was adjusted to given values with HCl (pH 6.0 and 6.5) or NaOH (pH 7.0, 7.5, 8.0, and 8.5). Reactions also contained 1 mmol/L or 0.1 mmol/L CaCl2 and 100 mmol/L NaCl, as indicated. At given times, 2-μL aliquots were removed and trypsin activity was determined using the N-CBZ-Gly-Pro-Arg-p-nitroanilide substrate at 150 μmol/L final concentration.

Immunohistochemistry
Pancreatic tissue from 5 guinea pigs, 15 patient samples without pancreatic disease near neuroendocrine tumors (average age, 59.5; female/male, 7.8), and 15 patients (average age, 56.6; female/male, 4:11) who had chronic pancreatitis (13 alcohol, 2 gallstone) were investigated. The human samples were obtained with the permission of the Regional Ethical Committee of Semmelweis University (#172/2003).

The pancreatic tissues were fixed in 10% neutral buffered formalin for 24 hours, followed by paraffin embedding, and were then cut and stained with H&E to establish the diagnosis. Paraffin-embedded, 3- to 4-μm-thick sections were used for immunohistochemistry to detect PAR-2 expression. The slides were treated for 30 minutes with target retrieval solution (Dako, Glostrup, Denmark) in a microwave oven, followed by incubation with the primary rabbit polyclonal antibody (Santa Cruz Biotechnology Inc, Heidelberg, Germany) and anti-GAPDH antibody (1:5000; AbDSerotec, Düsseldorf, Germany) and nuclear counterstaining was achieved by using lysis buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100 containing protease inhibitor complex [Sigma Aldrich Co, Budapest, Hungary]). Samples (50 mg) were homogenized, followed by centrifugation at 13,200 rpm at 4°C for 5 minutes. Measurements of protein concentration were performed using Bradford analysis.7 A total of 30 μg of protein samples were loaded in each lane, run on 10% sodium dodecyl sulfate/polyacrylamide electrophoresis at 200 V for 35 minutes, and then transferred to nitrocellulose membranes at 100 V, 4°C, for 75 minutes. For aspecific protein blocking, nonfat dry milk (5%, phosphate-buffered saline) was used for 30 minutes. Blots were incubated with polyclonal PAR-2 rabbit antibody (1:300; Santa Cruz Biotechnology Inc, Heidelberg, Germany) and anti-GAPDH antibody (1:5000; AbDSerotec, Kidlington, England) at 4°C overnight. After washing in 0.1% Tris, the secondary antibodies as anti-mouse G serum was used. The negative controls exhibited no signal. Normal skin epithelial cells were used as positive controls to confirm correct immunohistochemical staining for PAR-2 (results not shown).

The immunohistochemical reactions were digitalized with a Mirax MIDI slide scanner (3DHistech Ltd, Budapest, Hungary). Relative optical (RO) density was calculated using ImageJ program (National Institutes of Health, Bethesda, MD). Pixel values (PV) were normalized to erythrocyte density (PVNorm = PVMeasured / PV Erythrocyte) in all sections. RO-Density value was calculated from the RO-Density = log10(255/PVNorm) equation, assuming that the brightest value in the image equals 255.

Western blot analysis was used to determine the specificity of the PAR-2 antibody. Proteins were extracted from fresh-frozen guinea pig (n = 3) and human (n = 3) pancreatic tissue stored at −80°C. Isolation was performed by using lysis buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100 containing protease inhibitor complex [Sigma Aldrich Co, Budapest, Hungary]). Samples (50 mg) were homogenized, followed by centrifugation at 13,200 rpm at 4°C for 5 minutes. Measurements of protein concentration were performed using Bradford analysis.7 A total of 30 μg of protein samples were loaded in each lane, run on 10% sodium dodecyl sulfate/polyacrylamide electrophoresis at 200 V for 35 minutes, and then transferred to nitrocellulose membranes at 100 V, 4°C, for 75 minutes. For aspecific protein blocking, nonfat dry milk (5%, phosphate-buffered saline) was used for 30 minutes. Blots were incubated with polyclonal PAR-2 rabbit antibody (1:300; Santa Cruz Biotechnology Inc, Heidelberg, Germany) and anti-GAPDH antibody (1:5000; AbDSerotec, Kidlington, England) at 4°C overnight. After washing in 0.1% Tris, the secondary antibodies as anti-mouse GAPDH (1:2000; AbDSerotec, Düsseldorf, Germany) and horseradish peroxidase–conjugated anti-rabbit antibody (1:2000, Dako Cytomation, Glostrup, Denmark) were applied at room temperature for 90 minutes. Following 3 series of washings in Tris-buffered saline with Tween 20, signals were visualized by enhanced chemiluminescent detection.

Real-Time Reverse-Transcription Polymerase Chain Reaction
RNA extraction. Fifteen formalin-fixed, paraffin-embedded normal pancreatic tissue samples and 15 samples of chronic pancreatitis tissue were selected for real-time reverse-transcription polymerase chain reaction analysis. Total RNA was isolated from five 5- to 10-μm macrodissected sections (connective tissue excluded) using RNeasy FFPE Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. RNA concentrations were obtained using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific Inc, Waltham, MA).

Reverse transcription of RNA. Complementary DNA samples were prepared from 1 μg total RNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA) as specified by the manufacturer.

Primer design. Gene-specific primers were designed by AlleleID 6.01 primer design software (Premier Biosoft International, Palo Alto, CA) for real-time reverse-
transcription polymerase chain reaction. Isoform specificity and primer sizes were checked by BioEdit biological sequence alignment editor software (Tom Hall Ibis Therapeutics, Carlsbad, CA). Primer specificity was checked by BiSearch software (Hungarian Academy of Sciences, Institute of Enzymology, Budapest, Hungary). Primer specific amplification degree (58°C) was optimized by gradient polymerase chain reaction. The used primer sequences are shown in Supplementary Table 1.

Reverse-transcription polymerase chain reaction. Real-time reverse-transcription polymerase chain reaction analysis was performed using SYBR Green technology on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. β-actin was used as the internal control gene. Primer-specific amplification was controlled by 2% agarose gel electrophoresis, as well as by melting temperature analysis. The final 20 μL reaction mixture contained Power SYBR Green PCR Master Mix (Applied Biosystems), 10 pmol/L of forward and reverse primers, and 100 ng complementary DNA as template. Amplification conditions were as follows: incubation at 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds, 60°C for 60 seconds, and 72°C for 15 seconds, with subsequent melting analysis, heating to 95°C for 20 seconds, cooling to 45°C for 10 seconds, and then re-heating to 95°C.

Statistical Analysis

Data are expressed as means ± SEM. Significant difference between groups was determined by analysis of variance. Statistical analysis of the immunohistochemical data was performed using the Mann–Whitney U test. Probability values of $P < .05$ were accepted as being significant.

Supplementary References

Supplementary Figure 1. Effects of PAR-2-AP and trypsin on [Ca^{2+}]_{i} in microperfused guinea pig pancreatic ducts at pH 8.0. (A) Light (1) and fluorescent ratio images (2 and 3) of microperfused pancreatic ducts showing the effects of luminal administration of 10 μmol/L PAR-2-AP and 10 μmol/L PAR-2-ANT on [Ca^{2+}]_{i} at pH 8.0. Images were taken before (1 and 2) and after (3) exposure of the ducts to PAR-2-AP or trypsin. An increase in [Ca^{2+}]_{i} is denoted by a change from a “cold” color (blue) to a “warmer” color (yellow to red); see scale on the right. (B and C) Representative experimental traces and summary data of the changes in [Ca^{2+}]_{i} after treatment with PAR-2-AP. (D) Same protocol was used to evaluate the effects of trypsin. (E and F) Representative experimental traces and summary data of the changes in [Ca^{2+}]_{i} after treatment with trypsin. *P < .05 vs 1 μmol/L PAR-2-AP or 0.1 μmol/L trypsin, respectively. **P < .001 vs 10 μmol/L PAR-2-AP or 10 μmol/L trypsin, respectively.
Supplementary Figure 2. Effects of PAR-2-AP and trypsin on [Ca^{2+}]_{i} in microperfused guinea pig pancreatic ducts at pH 6.8. (A) Light (1) and fluorescent ratio images (2 and 3) of microperfused pancreatic ducts showing the effects of luminal administration of 10 μmol/L PAR-2-AP and 10 μmol/L PAR-2-ANT on [Ca^{2+}]_{i} at pH 6.8. Images were taken before (1 and 2) and after (3) exposure of the ducts to either PAR-2-AP or trypsin. The colors are described in Supplementary Figure 1; see scale on the right. (B and C) Representative experimental traces and summary data of the changes in [Ca^{2+}]_{i} at pH 6.8. (D) The same protocol was used to evaluate the effects of trypsin. (E and F) Representative experimental traces and summary data of the changes in [Ca^{2+}]_{i} at pH 6.8. n = 3–4. *P < .05 vs 1 μmol/L PAR-2-AP or 0.1 μmol/L trypsin, respectively. **P < .001 vs 10 μmol/L PAR-2-AP or 10 μmol/L trypsin, respectively.
Supplementary Figure 3. Summary of the effects of PAR-2-AP and trypsin on [Ca\textsuperscript{2+}]\textsubscript{i} in microperfused guinea pig pancreatic ducts at different extracellular pH values. (A) The elevation in [Ca\textsuperscript{2+}]\textsubscript{i} evoked by 1 \mu mol/L PAR-2-AP and (B) 0.1 \mu mol/L trypsin at different extracellular pH values (6.8; 7.4; 8.0). n = 3–4. * P < .05 vs at pH 6.8.

Supplementary Figure 4. The vicious trypsin cycle. If trypsin is present in the duct lumen, PAR-2 receptors on the duct cell are activated, leading to Ca\textsuperscript{2+} release from intracellular stores and an increase in cytosolic Ca\textsuperscript{2+} concentration. This causes inhibition of the luminal anion exchangers and CFTR Cl\textsuperscript{−} channels reducing bicarbonate secretion by the duct cell. The decrease in bicarbonate secretion will decrease luminal pH in the duct, which strongly accelerates the autoactivation of trypsinogen to trypsin. The activated trypsin will further inhibit bicarbonate transport by the duct cells, leading to a vicious cycle generating further decreases in luminal pH and enhanced trypsinogen activation with the potential for damaging the gland. The cycle may eventually be broken by the down-regulation of duct cell PAR-2 expression once pancreatitis is established. N, nucleus.

Supplementary Table 1. Nucleotid Sequences of the Primers Used in the Study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Product length (base pairs)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>GTACGCCAACAAGTGCTG (sense)</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>CTTCATTTGCTGGTGTCG (antisense)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAR-2</td>
<td>GGCACCATCCAGAAACCAATAG (sense)</td>
<td>128</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>GCAGAAAACCTCATCCAGAAAAGAC (antisense)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>