THE ROLE OF CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR Cl^- CHANNEL IN PANCREATITIS

Ph.D. Thesis

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Articles closely related to the subject of the thesis and cited in the thesis

1) **L. Judák**, P. Hegyi, Z. Rakonczay Jr., J. Maléth,M.A. Gray, V. Venglovecz. EtOH and its non-oxidative metabolites profoundly inhibit CFTR function in pancreatic epithelial cells which is prevented by ATP supplementation. *Pflugers Arch - Eur J Physiol.* 2013 Doi:10.1007/s00424-013-1333-x

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3) L. V. Kemény, A. Schnúr, M. Czepán, Z. Rakonczay Jr., E. Gál, J. Lonovics, Gy. Lázár, Zs. Simonka, V. Venglovecz, J. Maléth, **L. Judák**, I. B. Németh, K. Szabó, J. Almássy, L. Virág, A. Geisz, L. Tiszlavicz, D. I. Yule, T. Wittmann, A. Varró and P. Hegyi.Na^+/Ca^{2+} exchangers regulate the migration and proliferationof human gastric myofibroblasts.*Am J Physiol Gastrointest Liver Physiol* 2013;305(8):G552-63

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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. 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. 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.

Pancreatic ductal epithelial cells (PDECs) possess several transporters and ion channels, which play a crucial role in HCO_3^- secretion. The cystic fibrosis transmembrane conductance regulator (CFTR) protein is a cAMP-regulated Cl^- channel with multiple functions in epithelial cells. 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 classical view is that CFTR mainly secretes Cl^- and having a relatively low permeability to HCO_3^-. However, under stimulated secretion the channel permeability changes and become more permeable for HCO_3^- than Cl^-. It is well documented that insuffiecient function of CFTR increases the risk of acute pancreatitis (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 ethanol (EtOH) induces pancreatitis is not well understood. 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.

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. Several studies indicated that trypsin stimulates enzyme secretion from acinar cells via proteinase-activated receptor-2 (PAR-2). In contrast the effect of trypsin on ductal HCO_3^- secretion is contradictory, highly depends on the species and the localization of PAR-2. In these studies the effect of trypsin was investigated on the activity of the Cl^-/HCO_3^- exchanger, whereas no data is available regarding the effect of trypsin on CFTR.

2. AIMS

The main aims of this work were to investigate the role of CFTR Cl^- channel in PDEC physiology.

1. To investigate the direct effects of EtOH and its metabolites on CFTR Cl^- channel.

2.To examine the effects of trypsin on CFTR Cl^- channel.

3. MATERIALS AND METHODS

3.1. 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).

3.2. 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.

3.3. 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 $CaCl_2$, 1 $MgCl_2$, 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 $MgCl_2$, 10 HEPES, 5 ethylene glycol-bis(b- aminoethyl ether)-N,N,N8,N8-tetraacetic acid (EGTA) and 1 Na_2ATP (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 tipically 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 mVand normalised to cell capacitance (pF).

3.4. Measurement of intracellular ATP

Changes in intracellular ATP (ATP_i) were estimated using the Mg^{2+}-sensitive fluorescent dye, Magnesium Green-AM (MgGreen-AM). Most of the Mg^{2+} ions in the cells are bound to ATP. Since ATP has a higher affinity for Mg^{2+} than ADP, upon ATP hydrolysis the concentration of cytosolic free Mg^{2+} increases. Upon binding Mg^{2+}, 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 $CaCl_2$, 1 $MgCl_2$, 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/F_0) and expressed as relative fluorescence.

4. RESULTS

4.1. 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), with a mean reversal potential of −5.6 mV, which is very close to the Cl^- equilibrium potential (~ −5.7 mV). Administration of 5 μM forskolin (cAMP agonist) to the bathing solution increased whole cell currents by approximately tenfold in 70 % of cells. To confirm that the basal and stimulated currents were both mediated by CFTR, we tested $CFTR_{inh}$-172, a potent inhibitor of the channel. Administration of 10 μM $CFTR_{inh}$-172 reversibly and significantly blocked the basal currents by 58.2 ± 5.7 % (at + 60 mV) and forskolin-stimulated currents by 66.6 ± 6.4 % (at + 60 mV), providing further evidence that under our experimental conditions the whole cell currents were due to CFTR.

4.2. 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. 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, whereas 100 mM EtOH induced a robust increase within 5 min. In contrast, both 10 and 100 mM EtOH blocked the forskolin-stimulated whole cell currents. Both the stimulatory and inhibitory effects of EtOH were reversible on washout. In addition, block by EtOH was not voltage dependent.

The rate of the mannitol-induced increase in whole cell currents was similar to the rate of EtOHinduced 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 forskolinstimulated currents by 49.7 ± 10.2 %

(after 15 mins at + 60 mV), suggesting that the inhibitory effect of EtOH is independent of its osmotic effect.

4.3. Effects of EtOH metabolites on basal and forskolin-stimulated CFTR currents

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

The unsaturated FA, POA, at low concentrations (10 μM) did not affect the basal or forskolin-stimulated CFTR currents. However, at higher concentrations (100 and 200 μM), POA induced a dose-dependent and significant decrease in both the basal currents and forskolin-stimulated CFTR currents. 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.

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, strongly suggesting that formation of POA is a crucial step in the inhibitory effect of POAEE/EtOH.

4.4. EtOH, POAEE and POA cause ATPi depletion in PDECs, which is linked to CFTR inhibition

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. 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^{2+}-sensitive fluorescent dye, MgGreen-AM, which allows an indirect measurement of ATP_i. Administration of 100 mM EtOH caused a small ATP_i depletion in 75% of cells, whereas POA or POAEE (200 μM) induced a marked increase in MgGreen fluorescence intensity, whichreached a plateau about 5 min after the administration of the metabolites. 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.

In order to decide whether the inhibitory effect of EtOH, 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 and forskolin-stimulated currents 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_i.

4.5. 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, 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 and POAEE on forskolin-stimulated currents, as well as the effect of POA on the basal and forskolin-stimulated CFTR currents. 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.

4.6. Low concentration of EtOH stimulates whereas high concentrations of EtOH and fatty acids inhibit forskolin-stimulated CFTR currents 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. In both cases, inhibition was voltage-independent and irreversible.

4.7. Intracellular ATP 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). 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 % (unpublished data).

4.8. 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 or 10 µM trypsin 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) or PAR-2 antogonist (PAR-2-ANT; 10 µM) completely prevented

the inhibitory effect of trypsin on the forskolin-stimulated CFTR currents. These results suggest that trypsin inhibits CFTR currents by activation of PAR-2.

5. 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^-/HCO_3^- exchangers on the apical membrane to provide a daily supply of ~ 2.5 L of a HCO_3^- 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 HCO_3^- 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 humansEtOH 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 acitivity of CFTR. Previous studies have demonstrated that Ac, an oxidative metabolite of EtOH, strongly inhibits NF-κB binding activityand cholecystokinin-induced secretionin 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_i; 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_i 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_i in the ductal cells. POA caused a significantly greater decrease in ATP_i than POAEE. In addition, longer administration of POA did not cause a further increase in ATP_i (as based on Mg-Green fluorescence intensity), indicating that 200 μM POA likely induced nearly complete ATP_i 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. 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 inacutepancreatitisis 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 CFTRcurrents 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.

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