Regulation of CFTR and its Contribution to other Epithelial Cl^- Channels

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES
DER NATURWISSENSCHAFTEN (DR.RER.NAT.)
DER FAKULTÄT III FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN
DER UNIVERSITÄT REGENSBURG

vorgelegt von
Patthara Kongsuphol
aus Bangkok, Thailand

im Jahr 2011

Promotionsgesuch eingereicht am: 25. January 2011

Die Arbeit wurde angeleitet von: Prof. Dr. Karl Kunzelmann

Prüfungsausschuss:

Vorsitzender:	Prof. Dr. Ralph Witzgall
1. Gutachter:	Prof. Dr. Karl Kunzelmann
2. Gutachter:	Prof. Dr. Richard Warth
3. Prüfer:	Prof. Dr. Herbert Tschochner
Ersatzperson:	Prof. Dr. Michael Thomm

ZUSAMMENFASSUNG

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) ist ein cAMP-abhängiger Cl^--Kanal, der in der apikalen Membran von Epithelzellen exprimiert wird. CFTR spielt eine essentielle Rolle in der Elektrolyt- und Wasser-Homöostase. Mutationen im CFTR-Protein verursachen die Erkrankung Mukoviszidose (Cystische Fibrose), eine tödlich Erbkrankheit, die vor allem bei Kaukasiern auftritt. CFTR fungiert als Cl^--Kanal, reguliert aber auch andere Ionenkanäle und Transporter. Obwohl CFTR seit mehr als 20 Jahren untersucht wird, bleiben viele Fragen bezüglich seiner Funktionen offen. In der vorliegenden Arbeit wurden verschiedene Funktionen von CFTR untersucht, die zu einem besseren Verständnis der Rolle von CFTR in Epithelzellen führen sollten: Die Regulation von CFTR durch die Adenosinmonophosphat-abhängige Kinase (AMPK) *in vivo* und *in vitro*, die Rolle der CFTR- Mutation S573C für die Entwicklung einer Pankreatitis, sowie die neuartige Funktion von CFTR als Sensor der extrazellulären Protonenkonzentration und Regulator der Ca^{2+} abhängigen Cl^--Leitfähigkeit (CaCC).

Regulation von CFTR durch AMPK in vivo

Die Rolle der AMPK für den epithelialen Cl^- -Transport wurde *in vivo* mit Hilfe von AMPK-α1 defizienten Mäusen untersucht. Die Ergebnisse dieser Untersuchungen lassen auf eine wichtige Rolle von AMPK für die Regulation der Funktion von CFTR *in vivo* schließen. Ussingkammer-Experimente mit Präparaten des Kolons von AMPK-α1 defizienten Mäusen zeigten nach Aktivierung von CFTR durch cAMP einen erhöhten CFTR abhängigen Cl^- -Strom. Im Gegensatz zu gesunden Geschwistertieren, war dieser cAMP-abhängige Cl^- -Strom unempfindlich für Phenformin, einem Aktivator der AMPK. Zusätzlich bestätigten Messungen der rektalen Potenzialdifferenz (RPD) an AMPK-α1 defizienten Mäusen eine basale Erhöhung der RPD und eine verstärkte Aktivierung von CFTR durch cAMP gegenüber den Kontrolltieren. Dies sind die ersten Daten, die einen hemmenden Effekt der AMPK auf CFTR *in vivo* zeigen.

Mechanismus der Regulation von CFTR durch AMPK

Es wurde versucht die Phosphorylierungsstellen für AMPK im CFTR- Protein zu identifizieren und den Mechanismus der Regulation von CFTR durch AMPK *in vitro* zu untersuchen. Hierzu wurde die Methode der Expression in *Xenopus* laevis Oozyten verwendet. In diesem System können verschiedene CFTR-Mutationen exprimiert und der CFTR abhängige Cl^- -Strom mit Hilfe der Spannungsklemme untersucht werden. Im Gegensatz zu den früheren Beobachtungen konnte gezeigt werden, dass die Aminosäuren S737 und S768 in der R-Domäne von CFTR von AMPK und nicht von der Proteinkinase A (PKA) phosphoryliert werden. Mutationen dieser Serine zu Alanin führte zur einer basalen Erhöhung des CFTR- Stroms im nichtstimulierten Zustand. Aktivierung von CFTR durch die PKA führte zu einem 4- fach größeren CFTR Strom. Dieser Strom konnte weder durch den AMPK-Inhibitor Phenformin noch durch den AMPK-Aktivator Compount C beeinflusst werden. Zusammenfassend zeigten diese Experimente, dass AMPK durch Phosporylierung von S737 und S768 die Ruheaktivität und PKA-abhängige Aktivierung von CFTR inhibiert.

Weiterhin wurde gezeigt, dass die α-Untereinheit von AMPK mit dem C-Terminus von CFTR interagiert. Daher kann eine lokale Regulation von CFTR durch die AMPK vermutet werden, unabhängig von dem gesamtzellulären AMP/ATP Verhältnis. Aus den Ergebnissen lässt sich folgendes Modell ableiten: AMP wird lokal vom Shank2/PDE Komplex gebildet. Dieser Komplex ist mit CFTR über eine PDZ- Interaktionsdomäne assoziiert. Die lokale AMP Konzentration führt zur Aktivierung der AMPK, die ebenfalls am C-Terminus von CFTR bindet. Die Aktivierung der CFTR-nahen AMPK hält den CFTR-Kanal unter Ruhebedingungen geschlossen.

Metformin Behandlung erhöht das Risiko einer Pankreatitis bei CF Patienten

Metformin ist ein Aktivator der AMPK und wird zur Behandlung von Diabetes mellitus Typ II eingesetzt. Die Laktatazidose ist eine häufige sekundäre Komplikation der Metformin-Therapie, insbesondere bei Patienten mit Nierenfunktionsstörung, Alkoholmissbrauch oder Lebererkrankungen. Auch wurde von Patienten mit Nierenversagen berichtet, bei denen sich eine Pankreatitis nach Metformin-Therapie entwickelte. Dieser gab Anlass, die Wirkung von Metformin auf die Funktion von CFTR mit der Mutation S573C zu untersuchen, die eine Pankreatitis auslösen kann. Während der cAMP aktivierte Cl^--Strom des Wildtyp-CFTR von Metformin nicht gehemmt wird, ist der cAMP aktivierte Cl^--Strom der CFTR Mutante S573C

reduziert und durch Metformin hemmbar. Das Phosporylierungsmuster von wtCFTR und S573C-CFTR war dabei nicht verändert. Intrazelluläre Ansäuerung, z.B. durch eine Laktatazidose, führte ebenfalls zu geringer Hemmung von wtCFTR, aber zu fast vollständiger Inhibition von S573C-CFTR. Zusammenfassend zeigen diese Daten, dass bei Patienten mit der CFTR Mutation S573C die verringerte Aktivierung des CFTR Cl^--Stroms durch cAMP funktionell ausreichend ist. Während einer Metformin-Therapie besteht jedoch die Gefahr einer kritischen Hemmung von S573-CFTR, u. a. im Pankreas, was zu einer Pankreatitis führen kann.

CFTR ermöglicht die Messung einer extrazellulären Azidose und reguliert Ca^{2+} aktivierte Cl^- Kanäle

Im letzten Abschnitt der vorliegenden Arbeit wurden Hinweise gefunden für eine neue Rolle von CFTR bei der Messung der extrazellulären Protonenkonzentration. In *Xenopus* Oozyten konnte gezeigt werden, dass die Expression von CFTR notwendig ist, um über eine extrazelluläre Ansäuerung den endogenen Ca^{2+} aktivierten Cl^- Kanal (CaCC) zu aktivieren. Dieser Strom ist deutlich auswärts gleichrichtend und wird durch DIDS und NPPB inhibiert. In der Abwesenheit von intaktem CFTR wird dieser Strom nicht aktiviert. Die Expression von CFTR könnte zu einer Translokalisation eines H^+- Rezeptors in die Oozytenmembran führen. Bindung von extrazellulären Protonen aktiviert die Phospholipase C und führt zum Anstieg der intrazellulären H^+-Konzentration, was CaCC aktiviert. Die physiologische Bedeutung der CFTR induzierten Protonenempfindlichkeit und Aktivierung von CaCC ist unklar. Die CFTR abhängige Expression eines H^+- Rezeptors könnte für den Knochenmetabolismus und die Mineralisierung eine wichtige physiologische Rolle spielen. Osteoporose ist eine bekannte Komplikation der Mukoviszidose. Es ist bekannt, dass die Funktion von Osteoblasten und Osteoklasten von der extrazellulären Protonenkonzentration bestimmt wird und Kochenzellen CFTR exprimieren. Es ist daher möglich, dass bei Mukoviszidose eine Dysfunktion der Protonenmessung vorliegt, was zur Osteoporose führen könnte.

CONTENTS

CHAPTER 1.

Introduction

Epithelial cells form barriers that protect living organisms from harmful influences and allow transport of substrates and electrolytes between different compartments. In order to fulfill these tasks, epithelial monolayers are formed that are tightly regulated to allow selective transport between the apical and the basolateral compartment. Tight junctions between individual cells guarantee limited and selective paracellular transport of ions and water[1], while polarized distribution of ion channels, substrate transporters and water channels in apical and basolateral membranes allow selective and vectorial (unidirectional) transcellular transport. Depending on the tissue specific arrangement of these transport proteins in the luminal and basolateral membrane, the epithelium will have either secretory or absorptive properties. Secretion and absorption of electrolytes, i.e. ions such Na^+, K^+, Cl^-, HCO_3^-, and water, are main functions of epithelial cells present in kidney, airways, intestine, sweat duct and a number of exocrine glands[2]. Apart from electroneutral transport which will not be further discussed in the present introduction, electrogenic secretion or absorption of ions across epithelial cells is a major determinant of the ion flux across the epithelium. Depending on existing ion gradients across apical and basolateral membranes, the membrane potentials, and the arrangement of ion channels, transporters and pumps, secretion or absorption of ions will occur. Water will follow passively through the paracellular shunt or transcellularly through water channels.

A number of tightly controlled ion channels and transporters in both apical and basolateral membranes are required for proper electrogenic transport. In principle epithelial cells can be subdivided into absorptive and secretory cells (Figure1)[3]. In absorptive cells, present in airways and the intestinal epithelium, sodium ions (Na^+) are absorbed via the Epithelial Na^+ Channel (ENaC) while chloride ions (Cl^-) are transported paracellulary (Figure 1A). Na^+ is pumped out of the cell by basolateralllly located Na^+/potassium (K^+) ATPases, while K^+ recycles through basolateral K^+ channels. Luminal absorption of Na^+ and basolateral recycling of K^+ will generate a lumen negative transepithelial voltage, which drives paracellular absorption of Cl^-. In secretory cells, such as submucosal gland cells and epithelial cells in the base of colonic crypts, Cl^-, K^+, and Na^+ are taken up into the cell by basolaterally located $Na^+/K^+/2Cl^-$ cotransporters (NKCC1).

Na^+ is pumped out again by basolateral Na^+/K^+-ATPases, and K^+ exits via basolateral K^+ channels. Apical (luminal) Cl^- secretion is mediated by cAMP-regulated CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) Cl^- channels, and probably by Ca^{2+}-regulated TMEM16A Cl^- channels, while in some tissues such as colon and distal airways, K^+ is secreted by luminal K^+ channels. Na^+ will move paracellularly between the cells following the lumen negative transepithelial voltage produced by luminal Cl^- secretion and basolateral K^+ transport. (Figure1B). The present thesis will focus on the CFTR Cl^- channel, its regulation by protein kinase and role in cystic fibrosis.

A) Absorptive cell B) Secreting cell

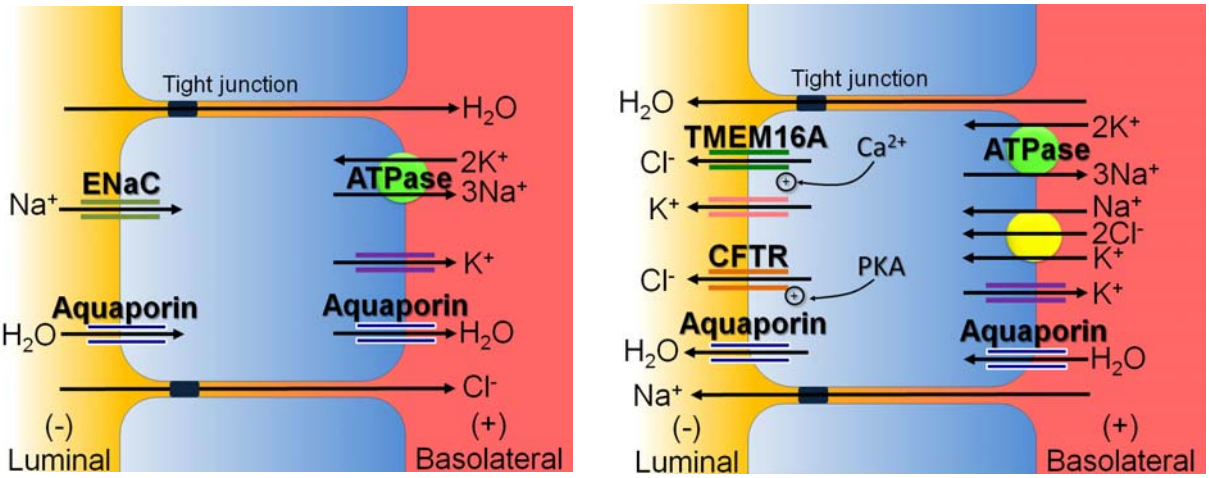

Figure 1. Simplified schemes of ion transport in absorptive (A) and secretory epithelial cells (B). A) In absorptive cells, Na^+ is absorbed via ENaC and exits the cell basolaterally via Na^+/K^+ ATPases, while K^+ is transported out through basolateral K^+ channels. Cl^- moves through the paracellular compartment. Water will move passively following ionic gradient through aquaporin water channels (transcellular) or through the gap (tight) junctional pathway (paracellular). B) In secretory cells, Cl^-, K^+ and Na^+ enter the cell via $Na^+/K^+/2Cl^-$ cotransporters (NKCC1) where Cl^- is secreted to the luminal side by CFTR while K^+ may be secreted by luminal K^+ channels. Na^+ is pumped out by basolateral Na^+/K^+ ATPases, while K^+ leaves the cells via basolateral K^+ channels. Na^+ is secreted paracellularly and water moves transcellularly and through the paracellular shunt pathway.

Cystic fibrosis (CF)

Cystic Fibrosis (CF) is the most common fatal genetic disorder among the Caucasian population. It is caused by defects in the cystic fibrosis transmembrane conductance regulator (CFTR) that plays a crucial role in epithelial Cl^- transport. A major secondary complication causing the high lethality in CF is due to chronic lung infections[4]. Malfunction or absence of CFTR in the airway

surface epithelium reduces Cl^- and water secretion into the lumen, probably causing airway dehydration, mucus hyperviscosity, and disruption of the mucociliary clearance. These complications lead to a vicious cycle of inflammation and bacterial colonization and infection. *Staphylococcus aureus* and *Haemophilus influenza* are common bacteria affecting CF patients, while infections by *Pseudonomas aeruginosa* indicate a turning point into a severe chronic inflammatory lung disease[4].

Other epithelial tissues expressing CFTR, including salivary glands, liver bile ducts, pancreatic ducts, small intestine, colon, vas deferens, the cervicouterine tract, and sweat glands[5,6], are also affected in CF. Similarly to the airway epithelium, some of these tissues also show signs of dehydration and are covered with a highly viscous mucus. The thick and sticky mucus blocks duct perfusion and destroys the tissues by the inflammatory processes. Thus pancreatitis affects around 85% of CF patients[5]. Because CFTR is expressed abundantly in intestinal epithelia, around 20% of infants suffering from CF, develop a meconium ileus[7]. In the ducts of the sweat gland, CFTR is essential for reabsorption of salt. Malfunction of CFTR elevates the sweat salt content, which is still a major diagnostic tool in CF[5].

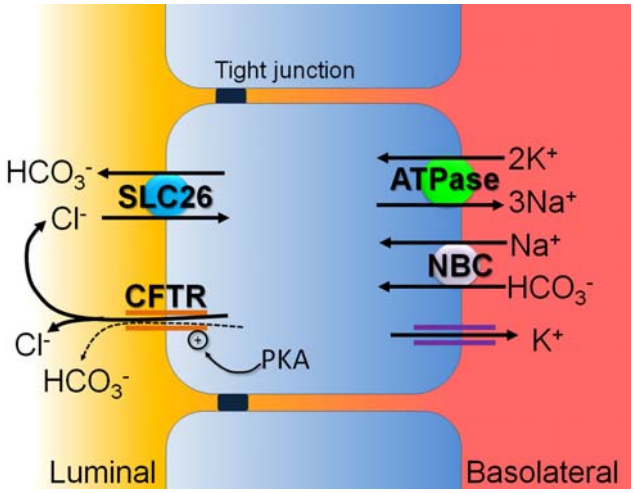

Figure 2. Schematic model represents HCO_3^- transport in pancreas. HCO_3^- is taken up basolaterally via the Na^+/HCO_3^- cotransporter (NBC). Moreover HCO_3^- is produced intracellularly with the help of the carboanhydrase (not shown). Na^+ is pumped out by the basolateral Na^+/K^+-ATPase, whereas K^+ exits the cell through basolateral K^+ channels. HCO_3^- is mainly secreted through the apical Cl^-/HCO_3^- exchanger and partly directly through CFTR. Cl^- taken up by apical Cl^-/HCO_3^- exchangers recycle to the luminal compartment through CFTR. (Model adapted from Shumaker *et al*[179])

CFTR does not only conduct Cl^- but is also permeable for HCO_3^-. This may play an important role in the airways as well as in the small intestine, where defective HCO_3^- transport may contribute to formation of the meconium ileus in cystic fibrosis. Also in the pancreas the importance of the transport of bicarbonate by CFTR is highly relevant. Secretion of HCO_3^- in the pancreatic duct alkalinizes the pancreatic juice and neutralizes the acid chyme that is entering duodenum. Although HCO_3^- is mainly secreted by the Cl^-/HCO_3^- exchanger (SLC26) located in the apical membrane of pancreatic epithelial cells, CFTR is necessary to recycle Cl^- over the apical membrane, thereby driving bicarbonate

secretion. As the bicarbonate concentration rises in the more distal part of the pancreatic duct, direct secretion of HCO_3^- through CFTR Cl^- channels will become more important[8-10] (Figure 2). In CF, the pancreatic tissue shows cystic degeneration and pancreatic fibrosis due to duct obstruction and pancreatitis. Degeneration of the pancreas, which indicates a severe state of the CF disease, causes a defect in secretion of pancreatic enzymes and consequently malnutrition of the patients[11].

CF can be categorized into six classes, according to CFTR production and function[4]. Class I: no CFTR synthesis, class II: inadequate processing of CFTR, class III: improper CFTR regulation, class IV: reduced CFTR conductance, class V: partially defective CFTR production, and class VI: accelerating CFTR turnover. More than 1,700 CFTR mutations are described meanwhile. Deletion of phenylalanine at position 508 (F508del) is a class II mutation that is most common to be found in CF patients. F508del causes misfolding of the protein and thus targeting for degradation within the endoplasmic reticulum (ER)[12].

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR): Properties and regulation

CFTR is a cAMP dependent Cl^- channel that is expressed in the apical membrane of epithelial cells[13]. The properties that distinguish CFTR from other Cl^- channels can be described as follow: *1)* small single-channel conductance (6-10 pS), *2)* linear current-voltage (I-V) relationship, *3)* anion permeability sequence of $Br^- \geq Cl^- > I^-$ *4)* time and voltage independence of the channel gating, and *5)* cAMP dependent regulation. CFTR consists of 5 domains including two membrane spanning domains (MSD), two nucleotide binding domains (NBD), and one regulatory domain (R domain) (Figure 3). Each MSD is composed of 6 transmembrane domains that connect the N-terminus with NBDs, R-domain and C terminus. The two MSDs-NBDs are joined by a unique R domain. CFTR regulation depends mainly on ATP-binding and hydrolysis at both NBDs and phosphorylation of the R domain.

CFTR nucleotide binding domains (NBD)

CFTR belongs to the adenosine-triphosphate (ATP) binding cassette (ABC) transporter family[13]. Similar to other members of the ABC transporter family, CFTR contains two NBDs which bind and hydrolyze ATP. For other members of ABC transporter, ATP is required for an uphill

transport of substrates. In contrast, Cl^- moves across CFTR following the electrochemical gradient. Thus ATP hydrolysis is not used to drive active uphill transport of Cl^-. However, ATP hydrolysis is required for channel gating[14-16]. Together with R domain phosphorylation by protein kinase A (PKA), binding and hydrolysis of ATP leads to conformational changes within CFTR and thus a change from the closed to the open state. However, the precise conformational changes influenced by ATP-binding and hydrolysis are still controversial[17-20] So far the model proposed by Vergani *et al*. is most widely accepted[21]. According to this binding of ATP molecules to each NBD at the core subdomain (head) dimerizes both NBDs in a 'head to toe' configuration (Figure 3). Upon this dimerization, CFTR will undergo a conformational change which will lead to opening of the channel. On the other hand, hydrolysis of ATP at NBD2 dissociates the dimerization[22-24], therefore causing closure of the channel.

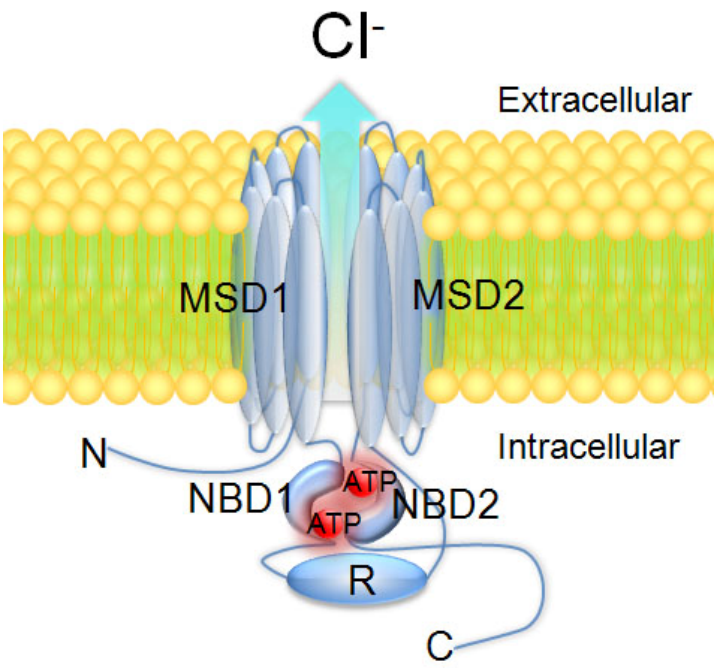

Figure 3. Model of the CFTR. CFTR consists of five major components including two membrane spanning domains (MSD), two nucleotide binding domains (NBD), and a regulatory (R) domain. CFTR is activated by two important regulatory steps, including binding of two ATP molecules at both NBDs and phosphorylation of the R domain by PKA.

CFTR-R domain phosphorylation

Although ATP binding and hydrolysis at both NBDs is necessary for channel gating, the most important regulatory step for CFTR gating comes through phosphorylation of the R-domain. Phosphorylation of CFTR is tightly controlled by the balance between kinases and phosphatases. Several kinases phosphorylate CFTR, including Protein Kinase A (PKA), Protein Kinase C (PKC), cGMP-dependent protein kinase (PKG), and $p60^{C\text{-}SRC}$. Although CFTR phosphorylation by PKG and $p60^{C\text{-}SRC}$ have not been widely studied, both have been shown to activate CFTR[25-27]. PKC is known to phosphorylate CFTR at serine residues S686 and S700 in vivo[28]. A study by Jia *et al*. suggests that pre-exposure of CFTR to PKC promotes PKA phosphorylation of CFTR[29]. It has been claimed that pre-phosphorylation by PKC is required for

gating of CFTR by PKA. Nonetheless the mechanisms of PKC-dependent regulation are still unclear and await further studies.

PKA is a cAMP dependent protein kinase. It is the most important kinase phosphorylating CFTR. *In vivo* and mass spectrometry experiments indicate that 8 serines, located in the R domain, including S660, S700, S712, S737, S768, S795, and S813, are phosphorylated by PKA[28,30,31]. PKA phosphorylation is crucial for CFTR activation. Studies by Winter and colleagues suggest that PKA phosphorylation may in part increase the affinity of ATP binding to both NBDs therefore increase the rate of channel opening[32,33].

Another kinase, Adenosine Monophosphate dependent Kinase (AMPK) has also been reported recently to interact and phosphorylate CFTR[34-38]. AMPK is a serine/threonine kinase that forms a heterotrimeric complex comprising a catalytic α subunit and regulatory β and γ subunits. AMPK is considered as a “low-fuel” sensor that responds to an increase in intracellular AMP/ATP ratio such as lowering of intracellular glucose, or stress conditions like hypoxia[39]. Once AMPK is activated, it phosphorylates many downstream targets, triggering catabolic pathways to regenerate ATP and switching off biosynthetic pathways and other processes that consume ATP. Phosphorylation of CFTR by AMPK inhibits CFTR Cl^- current[34,35,37,40]. However, the phosphorylation sites and mechanisms by which AMPK regulate CFTR are still obscure.

AMPK can be activated by several drugs including biguanidine compounds like phenformin and metformin. Phenformin and metformin activate AMPK, in part, by inhibiting complex I of respiratory chain, without interfering AMP/ATP ratio[41]. Phenformin and metformin are drugs used to combat type II diabetes by promoting insulin-stimulated glucose uptake into muscles and lowering hepatic glucose output[41,42]. Due to the high incidence of lactic acidosis, phenfomin has been withdrawn from clinical use and has been replaced by metformin. Pancreatitis is not a common complication of metformin treatment, however two cases of metformin-induced acute pancreatitis are described[43,44]. These patients are reported to have a background of renal failure. Treatment with metformin leads to accumulation of metformin within the body, thereby developing metabolic acidosis and acute pancreatitis.

Although CFTR has been extensively studied for more than 20 years, the role of CFTR R-domain phosphorylation and its role for channel gating are not completely elucidated[45-47]. Partial or complete removal of the R domain (708-853 or 768-830) constitutively activates CFTR in the presence of ATP, independently of PKA phosphorylation[33,48,49]. These data suggest that the R

domain inhibits CFTR in a similar fashion as described for the inhibitory “ball and chain” model in Shaker K^+ channels[50]. The inhibitory effects of the R domain can be released by removal of R domain or by PKA phosphorylation[31,48,51]. However, studies on single or multiple mutations of serines to alanine indicate that not all PKA-phosphorylated serine residues are responsible for CFTR activation. Only serines located at positions 660, 795, and 813 are responsible for activation of the channel[31,33,52-54]. Interestingly, mutations of serines 737 and 768 to alanines increase CFTR Cl^- currents[46,53,55-57]. These data indicate that S737 and S768 inhibit CFTR, they are, therefore, referred to as “inhibitory serines”.

Taken together the non-phosphorylated R domain inhibits CFTR. This inhibitory action of the R-domain on CFTR is released by PKA-dependent phosphorylation of several serine residues. However, the paradoxical effects of PKA-dependent and AMPK-dependent phosphorylation of the different serine residues within the R-domain are far from being understood. The purpose of the present study was therefore to gain a mechanistic insight into the control of CFTR by AMPK and to demonstrate regulation of Cl^- secretion by AMPK *in vivo.*

Compartmentalization and local regulation of CFTR

CFTR interacts with many proteins forming a macromolecular complex that locally regulates its activity[58]. The PDZ-binding domain located in CFTR’s C-terminus plays a crucial role in protein-protein interaction of CFTR with scaffold proteins, receptors, the cytoskeleton and a number of proteins that belong to the cellular signaling machinery. For instance, the PDZ-binding motif at the C-terminus of CFTR interacts with PDZ-domains of NHERF1 (Na^+/H^+ Exchanger Regulatory Factor 1). NHERF1 binds to β_2 Adrenergic Receptors (β_2-AR) located at the plasma membrane (Figure 4). When β_2-AR are activated by binding of adrenaline, it triggers stimulatory GTP-binding (Gs) proteins and activation of the adenylyl cyclase, thereby increasing intracellular cAMP, which finally activates the CFTR Cl^- channel (Figure 4)[59]. In addition, NHERF1 was also described to be important for activation of CFTR through Adenosie 2b Receptor (A2b-R) (Figure 4)[59]. NHERF1 contains an ERM (Ezrin, Radixin, Moesin) module which interacts with an ezrin/PKA complex. As a consequence, binding of CFTR to NHERF1 brings along PKA which will be located into close proximity of the R-domain of the CFTR channel (Figure 4)[58-61]. The nearby located adenylyl cyclase converts ATP into cAMP, while the phosphodiesterase (PDE) degrades cAMP and thus removes the signaling molecule. PDE, in particular PDE4D is also anchored to the macromolecular complex by a protein named Shank2, which binds to CFTR’s PDZ motif[62]. Competitive binding of NHERF1/PKA and Shank2/PDE4D protein complexes to the

PDZ motif of CFTR allows for spatiotemporal regulation of cAMP levels and thus local control of CFTR activity, independent of global changes in cAMP[61,62].

Thus CFTR phosphorylation and activation of Cl^- secretion is regulated by the NHERF1/Ezrin/PKA complex and generation of local cAMP levels, while dephosphorylation of CFTR and inactivation of Cl^- secretion is due to the activity of local phosphatases. Binding of Protein phosphatase 2A (PP2A) to CFTR's C terminus will dephosphorylate CFTR and consequently close the channel[61]. Apart from these well established and important mechanisms, other proteins have also been reported to bind and regulate CFTR. For instance, binding of SYN1a, SNAP23, and Munc18a reduce channel activity, whereas RACK1 and PKC activate CFTR[59]. Taken together, the available data indicate that macromolecular protein-complexes

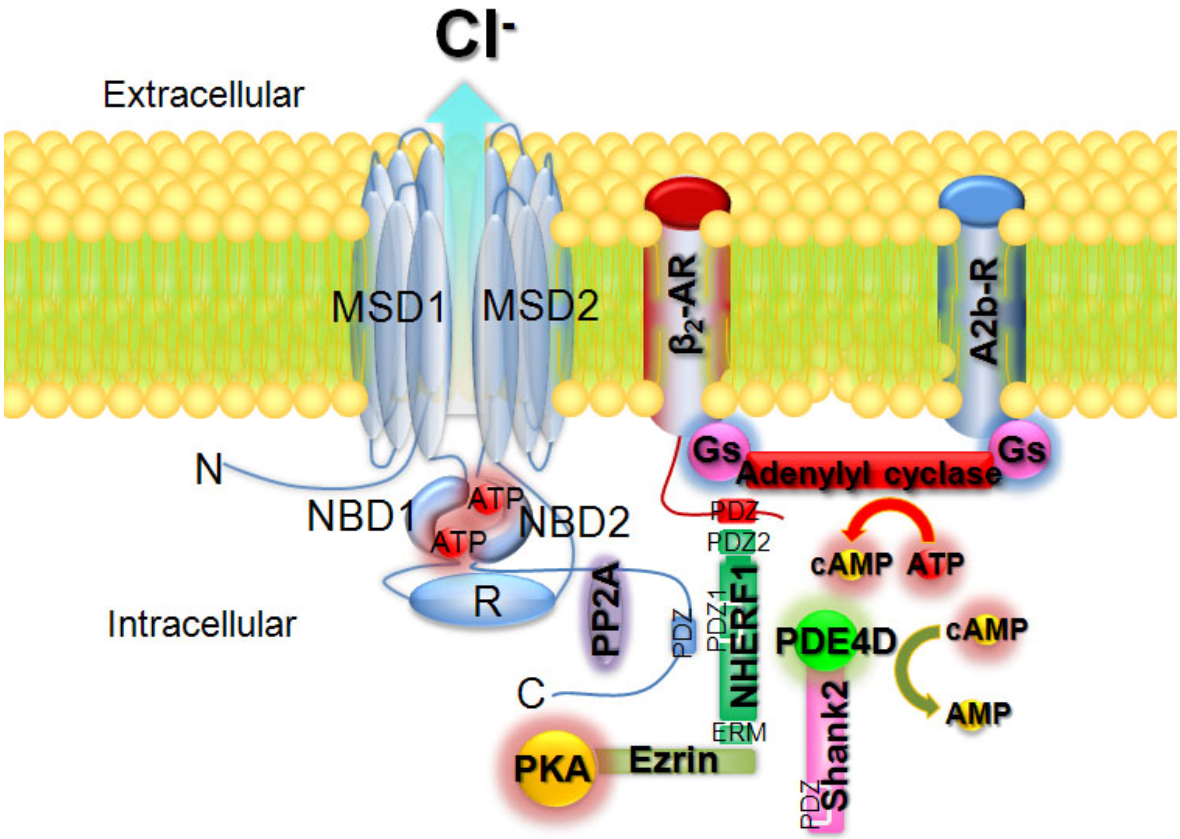

Figure 4. Compartmentalized control of CFTR. CFTR interacts with several binding partners mainly through CFTR's PDZ motif. NHERF1/β_2-AR/A2b-R and shank2/PDE4 protein complexes bind to the PDZ motif in a competitive manner, thus allowing for spatiotemporal control of cAMP levels in close proximity to CFTR. NHERF1 interacts with the ezrin/PKA complex, therefore binding of CFTR to NHERF1 places PKA into close proximity of the R-domain of the CFTR channel. Phosphatase 2A (PP2A) binds to the C-terminal end of CFTR, allowing for dephosphorylation of the R domain, and closure of the channel.

around CFTR allow for local control of channel activity, independent of global cellular changes of signaling molecules. Such a compartmentalized regulation of channel activity occurs independent in both apical and basolateral membranes of polarized epithelial cells, and has also been reported for Ca^{2+} dependent regulation of ion channels.

CFTR as a regulator of other channels

Although numerous studies describe the role of CFTR as an ATP/PKA regulated Cl^- channel, CFTR also controls the activity of other ion channels and transporters including ENaC[63,64], K^+ channels (such as ROMK1 and ROMK2)[65,66], anion exchangers[67-69], and water channels (aquaporin)[70]. Importantly CFTR is also known to regulate other Cl^- channel including the so called Outwardly Rectifying Cl^- Channel (ORCC)[71,72], and Calcium (Ca^{2+})-activated Cl^- channel (CaCC)[73,74]. Because CaCC has been proposed as one of the alternative therapeutic targets for the treatment of cystic fibrosis[4], studies on regulation of CaCC and the relationship between CFTR and Ca^{2+} dependent Cl^- secretion are of particular interest.

CaCC regulation by CFTR

Ca^{2+}-activated Cl^- channels (CaCC) are Cl^- channels that are activated by an increase in intracellular Ca^{2+}. CaCC are important for many physiological processes. For instance membrane excitability, olfactory transduction, regulation of the vascular tone, photoreception, and epithelial secretion are all controlled by CaCC[75,76]. The molecular identity of CaCC has long been a mystery until recently when three independent laboratories using three independent experimental approaches, identified the protein TMEM16A (also known as ANO1 or DOG1) as the major component of CaCC[77-79]. TMEM16A belongs to a family of 8 transmembrane domain proteins, together with 9 others members termed TMEM16B-H, TMEM16J, and TMEM16K (also called ANO2-10). Unique properties of CaCC comprise strong outward rectification at submaximal Ca^{2+} concentration (Figure 5), time and voltage dependence of the Cl^- current, and an anion permeability sequence of $I^->Cl^-$. In addition, studies on TMEM16A expression in HEK293 cells reveal a very small single channel conductance of CaCC[77]. Along with the discovery of TMEM16A as a major component of CaCC, TMEM16A-/- mice have been generated[80]. These mice demonstrate a very severe phenotype and usually die within 3-5 days. Their high lethality is predominantly due to defective development of cartilage rings of the trachea. Subsequent electrophysiological studies on TMEM16A knockout animals showed defective Ca^{2+} activated Cl^- currents in trachea, colon, hepatocytes, and pancreas of TMEM16A-

/- mice, supporting the concept of TMEM16A as a major component of CaCC[81]. TMEM16A was also found to be upregulated in Gastrointestinal Stromal Tumors (GIST). Because of its extreme abundance in GIST, TMEM16A is used as a marker for clinical diagnosis of GIST[82]. An upregulation of TMEM16A in GIST implies a role of CaCC in cancer development. However the function of TMEM16A in cancer is not known and needs to be further elucidated

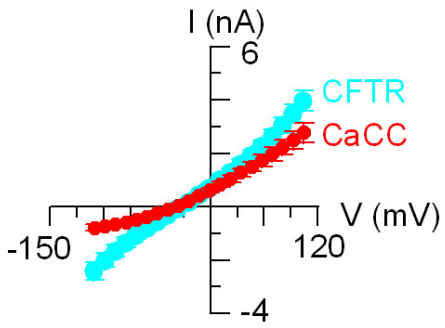

Figure 5. IV curve of CFTR and CaCC. I/V curves representing the current voltage relationships for CFTR and CaCC when expressed in HEK293 cells. CFTR displays a linear IV curve (blue) whereas CaCC shows strong outward rectification (red).

CFTR was reported to inhibit CaCC. Overexpression of CFTR in bovine pulmonary artery endothelial cells or *Xenopus* oocytes inhibited endogenous CaCC, while activation of CFTR completely abolishes current produced by CaCC[73,74]. However the mechanism underlying the inhibition of CaCC by CFTR is still poorly understood. On the other hand, CaCC activity was described to be enhanced in cystic fibrosis, where expression of functional CFTR is missing[83,84]. This finding correlates with another observation indicating that intracellular Ca^{2+} mobilization and Ca^{2+} signaling are increased in human CF airway epithelial cells[85-87]. Hence, augmented Ca^{2+} activated Cl^- currents in CF are most likely due to an increase in intracellular Ca^{2+} signaling.

Intention and outline of the present thesis

The present data indicate that CFTR is a multifunctional protein that shows a complex regulation and that interacts with numerous additional proteins. Although CFTR has been studied for more than 20 years, many open questions regarding its regulation by AMPK, the functional consequences of particular CFTR-mutations and its interaction with other proteins and interference with other cellular processes are still remaining. The present thesis therefore aimed in elucidating several of these mechanisms.

AMPK phosphorylation of CFTR has been shown to inhibit CFTR activity in human airway epithelial cells[34,35,37,40]. Although highly interesting, *in vivo* data on the regulation of CFTR by AMPK have never been presented. In chapter 2 of this thesis data are shown on the regulation of Cl^- secretion by AMPK *in vivo*. This study makes use of animals in which the relevant AMPK

has been knockout out. The data indicate that AMPK does regulate CFTR-dependent transport *in vivo.*

Chapter 3 continues to focus on AMPK-dependent regulation of CFTR. The phosphorylation sites for AMPK within CFTR have never been identified. Moreover, the mechanisms by which AMPK regulates CFTR activity were so far unknown. The chapter therefore describes how AMPK acts on CFTR, and elucidates the relationship between regulation by protein kinase A and AMPK. For the first time the phosphorylation sites for AMPK are described.

AMPK can be activated by several drugs including biguanidine compounds like phenformin and metformin. On the other hand biguanidines have been shown to induce pancreatitis in some patients. Pancreatitis is also common in cystic fibrosis and has been correlated with several CFTR mutations including S573C. Interestingly, S573C is a non-CF causing mutation, associated with pancreatitis[88,89]. The study described in chapter 4 of the present thesis examines the correlation of metformin-induced pancreatitis to the mutation S573C. Using the expression system in *Xenopus laevis* oocytes, the function of S573C-CFTR and its alteration by metformin is studied. It was found that the antidiabetic drug metformin alters regulation of CFTR by AMPK. Treatment of diabetes in patient carrying the S573C-CFTR mutation by metformin may therefore cause a rsik for pancreatitis.

In the last chapter 5 of this thesis, the focus is on CFTR-dependent sensing of extracellular acidic pH, which elicits a transient increase in intracellular Ca^{2+}. Since this study was performed in oocytes of *Xenopus* laevis, which express endogenous Ca^{2+} dependent Cl^- channels (CaCC), CaCC is activated during CFTR-dependent sensing of low extracellular pH. Similar to the previously described translocation of ß-adrenergic receptors to the apical membrane of epithelial cells, CFTR may also allow for translocation of proton receptors to the plasma membrane. It is speculated that H^+ sensing in bone cells is CFTR dependent and is impaired in cystic fibrosis, thus leading to osteoporosis.

CHAPTER 2.

Regulation of Cl^- Secretion by AMPK *In Vivo*

ABSTRACT

Previous *in vitro* studies suggested that Cl^- currents produced by the cystic fibrosis transmembrane conductance regulator (CFTR; ABCC7) are inhibited by the α1-isoform of the adenosine monophosphate (AMP) stimulated kinase (AMPK). AMPK is a serine/threonine kinase that is activated during metabolic stress. It has been proposed as a potential mediator for transport-metabolism coupling in epithelial tissues. All previous studies have been performed *in vitro* and thus little is known about regulation of Cl^- secretion by AMPK *in vivo*. Using AMPKα1-/- mice and wild type littermates, we demonstrate that phenformin, an activator of AMPK, strongly inhibits cAMP activated Cl^- secretion in mouse airways and colon, when examined in *ex vivo* in Ussing chamber recordings. However, phenformin was equally effective in AMPKα1-/- and wt animals, suggesting additional AMPK-independent action of phenformin. Phenformin inhibited CFTR Cl^- conductance in basolaterally permeabilized colonic epithelium from AMPKα1+/+ but not AMPKα1-/- mice. The inhibitor of AMPK compound C enhanced CFTR mediated Cl^- secretion in epithelial tissues of AMPKα1+/+ mice, but not in AMPKα1-/- mice. There was no effect on Ca^{2+} mediated Cl^- secretion, activated by ATP or carbachol. Moreover CFTR-dependent Cl^- secretion was enhanced in the colon of AMPKα1-/- mice, as indicated in Ussing chamber *ex vivo* and rectal PD measurements *in vivo*. Taken together, these data suggest that epithelial Cl^- secretion mediated by CFTR is controlled by AMPK *in vivo*.

Key words: Cystic fibrosis transmembrane conductance regulator, CFTR, AMPK

Published in: **Kongsuphol P**, Hieke B, Ousingsawat J, Almaca J, Viollet B, Schreiber R, Kunzelmann K. Regulation of Cl- secretion by AMPK in vivo. *Pflügers Archiv : European Journal of Physiology* 457, 1071-8(2009).

Own experimental contribution: All open and real short circuit Ussing chamber experiments in mouse trachea and colon, genotyping and related work.

Own writing contribution: Methods, Results and parts of Introduction and Discussion.

INTRODUCTION

The cystic fibrosis transmembrane regulator (CFTR) is a Cl^- channel at the apical surface of many epithelia that is defective in the severe inherited disease cystic fibrosis[90]. The activity of CFTR is regulated by binding of ATP and heterodimerization of both nucleotide binding domains along with phosphorylation by protein kinase A (PKA)[91-93]. Recently a new kinase, the adenosine monophosphate dependent kinase (AMPK) has emerged that phosphorylates CFTR at an unknown location and inhibits its single channel open probability[35,38]. AMPK is a ubiquitous serine/threonine kinase with orthologous in all eukaryotes. The enzyme exists as a heterotrimer with a catalytic α subunit and regulatory β and γ subunits. In response to metabolic stress and rise in the cellular AMP levels, AMPK phosphorylates numerous proteins and activates catabolic pathways that generate ATP, while inhibiting cell growth, biosynthesis and other processes that consume ATP[39]. Thus AMPK is regarded as a 'low-fuel' sensor[39,94]. It acts on a wide range of substrates and cellular pathways, including regulation of metabolic pathways controlling glycolysis, fatty acid synthesis and oxidation, as well as cellular glucose uptake, and cholesterol synthesis. Moreover it controls signaling pathways involved in apoptosis, cell cycle, transcriptional regulation, and inflammation[95]. Recent reports also suggest that AMPK affects the activity of proteins involved in electrolyte transport, such as CFTR and the epithelial Na^+ channel ENaC[38,96,97].

A mouse knockout model for AMPKα1 has been generated some time ago[98]. The animals demonstrate normal survival and did not present any obvious phenotype. It was shown that in skeletal muscle AMPKα2 is the predominant catalytic subunit of AMPK and is responsible for glucose uptake although during contraction, the two alpha-isoforms seem to substitute for each other in terms of activity[98]. However, the results of another study did not support an essential role of either AMPKα1 or AMPKα2 in regulating exercise-induced gene activation in skeletal muscle[99]. Nevertheless, in a subsequent report the authors demonstrated that AMPKα1 but not AMPKα2 is necessary for regulation of twitch-contraction stimulated glucose uptake[100]. In general defects in glucose homoeostasis were not observed in AMPK*α*1−/− mice, while AMPK*α*2−/− mice show high plasma glucose levels and low plasma insulin concentrations in the fed period and during the glucose tolerance test[101]. Apart from these studies not much is known about the contribution of AMPK to membrane transport *in vivo*. At a recent conference data were presented that indicate a mild renal phenotype in AMPK*α*1−/− but not in AMPK*α*2−/− mice and suggested regulation of expression and function of the renal $Na^+/K^+/2Cl^-$-cotransporter NKCC2 in the thick ascending limb of the loop of Henle by AMPK*α*1[102].

The catalytic isoform AMPKα1 was shown to co-localize with apical CFTR in rat nasal epithelial cells and colonic epithelial cells[34,38]. Inhibitory regulation of CFTR by AMPK has been demonstrated in *Xenopus* oocytes[38] and cultured epithelial cells from colonic carcinoma and airways[34,35,103]. Moreover, lack of functional CFTR expression has been suggested to up-regulate AMPK activity in epithelial cells carrying the CF defect. It was concluded that AMPK activation in CF airway epithelial cells is an adaptive response that reduces inflammation. Thus therapies have been proposed to activate AMPK in CF airways in order to reduce excessive airway inflammation[36]. However, up to now no data were presented the existence of AMPK-regulation of CFTR in native epithelial tissues. Using pharmacological tools, epithelial tissues from AMPKα1 knockout animals[98] and wt littermates, we demonstrate regulation of CFTR by AMPK in the native epithelium, which could be particularly relevant under non-stimulated conditions.

MATERIALS AND METHODS

Animals, M1 cells and Ussing chamber experiments: Generation of the AMPKα1 knockout mice has been described in a previous report. A LacZ gene was knocked in the AMPKα1 gene using gene trp startegy, where LacZ is under the control of the AMPKα1 promoter[98]. For the Ussing chamber measurements, the nasal epithelium, trachea, and colon were removed from AMPKα1 knockout animals and wild type littermates[98], euthanized by ethically approved institutional procedures. For genotyping, mouse tail was digested using Viagen DirectPCR-Tail (Viagen Biotech, Inc. CA, USA). The product was subjected to PCR for genotyping using primers 5'-AGC CGA CTT TGG TAA GGA TG-3' (s) and 5'-CCC ACT TTC CAT TTT CTC CA-3' (as) for wt and 5'-GGG CTG CAG GAA TTC CAT ATC AAG C-3' (s) and 5'-CCT TCC TGA AAT GAC TTC TGG TGC-3' (as) for AMPK α1-/-.

Tissues were put immediately into ice-cold buffer solution of the following composition (mmol/l): NaCl 145, KCl 3.8, D-glucose 5, MgCI2 1, HEPES 5, Ca^{2+} gluconate 1.3. After stripping the colonic mucosa and opening of the tracheas by a longitudinal cut, tissues were mounted into a micro Ussing chamber with a circular aperture of 0.95 mm^2. Mouse M1 kidney collecting duct cells (kindly provided by Prof. Dr. C. Korbmacher, Physiologisches Institut, Universität Erlangen, Germany) were grown to confluence on permeable supports and mounted into the Ussing chamber as described previously[63]. Luminal and basolateral sides of the epithelium were perfused continuously at a rate of 5 ml/min. The bath solution containing (mmol/l) NaCl 145,

KH_2PO_4 0.4, K_2HPO_4 1.6, D-glucose 5, $MgCl_2$ 1, HEPES 5, and Ca-gluconate 1.3, was heated to 37°C, and pH was adjusted to 7.4. Experiments were carried out under open-circuit conditions. Values for transepithelial voltages (V_{te}) were referred to the serosal side of the epithelium. Transepithelial resistance (R_{te}) was determined by applying short (1 s) current pulses (ΔI = 0.5 µA) and after subtracting the resistance of the empty chamber, using Ohm's law ($R_{te} = \Delta V_{te} / \Delta I$). Transepithelial resistances were 63 ± 3.8 Ωcm^2; n = 12 (trachea), 31 ± 2.1; n = 13 (colon) and 669 ± 45 Ωcm^2; n = 38 (M1). For real short circuit measurements basolateral membranes of M1 cells or mouse colonic epithelium were permeabilized with 75 µg/ml nystatin to eliminate the electrical resistance of basolateral membranes.

Rectal potential different (RPD): For RPD measurements, mice were anaesthetized by intraperitoneal injection of 20 µl (75 mg/ml) ketamine and 5 mg/ml xylazine. A catheter (polythene tube with OD 1 mm) perfused with standard ringer solution (3 ml/min) was attached to an AgCl electrode and inserted 2 cm into the rectum. The catheter was continuously perfused with Ringer solution and forskolin (2 µM), ATP (10 µM) and Ba^{2+} (5 mM) were applied via the catheter.

Immunocytochemistry: The Colon was fixed for 20 min in 0.1 M PBS with 4% paraformaldehyde. Sections were embedded in paraffin, cut in 5 µm serial sections (Leica microtome RM 2165, Wetzlar, Germany) and mounted on slides. Dehydrated sections were heat-induced for epitope retrieval for 20 min. After washing in PBS, sections were blocked and incubated in primary antibodies at 4 ºC overnight. Sheep-anti mouse AMPKα1 and AMPKα2 antibodies (Kinasource) were used in a 1:50 dilution. The secondary antibody (Alexa Fluoro 350 Donkey anti-Sheep IgG was used at a dilution of 1:200 (Molecular Probes).

Western blotting: Lysates of isolated proximal and distal colonic crypt cells were resolved by 7% SDS-PAGE, transferred to Hybond-P (Amersham, Freiburg, Germany) and incubated with the antibodies described above. Bands were visualized with goat anti-rabbit IgG conjugated to horseradish peroxidase (Acris, Hiddenhausen, Germany) and ECL using a Fluor-STM MultiImager (Bio-Rad, Hercules, USA).

Materials and statistical analysis: All compounds used were of highest available grade of purity and were obtained from Sigma or Calbiochem. Student's *t*-test and, where appropriate, ANOVA was used for statistical analysis. Data are shown as mean values ± SEM. A *p* value of <0.05 was regarded as significant.

RESULTS

Expression of AMPK in mouse colon: The mouse colonic epithelium is the predominant site for CFTR expression[104]. We therefore analyzed expression of $AMPK_{\alpha1}$ and $AMPK_{\alpha2}$ in proximal and distal colon of $AMPK_{\alpha1}$-/- and $AMPK_{\alpha1}$+/+ animals (Figure 1). Western blot analysis of lysates from isolated colonic crypts and immunohistochemistry demonstrated pronounced expression of $AMPK_{\alpha1}$ in the proximal colon of $AMPK_{\alpha1}$+/+ animals (Figure 1B,C). Expression of $AMPK_{\alpha1}$ was weaker in colonic crypts of the distal colon and no expression of $AMPK_{\alpha1}$ was detected in the co lon of $AMPK_{\alpha1}$-/- animals (Figure 1B). $AMPK_{\alpha2}$ was neither found in the proximal or distal colon of wt or $AMPK_{\alpha1}$ knockout mice (Figure 1B), but was detected in muscle tissue (not shown). Moreover, expression of the LacZ transgene under the $AMPK_{\alpha1}$ promoter was demonstrated in the distal colon of $AMPK_{\alpha1}$+/- animals (Fig1D).

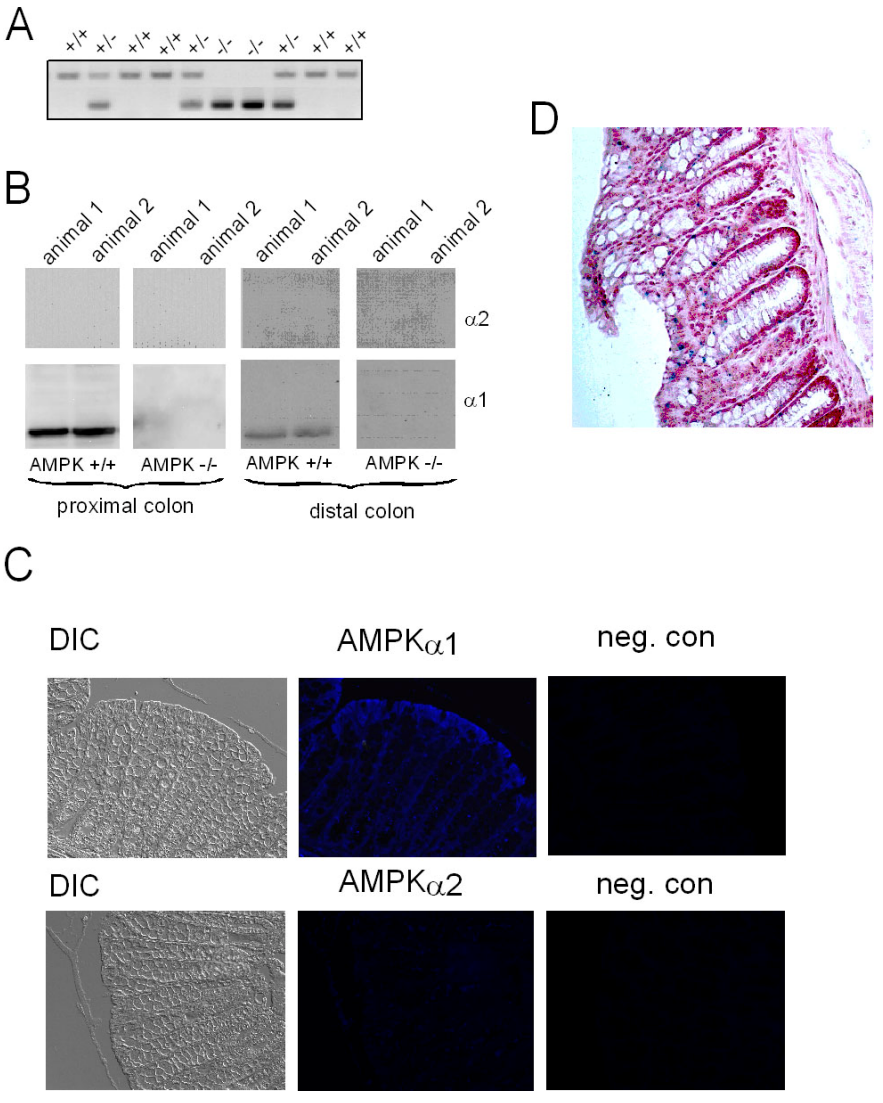

Figure 1: AMPKα1 but not AMPKα2 is expressed in mouse colonic epithelium: A) RT-PCR analysis of AMPKα1 +/+, AMPKα1 -/- and heterozygous animals. B) Western blot analysis of AMPKα1 and AMPKα2 in proximal and distal colon of AMPKα1 +/+, and AMPKα1 -/- animals. C) Immunohistochemistry of AMPKα1 and AMPKα2 in AMPKα1 +/+ mice. Negative control indicates staining without primary antibody. D) Expression of the LacZ transgene under the $AMPK_{\alpha1}$ promoter in distal colon of $AMPK_{\alpha1}$+/- animals.

Ion transport by CFTR is regulated by AMPK in mouse colon: We examined the effects of the AMPK-inhibitor compound C (80 µM, 30 min) and the AMPK-activator phenformin (1 mM, 1h) on ion transport in non-stimulated tissues, after mounting the tissues in a perfused Ussing chamber. Compound C slightly but significantly enhanced baseline transport, suggestive of basal AMPK activity in the non-stimulated tissue, particularly in the colon of $AMPK_{\alpha1}$+/+ mice (Figure 2A). This is further confirmed by the results obtained in non-stimulated epithelial tissues of $AMPK_{\alpha1}$-/- animals (Figure 2B). Here compound C had no effects on ion transport.

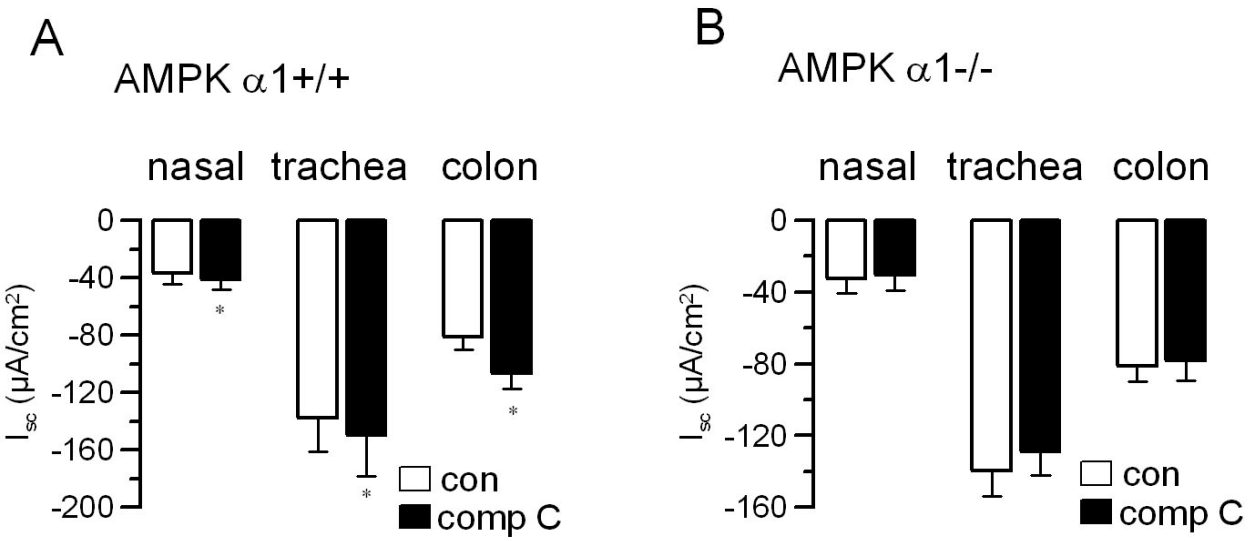

Figure 2: Enhanced basal ion transport by inhibition of AMPK in AMPKα1 +/+ but not in AMPKα1 -/- mice: Summary of equivalent short circuits currents (I_{sc}) measured in isolated non-stimulated nasal epithelium, trachea and colonic epithelium of AMPKα1 +/+ (A) and AMPKα1 -/- (B) mice. Inhibition of AMPK by compound C (80 µM, 1 h) slightly but significantly enhanced I_{sc} in epithelial tissues of AMPKα1 +/+, but not AMPKα1 -/- animals. (number of animals). *significant effects when compared to control (paired *t*-test).

Enhanced CFTR Cl^- secretion in $AMPK_{\alpha1}$-/- animals: We compared regulation of CFTR by AMPK in epithelial tissues from nose (Figure 3A,B), trachea (Figure 3C,D) and colon (Figure 3E,F) of $AMPK_{\alpha1}$+/+ animals, by stimulating Cl^- secretion with IBMX (100 µM) and forskolin (2 µM) (I/F) in the absence or presence of compound C. In all tissues, I/F induced a negative voltage deflection indicating activation of CFTR Cl^- conductance (Figure 3). Activation of CFTR in nasal, tracheal and colonic epithelium was enhanced after inhibition of AMPK by compound C (Figure 3). We compared CFTR dependent Cl^- transport activated by IBMX and forskolin in wt-animals and $AMPK_{\alpha1}$-/- mice. We found a slight but significant increase in I_{sc} by compound C in

epithelia from $AMPK_{\alpha1}$+/+ animals. This was not observed in $AMPK_{\alpha1}$-/- mice. Moreover, CFTR-dependent Cl^- secretion was enhanced in the colon of $AMPK_{\alpha1}$-/- mice (Figure 3F). The differences between $AMPK_{\alpha1}$-/- and wild type littermates were most pronounced in the colon, probably because of the important role of CFTR for Cl^- secretion in the mouse intestine, while CFTR was shown to be less essential in mouse airways[105,106].

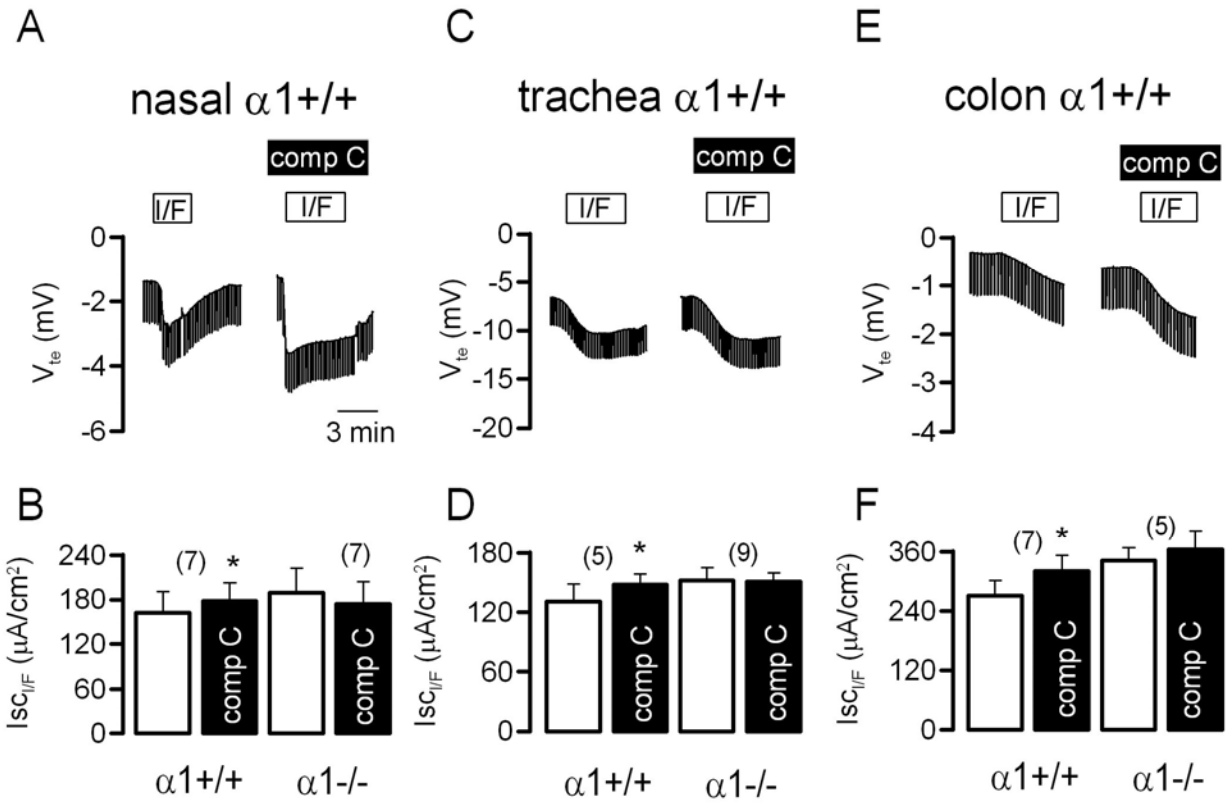

Figure 3: Enhanced CFTR-mediated ion transport by inhibition of AMPK in AMPKα1 +/+ but not in AMPKα1 -/- mice: Original recordings of the transepithelial voltage and comparison of the IBMX (100 µM)/forskolin (2 µM) induced equivalent short circuits currents (I_{sc}) measured in *ex vivo* tissues of nose (A,B), trachea (C,D) and colon (E,F) of AMPKα1 +/+ and AMPKα1 -/- mice. Black bars indicate stimulation of Cl^- secretion in the presence of compound C (80 µM, 1 h). (number of animals). *significant effects when compared to control (paired *t*-test).

We further examined whether AMPK affects Ca^{2+} dependent Cl^- secretion in mouse trachea and colon, when activated by ATP (100 µM) and carbachol (CCH, 100 µM), respectively. Ion transport activated by Ca^{2+} was indistinguishable between wt and $AMPK_{\alpha1}$ knockout animals (Figure 4). There was also no effect of compound C on Ca^{2+} activated Cl^- conductance in trachea or colon (Figure 4B,D). Thus no clear evidence exists for a regulation of Ca^{2+} dependent Cl^- secretion by AMPK. We further tried to make use of the activator of AMPK, phenformin (1

mM, 1h incubation). However, phenformin inhibited CFTR-dependent Cl^- secretion in all epithelial tissues examined from AMPK$_{\alpha1}$-/- and AMPK$_{\alpha1}$+/+ animals (Figure 5). Moreover, Ca^{2+} dependent Cl^- secretion activated by ATP or carbachol was inhibited by phenformin in wt and knockout animals (data not shown). We therefore suggest that phenformin has a rather non-specific effect on cellular electrolyte transport and may also inhibit other transport proteins such as the Na^+/K^+-ATPase[107]. Thus phenformin was not useful in our study to examine AMPK-dependent regulation of CFTR in AMPK$_{\alpha1}$-/- and AMPK$_{\alpha1}$+/+ animals.

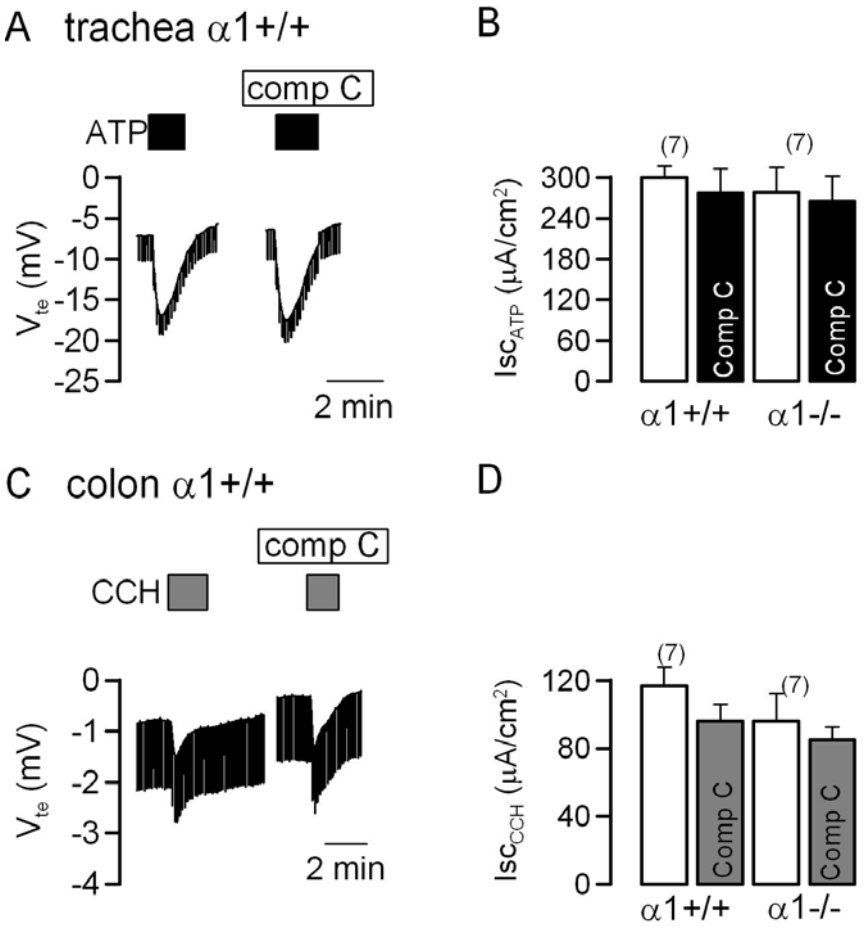

Figure 4: Lack of evidence for regulation of Ca^{2+} dependent Cl^- secretion by AMPK: Original recordings of the transepithelial voltage and summaries of the short circuit currents activated through Ca^{2+} dependent stimulation of trachea (A,B) and colonic (C,D) epithelium of AMPKα1 +/+ and AMPKα1 -/- mice. Negative transient voltage deflections were induced by luminal ATP (100 μM; trachea) and basolateral carbachol (100 μM; colon). The summaries of the Ca^{2+} activated equivalent I_{sc} indicate no effect of compound C and no difference between AMPKα1 +/+ and AMPKα1 -/- animals. (number of animals).

This finding was further substantiated by additional experiments performed in the mouse collecting duct cell line M1. Activation of ion transport by IBMX and forskolin was not affected by compound C but was inhibited by phenformin (data not shown).

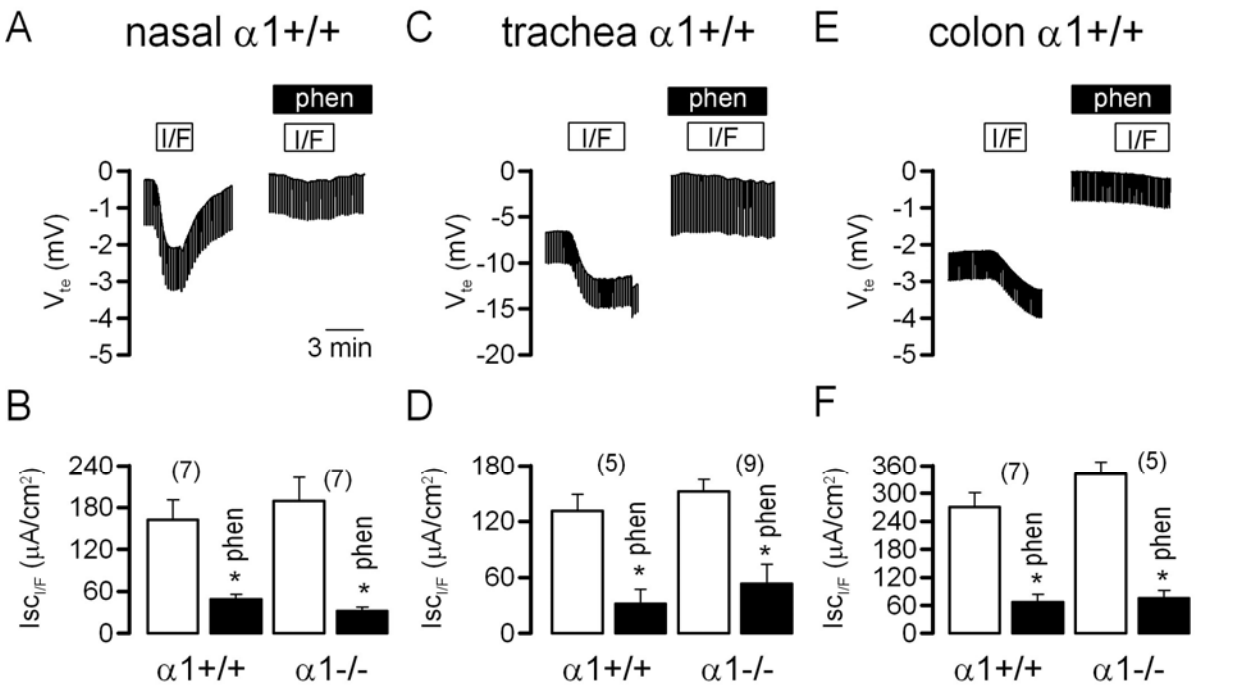

Figure 5: Phenformin inhibits CFTR-mediated ion transport independent of AMPKα1 expression: Original recordings of the transepithelial voltage and comparison of the IBMX (100 µM)/forskolin (2 µM) induced equivalent short circuits currents (I_{sc}) measured in *ex vivo* tissues of nose (A,B), trachea (C,D) and colon (E,F) of AMPKα1 +/+ and AMPKα1 -/- mice. Black bars indicate stimulation of Cl^- secretion in the presence of phenformin (1 mM, 1 h). (number of animals). *significant effects when compared to control (paired t-test).

To eliminate the contribution of basolaterally located proteins to electrolyte transport, the basolateral membranes of mouse colonic *ex vivo* tissues were permeabilized using nystatin (75 µg/ml). The epithelium was exposed to a transepithelial Cl^- gradient, in which 140 mmol/l Cl^- on the basolateral side have been replaced by equal amounts of gluconate. In these experiments, phenformin inhibited I_{sc} in colonic epithelium of $AMPK_{\alpha1}$+/+ but not $AMPK_{\alpha1}$-/- mice (Figure 7A). Moreover, I/F induced Cl^- conductance appeared to be larger in the $AMPK_{\alpha1}$-/- colon, although the difference did not reach significance (Figure 7B). Similar experiments were performed on mouse collecting duct cells grown on permeable supports. IBMX and forskolin activated a pronounced I_{sc} that was inhibited by 5 µM of the specific CFTR-inhibitor $CFTR_{inh}172$ or by phenformin (1-3 mM), indicating inhibition of CFTR in the luminal membrane of M1 cells by AMPK (Figure 6).

We further performed measurements of the rectal potential difference (RPD) *in vivo*, using a perfused rectal catheter and a reference electrode under the skin of anaesthetized mice. The RPD of $AMPK_{\alpha1}$-/- animals was significantly enhanced when compared to the $AMPK_{\alpha1}$+/+

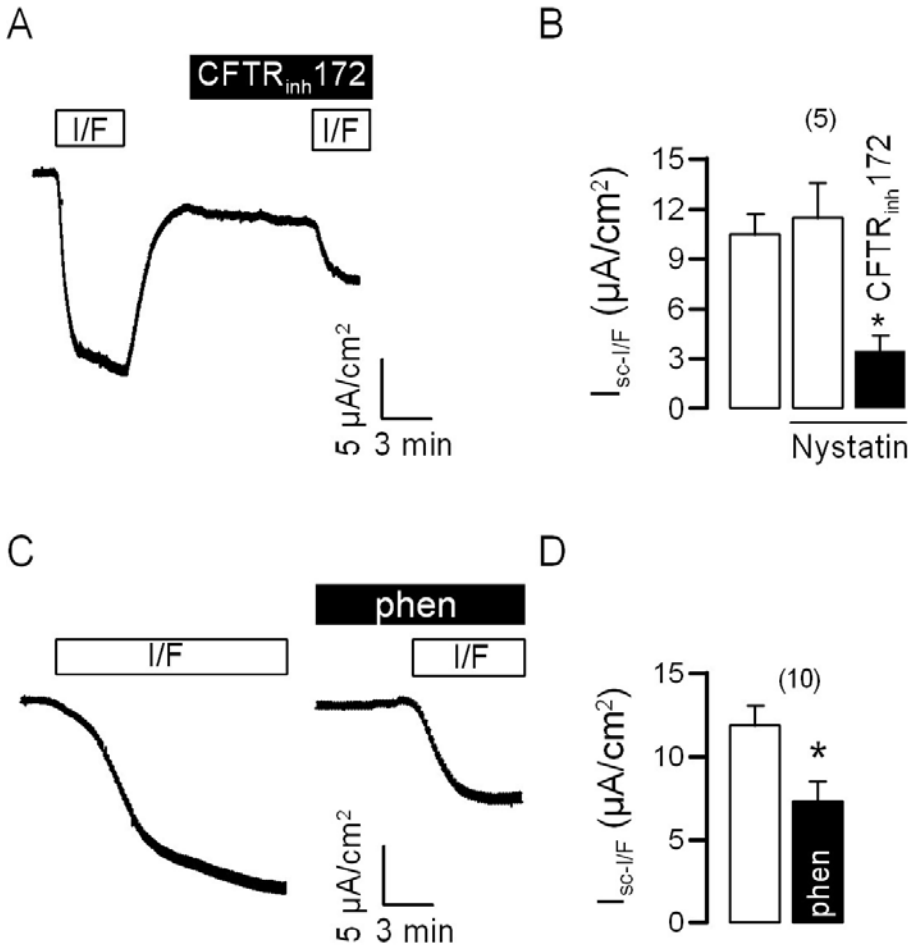

Figure 6: Effect of AMPK on ion transport in mouse collecting duct cells. Ion transport assessed in M1 cells after permeabilization of the basolateral membrane with 75 µM nystatin. A,B) Activation of apical CFTR-conductance is reduced in the presence of the specific inhibitor CFTRinh172 (5 µM). C,D) Activation of apical CFTR-conductance is reduced in the presence of the AMPK inhibitor phenformin (1 mM). (number of monolayers). *significant effects when compared to control (paired t-test).

rectum (Figure 7C,D). After perfusion of the rectum with 2 µM forskolin, the RPD increased significantly in both AMPK$_{\alpha1}$+/+ mice and AMPK$_{\alpha1}$-/- mice. The forskolin induced increase in RPD was significantly enhanced in the AMPK$_{\alpha1}$-/- rectum, suggesting enhanced CFTR-Cl^- conductance in the large intestine of mice lacking AMPK$_{\alpha1}$ expression (Figure 7E). Finally, activation of luminal K^+ secretion by perfusion with ATP (10 µM)[108], or inhibition of luminal K^+ channels with barium (5 mM) caused similar changes in both AMPK$_{\alpha1}$+/+ mice and AMPK$_{\alpha1}$-/- mice, indicating AMPK-inhibition of CFTR Cl^- channels but not of Ca^{2+} dependent K^+ transport in the large intestine of mouse (Figure 7F).

DISCUSSION

Evidence for AMPK-dependent regulation of CFTR in vivo: AMPKα1 is co-localized with apical CFTR in epithelia of airways and colon [34,35]. Inhibition of CFTR-dependent Cl^- secretion has been demonstrated in various *in vitro* models including *Xenopus* oocytes [38] and cultured

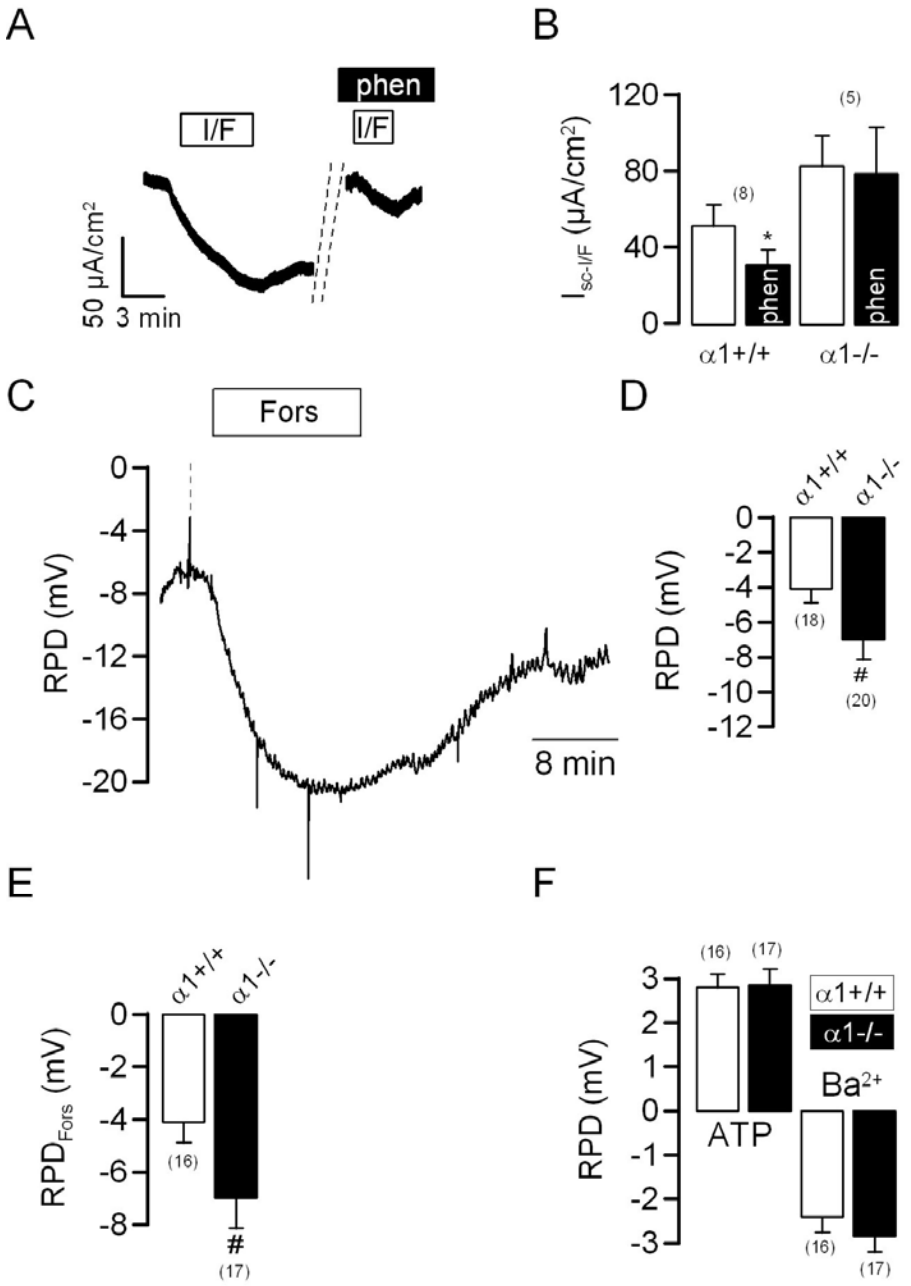

Figure 7: Enhanced CFTR-dependent ion transport in AMPKα1 -/- animal. A) Original recording of the real short circuit current assessed in basolaterally permeabilized (75 µM nystatin) colonic epithelium of an AMPKα1 +/+ animal. A,B) Activation of CFTR-dependent Cl^- secretion in the colon of AMPKα1 +/+ animals is inhibited in the presence of phenformin (1 mM, 1h), but not in the colon of AMPKα1 -/- mice. C) Original recording from a measurement of the rectal potential difference *in vivo* using a perfused catheter. Both baseline RPD (D) and forskolin (10 µM) induced RPD-increase (E) are enhanced in AMPKα1 -/- mice. F) Ca^{2+} (100 µM ATP) and Ba^{2+} (5 mM) induced RPD-changes are identical in AMPKα1 +/+ and AMPKα1 -/- animals. (number of animals). *significant effects when compared to control (paired t-test). #significant effect when compared to AMPKα1 +/+ mice (ANOVA).

epithelial cells from large intestine and airways[34,35,103]. The present experiments, for the first time, provide evidence that AMPK-dependent regulation also takes place *in vivo*. Most of the results presented here were obtained from *ex vivo* tissues. At the first glance the data suggest a rather limited impact of AMPK on CFTR-dependent transport. However several aspects have to be taken into account to fully appreciate the present results: i) We have no real control over the metabolic status in the tissues examined *ex vivo*. ii) The drugs used in the present study may only partially inhibit or activate AMPK activity, since we had to limit both incubation time and

concentrations. iii) Compensatory mechanisms may take place in the $AMPK_{\alpha1}$-/- animals, which could mask the regulatory impact of AMPK on CFTR. iv) Mouse colon shows strong CFTR expression while little CFTR is expressed in the airways[106].

Other factors should to be taken into account that might affect epithelial transport properties in $AMPK_{\alpha1}^{-/-}$ animals indirectly. These animals show additional pathological features which are currently not well understood. We found that they also have a reduced hematocrit and enlarged spleens, which points out to abnormal hematopoeisis. As reported recently, the major phenotypic changes observed in AMPK1-KO mice is a splenomegaly, possibly due to the massive amplification of erythroid nucleated cells. Moreover, immunohistology revealed abnormal development of the red pulp with a significant increase in the number of erythroid precursors[109].

Phenformin was inhibiting CFTR dependent Cl^- secretion also in the $AMPK_{\alpha1}$-/- animals, although there is no evidence for expression of the $AMPK_{\alpha2}$ isoform in epithelial cells from airways and the intestine. Woollhead and collaborators demonstrated that both phenformin and AICAR have additional pharmacological effects on the Na^+/K^+ -ATPase, which are probably independent of AMPK[107]. In their experiments phenformin and AICAR inhibited the Na^+/K^+ -ATPase, which reduced reduces Na^+ absorption by the epithelial Na^+ channel ENaC. There are currently no detailed reports on AMPK regulation of the Na^+/K^+ - ATPase; although reduced ATP supply due to cellular energy depletion clearly inhibits the function of the Na^+/K^+ - pump. The Na^+/K^+ - ATPase is the largest consumer of cytosolic ATP. Renal epithelial cells use up as much as 50% of their total mitochondrial oxidative metabolism during ion transport[110]. Since the Na^+/K^+ -ATPase is equally important for Cl^- secretion as for Na^+ absorption, blockage of the pump by phenformin probably explains inhibition of CFTR-mediated Cl^- secretion in the present study. Thus, the effects of phenformin in *ex vivo* tissues of $AMPK_{\alpha1}$-/- animals are unrelated to $AMPK_{\alpha1}$-function and therefore cannot be taken as a measure for AMPK activity. Nevertheless, the present experiments with the AMPK inhibitor compound C clearly supply evidence for inhibition of CFTR by AMPK in the native tissue, as CFTR dependent Cl^- secretion was inhibited by activation of AMPK in native airways and intestinal epithelia *ex vivo*. Moreover, inhibitory regulation of electrolyte secretion by AMPKα1 *in vivo* is clearly suggested by the rectal PD measurements.

Physiological role of AMPK-regulation of CFTR: Is CFTR Cl^- secretion turned off under cellular ATP depletion? This concept has been proposed recently. It would allow adjusting energetically

expensive electrogenic Cl^- secretion to the actual energy supply[38,96]. Assuming such a scenario, we would expect pronounced activity of AMPK particularly in heavily transporting epithelia, i.e. after maximal increase of intracellular cAMP. Under such conditions ATP consumption by the Na^+/K^+-ATPase should be rather high, the ATP: AMP ratio should drop and thus activate AMPK. However, Data from our laboratory suggests the opposite, namely reduced inhibitory effects of AMPK on CFTR Cl^- conductance during maximal activation of CFTR[111]. At any rate, CFTR Cl^- secretion was clearly enhanced in the colonic epithelium of AMPKα1 knockout animals and the differences to wt animals was particularly evident in measurements of the rectal potential difference (Figure 7). It is therefore concluded that AMPK is a physiologically relevant regulator of CFTR-dependent Cl^- transport.

ACKNOWLEDGEMENTS

Supported by DFG SFB699 A6 and DFG KU756/8-1. We acknowledge the expert technical assistance by Ms. E. Tartler and Ms. A. Paech. We thank Prof. Dr. F. Schweda, Prof. Dr. R. Warth, Dr. M. Reichold and S. Bandulik for critical discussions and their help with assessment of *in vivo* parameters. We also thank Dr. A, Mehta for fruitful discussions.

CHAPTER 3.

Mechanistic Insight into Control of CFTR by AMPK

ABSTRACT

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP and protein kinase A (PKA) regulated Cl^- channel in the apical membrane of epithelial cells. The metabolically regulated and adenosine monophosphate stimulated kinase (AMPK) is colocalized with CFTR and attenuates its function. However, the sites for CFTR-phosphorylation and the precise mechanism of inhibition of CFTR by AMPK remain obscure. We demonstrate that CFTR normally remains closed at baseline, but nevertheless opens after inhibition of AMPK. AMPK phosphorylates CFTR *in vitro* at two essential serines (S737 and S768) in the R-domain, formerly identified as 'inhibitory' PKA sites. Replacement of both serines by alanines i) reduced phosphorylation of the R-domain, with S768 having dramatically greater impact, ii) produced CFTR channels that were partially open in the absence of any stimulation, iii) significantly augmented their activation by IBMX/forskolin and iv) eliminated CFTR inhibition post AMPK activation. Attenuation of CFTR by AMPK activation was detectable in the absence of cAMP-dependent stimulation but disappeared in maximally stimulated oocytes. Our data also suggest that AMP is produced by local phosphodiesterases in close proximity to CFTR. Thus we propose that CFTR channels are kept closed in non-stimulated epithelia with high baseline AMPK activity but CFTR may be basally active in tissues with lowered endogenous AMPK activity.

Key words: Cystic fibrosis transmembrane conductance regulator, phosphorylation, AMP, PKA, AMP-activated protein kinase, ion transport.

Published in: **Kongsuphol P**, Cassidy D, Hieke B, Treharne KJ, Schreiber R, Mehta A, Kunzelmann K. Mechanistic insight into control of CFTR by AMPK. *The Journal of Biological Chemistry* 284, 5645-53(2009).

Own experimental contribution: All double electrode voltage clamp (DEVC) experiments and related work

Own writing contribution: Methods, Results and parts of Introduction and Discussion.

INTRODUCTION

The cystic fibrosis transmembrane regulator (CFTR) gene is mutated in patients with cystic fibrosis. CFTR has an adapted ABC transporter structural motif thereby creating an anion channel at the apical surface of secretory epithelia[90]. The consequent CFTR-mediated ion transport is tightly controlled by ATP binding and phosphorylation by protein kinase A (PKA). However a number of other protein kinases including PKC, Ca^{2+}/calmodulin-dependent kinase, and cGMP-dependent kinase also control CFTR's activity[3,17,46]. These kinases converge on the regulatory domain of CFTR that is unique not only within the large ABC transporter family but among all known sequences, and may be considered as a "phosphorylation control module". Regulation of CFTR by an inhibitory kinase, the adenosine monophosphate dependent kinase (AMPK), has been described recently but the regulatory sites within CFTR, the mechanism of regulation and the physiological relevance have all remained obscure[34,35,38,103]. Additionally, CFTR mutation is linked to inflammation and a lack of functional CFTR expression has itself been suggested to up-regulate AMPK activity in epithelial cells carrying the cystic fibrosis (CF) defect. Pharmacologic AMPK activation was shown to inhibit secretion of inflammatory mediators[36]. Thus AMPK may play multiple roles in CF pathophysiology making the mechanism of interaction an important problem in biology.

AMPK is a ubiquitous serine/threonine kinase that exists as a heterotrimer with a catalytic α subunit and regulatory β and γ subunits, each with multiple isoforms. In response to metabolic depletion and a consequent increase in the cellular AMP to ATP ratio, AMPK phosphorylates numerous proteins and activates catabolic pathways that generate ATP, while inhibiting cell growth, protein biosynthesis and a number of other ATP-consuming processes, thereby operating as a cellular 'low-fuel' sensor[39,94]. AMPK also controls signaling pathways involved in apoptosis, cell cycle, and tissue inflammation[95]. Because AMPK is a cellular metabolic sensor that inhibits CFTR and limits cAMP activated Cl^- secretion, a coupling of membrane transport by CFTR to cellular metabolism has been proposed[96]. However, AMPK activity can also increase without detectable changes in the cytosolic AMP to ATP ratio, suggesting a contribution of additional AMP-independent signals for regulation of CFTR by AMPK[40]. Drugs used to combat type 2 diabetes, such as phenformin and metformin, act in this manner to activate AMPK, AMP-independently. It is also likely that cytosolic AMP is compartmentalized depending on the distribution of AMP generating enzymes such as phosphodiesterases that convert cAMP to AMP. The concept of spatiotemporal control of cAMP signaling by anchored protein complexes is well established[61]. CFTR is known to form such macromolecular complexes with a number of

interacting partners[59,60,112]. For example, competitive interaction of NHERF1-PKA and Shank2-PDE4D with CFTR has been demonstrated recently[62]. In addition, Barnes and coworkers demonstrated that phosphodiesterase 4D generates a cAMP diffusion barrier local to the apical membrane of the airway epithelium [113]. It is therefore likely that activatory pathways through cAMP and inhibitory AMP/AMPK-signaling occur in a local CFTR-organized compartment. Here we explore the functional links between CFTR, inhibition of phosphodiesterases and AMPK focusing on the effects of mutating putative AMPK targets within the R domain on CFTR function.

MATERIALS AND METHODS

cRNAs for CFTR and double electrode voltage clamp: Oocytes were injected with cRNA (10 ng, 47 nl double-distilled water) encoding wtCFTR, L1430A/L1431A, F508del-CFTR, G551D-CFTR, S768A, S737A, S768D, S737D, E1474X, and AMPKα1. All mutants were generated by PCR and correct sequences were confirmed by restriction digest and by sequencing. Water injected oocytes served as controls. 2 - 4 days after injection, oocytes were impaled with two electrodes (Clark Instruments Ltd, Salisbury, UK), which had a resistance of < 1 MΩ when filled with 2.7 mol/l KCl. Using two bath electrodes and a virtual-ground head stage, the voltage drop across the serial resistance was effectively zero. Membrane currents were measured by voltage clamping (oocyte clamp amplifier, Warner Instruments LLC, Hamden CT) in intervals from -60 to +40 mV, in steps of 10 mV, each 1 s. The bath was continuously perfused at a rate of 5 ml/min. All experiments were conducted at room temperature (22 °C).

Oocyte staining: Oocytes were incubated for 60 min in ND96 solution (in mM: 96 NaCl, 2.0 KCl, 1.8 CaCl2, 1.0 MgCl2, 5.0 HEPES, pH 7.4), fixed for 60 min with 3% paraformaldehyde (in TBS, pH 8.0) and washed in TBS. After embedding in optimum cutting temperature compound (Sakura Finetek Europe, Zoeterwoude, NL), oocytes were cut to 20 µm slices with a cryostat (Leica CM3050 S, Wetzlar, Germany). Sections were put in either TBS or phosphate buffered saline (PBS; (mM) 137 NaCl, 1.8 KH2PO4, 10.3 Na2HPO4, pH 7.4), incubated for 5 min in 0.1% (w/v) SDS in PBS and washed 2 times with either TBS or PBS. Sections were incubated for 60 min in TBS or PBS (5% BSA) and for 60 min at 37°C with the anti-Flag M2 antibody diluted 1:50 in 2% BSA/TBS or a goat polyclonal casein kinase IIα antibody (Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:25 in 2% BSA/PBS. Afterwards sections were washed twice in PBS and incubated for 1 h with secondary antibodies (donkey anti-mouse IgG- Alexa Fluor 488

conjugated and donkey anti-goat IgG-Alexa Fluor 546 conjugated; Molecular Probes, Eugene, OR) at a dilution of 1:1000 in 2% BSA/PBS. Sections were washed 2 times with PBS for 5 min and covered with DakoCytomation fluorescent mounting medium (DakoCytomation, Inc., Carpinteria, CA). Images were obtained using a Zeiss Axiovert 200M microscope with a × 63 objective (Carl Zeiss, Inc., Jena, Germany).

Immunofluorescence: Nasal ciliated epithelial cells harvested from the inferior turbinate of patients undergoing unrelated surgery (approved by local ethical committee) were maintained in cell culture medium M199 prior to fixation in 4% paraformaldehyde. Cells were permeabilized using 1% Triton X-100, washed 3 times in PBS, then blocked in 1 mM glycine for 15 min, followed by 5% donkey serum for 15 min. Pelleted cells were resuspended in PBS containing primary antibodies (goat anti-CK2α (Santa Cruz) and mouse anti-CFTR NBD1 (Neomarkers) at a 1:100 dilution) and incubated at room temperature, with shaking, overnight. After 3 washes in PBS, pelleted cells were resuspended in PBS containing FITC-labeled anti-goat and rhodamine-labeled anti-mouse IgG secondary antibodies (Jackson, 1:100). After 2 h incubation with shaking the cells were washed five times in PBS and resuspended in 15 µl anti-fade mountant (6% n-propyl gallate in 70% glycerol, 100 mM Tris/HCl, pH 7.4) for mounting on glass slides. Coverslips were sealed with nail varnish for image capture using a Zeiss 510 laser scanning confocal microscope.

In vitro phosphorylation: Recombinant R domain was phosphorylated with recombinant AMPK (Calbiochem 171536) or PKA (NEB P6000S) and peptides were separated subsequently by two-dimensional electrophoresis as described earlier[114]. In brief, for AMPK phosphorylation, onto approx 20 µl packed volume of substrate beads (approx 2 µg protein), 20 µl of kinase reaction buffer was added. 32 mM Hepes pH 7.4, 0.65 mM DTT, 0.012 % Brij-35, 200 µM AMP (if included in that particular assay), 100 µM ATP, 10 mM MgAc and approx 500-800 cpm/pmol [gamma32P]-ATP. 0.1 units of enzyme activity was added per reaction (as defined by the manufacturer's SAMS assay) and incubated at 30 °C for 15 minutes before being washed 3 times with 1 ml ice-cold 50 mM Tris pH 7.5. The beads were then resuspended in SDS sample buffer and separated on 10 % SDS PAGE, coomassie stained to visualize protein, dried and analyzed via autoradiography. For PKA reactions, manufacturer's 10x buffer was diluted adding 0.1 U of PKA with 100 µM ATP (approximately 500-800 c.p.m.). For mapping purposes the gamma ATP concentration was increased 10 fold and the ATP concentration reduced to 50 µM to increase the incorporation of radioactive signal in the tryptic peptides. The S768A mutant of CFTR abrogated almost entirely phosphorylation by AMPK.

Materials and statistical analysis: All compounds used were of highest available grade of purity (SIGMA or Calbiochem). R-domain construct was kindly supplied by Dr. J. Hanrahan (Department of Physiology, McGill University, Montréal, Canada). The construct encodes for a His tagged R-domain of human CFTR containing amino acids 635 to 837. Compounds were applied after fully activating CFTR unless otherwise stated. Alternatively oocytes were stimulated with I/F in the absence or presence of compounds. Student's *t*-test was used for statistical analysis. A p value of <0.05 was regarded as significant.

RESULTS

CFTR is inhibited by baseline activity of AMPK: Figure 1 shows that cyclic AMP/PKA activated whole cell Cl^- currents in *Xenopus* oocytes expressing CFTR. The CFTR current was slightly but significantly inhibited by activators of AMPK such as membrane permeable 5-aminoimidazole-4-

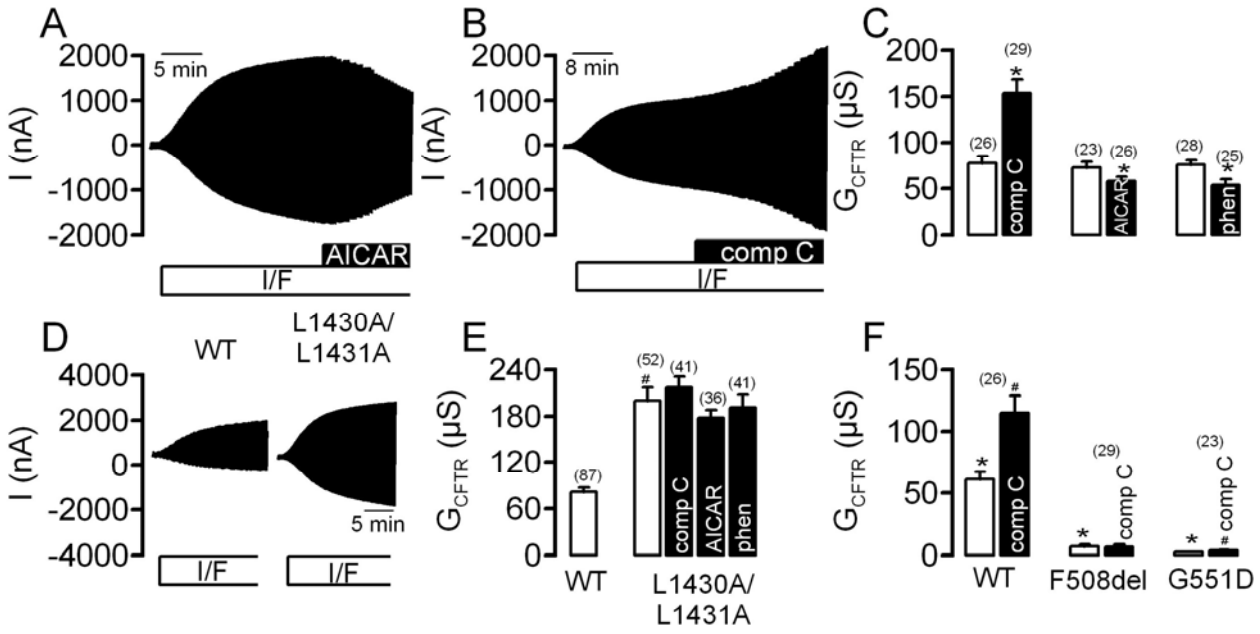

Figure 1: Effect of AMPK on CFTR. A,B) Whole cell currents activated by IBMX (1 mM) and forskolin (2 μM) in CFTR expressing oocytes showing the small effects of the AMPK activator AICAR (1 mM) (left) and compound C (right). C) Summary of the effects of compound C (80 mM), AICAR (1 mM) and phenformin (1 mM) on CFTR whole cell conductances activated by IBMX and forskolin. D) Whole cell currents activated by IBMX and forskolin in wtCFTR and L1430A/L1431A-CFTR expressing oocytes. E) Summary of whole cell conductance generated by wtCFTR and L1430A/L1431A-CFTR and effects of activators and inhibitors of AMPK. F) Comparison of the effects of compound C (80 μM) on whole conductances generated by wtCFTR, F508del-CFTR, and G551D-CFTR. Membrane currents were measured by voltage clamping in intervals from -60 to +40 mV, in steps of 10 mV. ∗ significant difference when compared to control. # significant difference when compared to wtCFTR. Data are shown as mean ± SEM, (number of experiments).

carboxamide 1-beta-D-ribofuranoside (AICAR, 80 μM for 1 hour)[115] or phenformin (1 mM, 1 h)

(Figure 1A,C). In contrast to these small inhibitory effects of AICAR and phenformin, CFTR whole cell conductance was doubled by the AMPK-inhibitor compound C, suggesting the presence of a latent constitutive inhibition of CFTR by a tonic baseline AMPK activity (Figure 1B,C). This is consistent with the recognized and substantial activity of AMPK in the absence of AMP (note the designation of the kinase is AMP-activated and not AMP-dependent). Interestingly, the application of compound C alone also activated CFTR without the need for PKA stimulation. To exclude a non-specific effect of compound C, we expressed a CFTR mutant (L1430A/L1431A), which has been proposed to eliminate binding of AMPKα1 to a C-terminal region of CFTR[38]. This mutant showed a much higher conductance (Figure 1D,E), which was not further augmented by compound C or inhibited by the two recognized AMPK activators, AICAR or phenformin (Figure 1E). Also two common CFTR-mutants, F508del-CFTR and G551D-CFTR could not be further activated by inhibition of AMPK with compound C (Figure 1F).

This enhanced activity of L1430A/L1431A–CFTR was similar to that seen with wild type CFTR first exposed to compound C and then activated by PKA. Thus local AMPK bound to CFTR is likely to be essential for inhibition of the maximum CFTR current post PKA stimulation. The proximity of the two proteins was further confirmed as shown in Figure 2A. CFTR (red) and AMPKα1 (green) are colocalized in an apical compartment of human nasal epithelial cells, thus confirming previous results[38]. Activation of AMPK in *Xenopus* oocytes by phenformin did not change the expression of CFTR in membranes of *Xenopus* oocytes (Figure 3), confirming previous results indicating inhibition of CFTR's open probability by AMPK [35].

Inhibitory R-domain sites are targeted by AMPK: It is not known where AMPK phosphorylates the CFTR protein[38] but the R domain of CFTR contains multiple phosphorylation sites for PKA. Interestingly two of these sites, namely S737 and S768, have been identified as 'inhibitory' R-domain sites, i.e. when mutated to alanines they augment CFTR's open probability relative to wild type[53,55]. We hypothesized that these sites might be phosphorylated by AMPK (rather than 'inhibited' by PKA) given some sequence homology with the expected consensus sequence for AMPK in local amino acids and therefore examined *in vitro* phosphorylation of isolated singly or doubly mutant or wild type R-domain protein. Figure 2B demonstrates that AMPK indeed phosphorylates the R-domain *in vitro* and this AMPK phosphorylation is largely reduced in the R-domain mutants S737A and S768A (compare lanes 2 and 3 in Figure 2B lower panel). The data also suggest that serine 768 has a much greater reductive impact because S768A almost

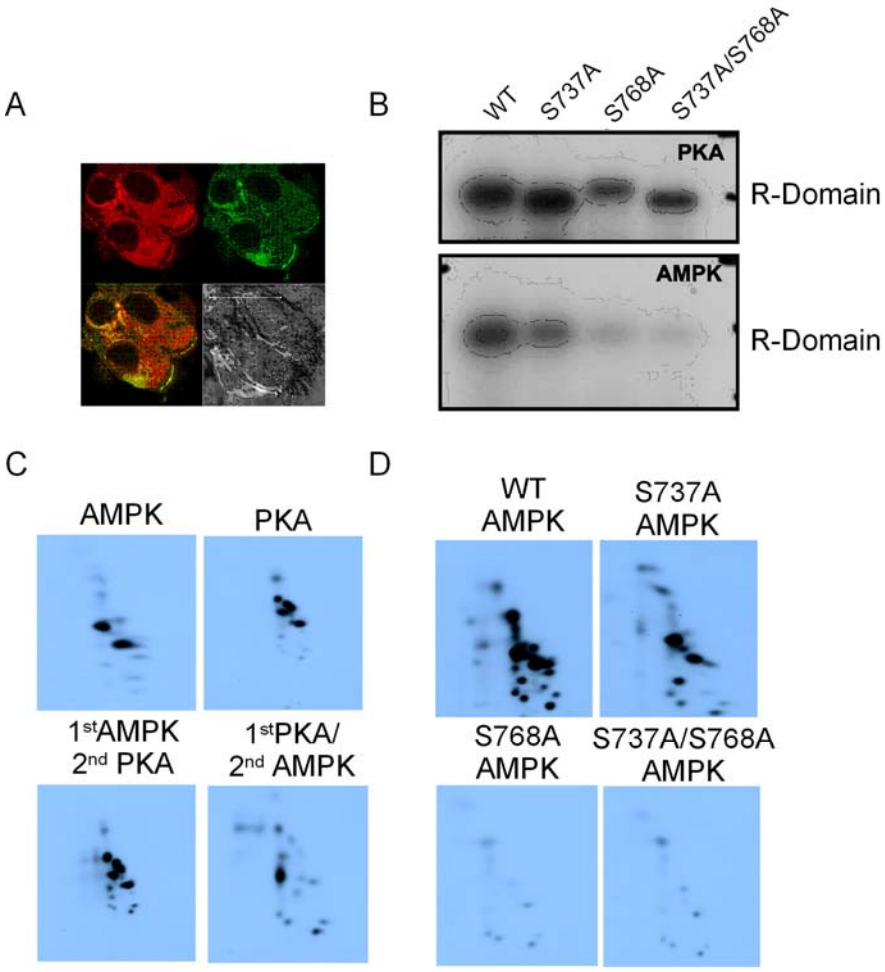

Figure 2: Expression and phosphorylation of CFTR. A) Immunostaining of CFTR (red) and AMPKα1 (green) in human nasal epithelial cells. B) Phosphorylation of the R domain with PKA and AMPK. AMPK phosphorylation was largely reduced or abolished in S737A and S768A, respectively (lower panel). When PKA alone was used to phosphorylate the R domain, a complex gel shift with some diminution of phosphorylation was found after mutation of the two serines. This shift was not observed when AMPK alone was the phosphorylating kinase but serine mutation abrogated the phosphorylation (lower panel). C) Order dependence of AMPK and PKA in R domain phosphorylation. PKA and AMPK dependent phosphorylation of R domain creates distinct peptides patterns. Recombinant R domain phosphorylated with recombinant AMPK in the presence of unlabelled ATP (lower left) followed by PKA and labelled ATP gives rise to some measurable incorporation. However when the PKA was added first, AMPK could only phosphorylate to a level approximately 4 fold less than that with PKA or AMPK alone. D) Effect of mutating serines S737 and S768 at AMPK phosphorylation sites. The S768A mutant abrogates almost all the phosphorylation. Electronic autoradiography was done at identical gain settings.

abolished all the phosphorylation whereas some was preserved with S737A. Crucially, the double mutant labeling was similar to S768A alone. We quantified data from 3 independent experiments and found that the S737A mutation leads to a small 23 ± 8 % reduction in counts, while S768A leads to a major 81 ± 5 % (mean ± range) reduction similar to the double mutant (87% ± 4 %) when compared to the wt (100%). Thus the data confirm that AMPK targets S768 preferentially and also phosphorylates S737. The specificity of this finding was confirmed towards AMPK because no such dramatic inhibition of labeling of the R domain was found when PKA was used to phosphorylate the R domain *in vitro* (compare upper and lower panels in Figure 2B). The gel shifts observed with PKA (but not AMPK) in the presence of these mutants are unexplained but are

consistent with a selective change in R domain structure after PKA phosphorylation[55,116]. Correspondingly, Figure 2C reveals that PKA and AMPK dependent phosphorylation of the R domain created distinct peptide patterns, as described below in detail.

The combined biochemical and physiological data shown below suggest a complex relationship between the PKA and AMPK driven phosphorylation of the R domain. To test this we undertook phospho-peptide mapping of the AMPK and PKA dependent phosphorylation of the R domain.

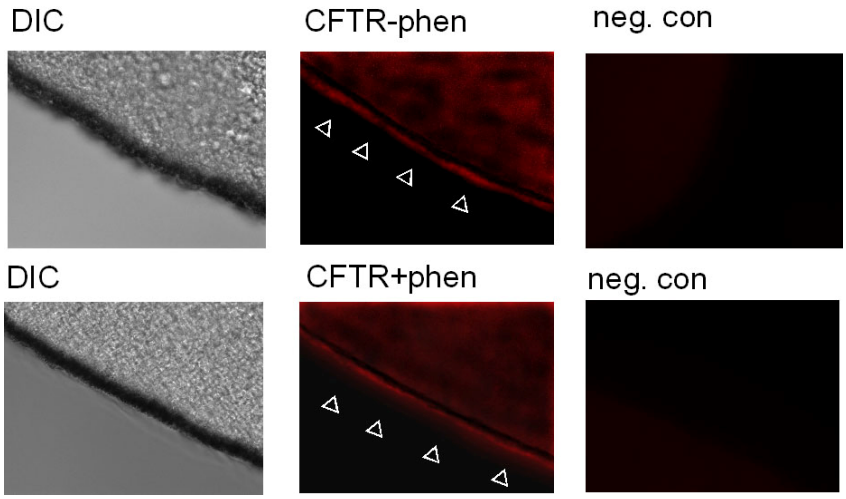

Figure 3: Expression of CFTR in the membrane of *Xenopus* oocytes. Immunohisto-chemistry of CFTR expression (arrows) in the absence or presence (2 hours treatment) of phenformin (2 mM). Negative control indicates staining without primary antibody. Phenformin does not detectably alter membrane expression of CFTR.

In such experiments, it must be remembered that the interpretation rests on dramatic changes in phosphorylation because it is not possible to match exactly the conditions that might pertain *in vivo*. First, we tested the effects of each kinase separately and found only two major phosphopeptide spots with AMPK but 4 phosphopeptide spots with PKA (Figure 2C, left uppermost panel pair). Next, we added the kinases sequentially in an attempt to mimic the physiological protocols used in the oocytes to stimulate or inhibit CFTR ion transport. These experiments were undertaken using unlabelled ATP in each first kinase run. To ease interpretation, we washed out residual kinase between runs to prevent any spill-over effects or kinase-kinase interactions (Figure 2C, lower panels). First, note the remarkable similarity between AMPK first, PKA second when compared to PKA alone (compare upper right with lower left in panel 2C). We conclude that AMPK (as first cold-label) cannot abrogate the subsequent 4-spot 'hot' PKA-mediated pattern. AMPK when added second, cannot generate the expected two spot pattern observed with AMPK alone (Fig 2C lower right compared to upper left) suggesting either inaccessibility of the necessary site(s) based on the gel shift data (Figure 2B upper panel) that was either suggestive of a structural alteration or the unknown effects of some other phosphorylated site. Thus *in vitro* rank order matters for these kinases where specific site(s) on

the R domain labeling is concerned. We interpret these data to imply that a selective, major (>80%) reduction in R domain phosphorylation occurs when the S768 OH group is no longer available for phosphorylation which could either mean that this is a key AMPK site by itself or that S768 is 'permissive' i.e. is involved in docking/orienting AMPK or, alternatively is required for labeling some other site on CFTR.

Next we tried to explore the different roles of the two serines with respect to AMPK but found that attempts to recreate the wild type pattern of spots in the presence of serine mutated R domain always showed a profound loss of label incorporation at short incubation times despite identical R domain protein loading suggesting that these two serines were the major R domain targets for AMPK. We therefore used prolonged incubation times to maximize the counts and these data are shown in Figure 2D. It is clear that S768A (lower left panel) has a more profound inhibitory effect on R domain phosphorylation compared to S737A (upper right) given that all these experiments were run simultaneously with similar concentrations of R domain protein and kinase, and each imaged for identical lengths of time. Broadly, prolonged incubation that previously created two major spots found with AMPK alone was now supplemented by multiple small spots that almost disappeared after S768A mutation, but much less so after S737A mutation (Figure 2D, compare lower two panels with upper). Once again the critical role of S768 is reiterated relative to S737.

When expressed in oocytes, CFTR bearing the R domain mutants S737A and S768A as well as the double mutant S737A/S768A (Figure 4A) produced dramatically enhanced conductances (S768A> S737A; Fig 4B) upon stimulation with forskolin (2 μM) and IBMX (1 mM). In contrast, the S768D mutation, mimicking phosphorylation at S768, produced a whole cell conductance that was significantly smaller than even wtCFTR (note that conductance not lowered with S737D; see also Figure 4D for phenformin sensitivity of these mutants). The large Cl^- conductances generated by S737A/S768A were inhibited by 5 μM of the specific chloride channel blocker CFTRinh-172 (Figure 4C), or alternatively, the PKA inhibitor KT5720 and Cl^- replacement by gluconate (not shown). Once again the differential roles of these two serines were observed because the conductance observed with S737A was almost 100 μS lower than that found with S768A. Furthermore, there remained a non-significant but interesting trend towards the retention of either inhibition or stimulation (with phenformin and compound C respectively) with the S737A but not with the S768A (compare second and third panels of Figure 4D with the corresponding wild type result in the first panel of the figure). Overall the data suggest that S737A, S768A and the double mutant S737A/S768A were no longer sensitive to

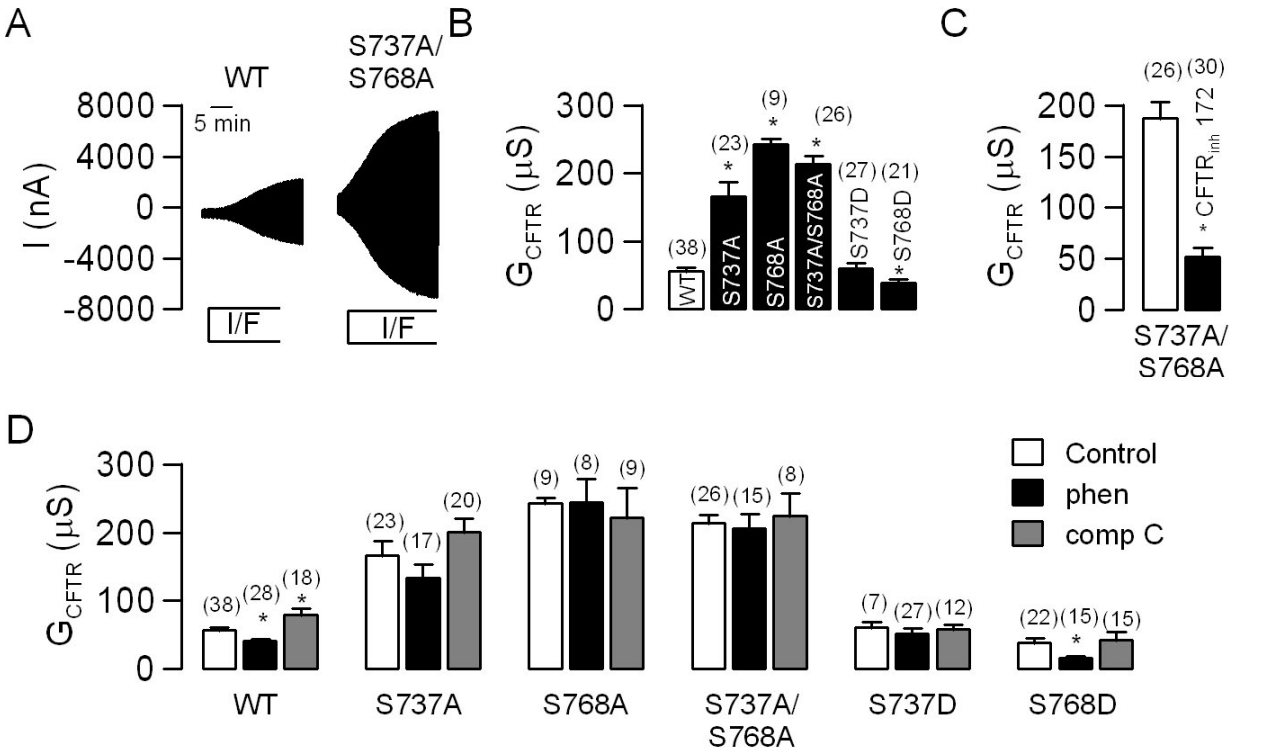

Figure 4: Effects of AMPK on wtCFTR and R domain mutants. A) Whole cell currents activated by IBMX (1 mM) and forskolin (2 µM) in wtCFTR and S737A/S768A-CFTR expressing oocytes. B) Summary of whole cell conductances generated by wtCFTR and different CFTR-mutants. C) Summary of the whole cell conductance activated by S737A/S768A-CFTR and inhibition by the CFTR blocker $CFTR_{inh}172$. D) Summary of CFTR whole cell conductances generated by wtCFTR and different CFTR-mutants, and effects of phenformin and compound C. Membrane currents were measured by voltage clamping in intervals from -60 to +40 mV, in steps of 10 mV. ∗ significant difference when compared to control. # significant difference when compared to wtCFTR. Data are shown as mean ± SEM, (number of experiments).

stimulation or inhibition of AMPK indicating that phosphorylation at both serines is required (Figure 4D, middle panels). In contrast the residual CFTR conductances generated by S768D (but not S737D) were not only further inhibited by phenformin, but neither phospho-mimic mutant could be augmented by the AMPK inhibitor compound C. Thus the presence of a fixed negative charge at S768 maintains sensitivity to AMPK-mediated inhibition whereas this is lost when S737 has a similar negative charge. Yet, unlike wild type CFTR, neither D mutant is able to enhance their conductance when AMPK is inhibited (Figure 4D). Thus negative charge at serine 768 is discriminant between inhibition and activation by AMPK.

In contrast to wtCFTR the CFTR mutants S737A (not shown), S768A and S737A/S768A produced a high Cl^- conductance under basal conditions, i.e. in the absence of IBMX and forskolin (Figure 5A,B). Enhanced basal conductance of the mutants (but not wild type) was inhibited by replacement of extracellular Cl^- with gluconate (Figure 5B). Yet both the basal conductance of the mutants and the basal conductance of wtCFTR after compound C were blocked by CFTRinh-172 (Figure 5C, rightmost columns) suggesting both were indeed CFTR mediated. As described above for activated whole cell Cl^- conductance, the enhanced baseline conductance exhibited by these mutants was also insensitive to phenformin and compound C, consistent with a loss of AMPK sensitivity (Figure 5C). Moreover, the PKA inhibitor KT5720 (50 µM) inhibited this enhanced baseline CFTR conductance generated by S737A/S768A

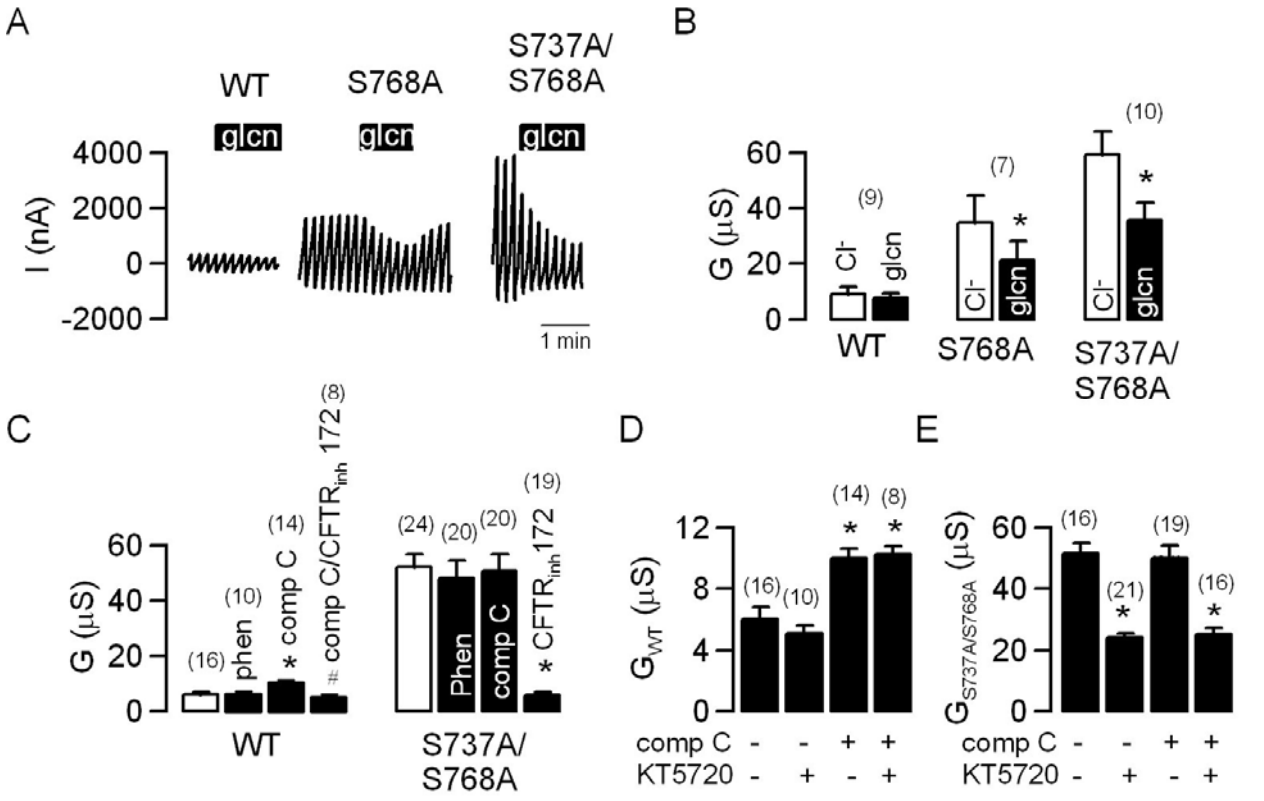

Figure 5: S737A/S768A-CFTR generates a baseline conductance. A) Effect of extracellular Cl^- replacement by gluconate (glcn) on whole cell currents generated by wtCFTR, S768A-CFTR and S737A/S768A-CFTR in the absence of IBMX and forskolin. B) Summary of the corresponding whole cell conductances. C) Summary of the whole cell conductances generated by wtCFTR and S737A/S768A-CFTR in the absence of stimulation with IBMX and forskolin and effects of phenformin, compound C and $CFTR_{inh}172$. D,E) Baseline whole cell conductances generated by wild type CFTR (D) and S737A/S768A-CFTR (E) and effects of compound C and the PKA inhibitor KT520. Membrane currents were measured by voltage clamping in intervals from -60 to +40 mV, in steps of 10 mV. ∗ significant difference when compared to control. Data are shown as mean ± SEM, (number of experiments).

irrespective of the presence of compound C (Figure 5E). In contrast, KT5720 did not affect the very low conductance produced by unstimulated wtCFTR (Figure 5D), suggesting this was PKA-independent (note also the corresponding insensitivity to gluconate replacement, Fig 5A,B left panels). We interpret this finding to suggest that the sensitivity of S737A/S768A-CFTR towards PKA inhibition is retained which implies that PKA is now active after the loss of AMPK sensitivity. The combined data posit a feedback loop between PKA and AMPK.

Physiological role of AMPK-regulation: We further examined the conditions under which regulation of CFTR by AMPK occurs. To that end we gradually increased CFTR activity by stimulating wtCFTR expressing oocytes either leaving phosphodiesterases intact or by inhibiting them differentially (left to right Fig 6A) as follows i) 2 µM forskolin (21 ± 3.7 µS), ii) 2 µM forskolin and 1 mM IBMX (61 ± 6.8 µS), or iii) 2 µM forskolin, 5 mM IBMX and 3 mM $8Br^-$-cAMP (120 ± 9.8 µS), in the presence or absence of compound C to detect inhibitory effects of AMPK (Figure 6A). Interestingly, at increased PKA-stimulation, the compound C effect was reduced and was undetectable at maximal stimulation, confirming interference between phosphorylation by AMPK and by PKA (upper panel). Thus the data suggest that one physiological role of the tonic basal AMPK activity may be to keep the CFTR channel shut, as evidenced by the rise in basal conductance with compound C alone in the absence of secretagogue stimulation (Figure 5), rather than limiting excessive activation as suggested previously. The initial recovery from PKA-stimulation was equal in S737A/S768A (1.1 ± 0.3 µS/min) and wtCFTR (1.1 ± 0.1 µS/min) (Figure 6C), but was enhanced in ooctyes coexpressing kinase-dead AMPKα1-K45R (2.8 ± 0.4 µS/min), while overexpression of wtAMPKα1β1γ1 literally eliminated CFTR currents (Figure 6B). In these experiments we stimulated oocytes with only 20 µmol/l forskolin that creates a transient pulse of cAMP capable of being degraded by phosphodiesterases. Although AMPK largely antagonizes activation of CFTR, the mutated serines S737A and S768A do not seem to influence the recovery time from forskolin stimulation. AMP may be generated in close proximity of CFTR, probably by local phosphodiesterases. It has been shown that PDE4D is translocated to CFTR via the PDZ protein Shank2 that binds to the C-terminal PDZ binding domain of CFTR[62]. Thus negative regulation of CFTR via Shank2 occurs in competition with positive regulation by other PDZ proteins, such as NHERF1, which translocates protein kinase A close to the R domain[117]. We eliminated the PDZ binding domain of CFTR (E1474X-CFTR) and gradually increased stimulation of the oocytes as shown in Figure 6D. As expected, we found that stimulation with 3 mM $8Br^-$-cAMP (which acts both through stimulation of PKA and by blocking PDE activity) was able by itself to activate wtCFTR maximally, i.e. no further activation

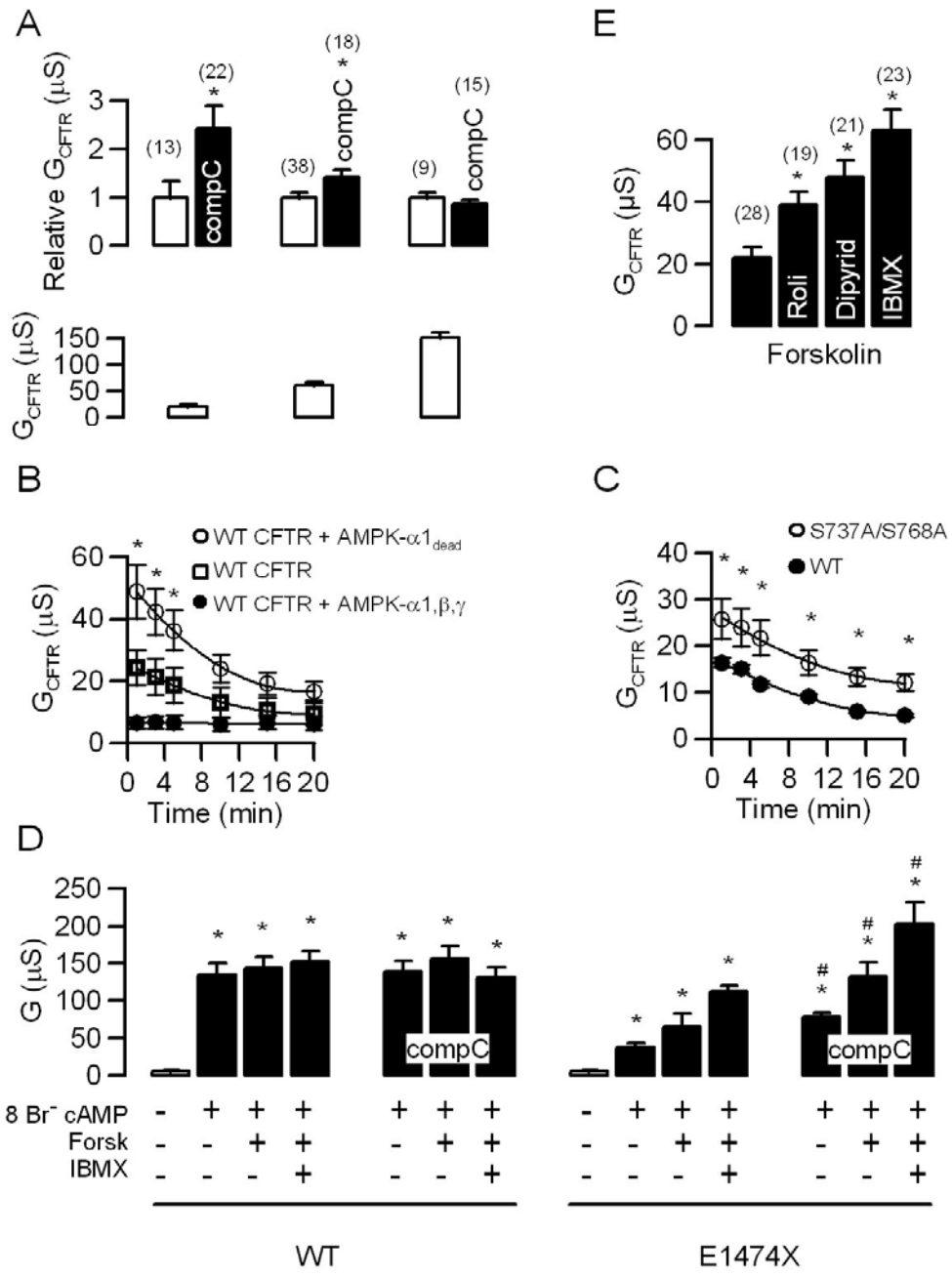

Figure 6: Functional role of AMPK-regulation of CFTR. A) Activation of CFTR whole cell conductance by stimulation with 20 µM forskolin (left column pair, raw baseline data in lower panel), 1 mM IBMX 80 µM forskolin (middle column) and 5 mM IBMX, 2 µM forskolin and 3 mM 8Br^--cAMP (right column). Increase of CFTR whole cell conductance by compound C (80 µM) relative to CFTR-baseline conductance (upper panels). B) Inactivation of CFTR whole cell conductances in oocytes expressing CFTR, CFTR and AMPKα1β1γ1, or the catalytically dead mutant AMPKα1-K45R. C) Inactivation of conductances generated by wtCFTR and S737A/S768A-CFTR. D) CFTR whole cell conductances activated by 3 mM Br^--cAMP, 3 mM Br^--cAMP and 2 µM forskolin, or 3 mM Br^--cAMP and 2 µM forskolin and 1 mM IBMX, and effects of compound C. E) Activation of CFTR by forskolin (2 µM) and additional stimulation by rolipram (50 µM), dipyridamol (80 µM) and IBMX (1 mM). Membrane currents were measured by voltage clamping in intervals from -60 to +40 mV, in steps of 10 mV. ∗ significant difference when compared to control. # significant difference when compared to wtCFTR. Data are shown as mean ± SEM, (number of experiments).

by additional stimulation with forskolin (2 µM) or IBMX (5 mM). Also, consistent with Figure 6A, right panel, inhibition of AMPK by compound C had no further enhancing effects on wtCFTR activity. In marked contrast 3 mM $8Br^-$-cAMP alone was unable to promote full activation of E1474X-CFTR but instead required co-stimulation by $8Br^-$ cAMP, forskolin and IBMX, and compound C further augmented whole cell conductance (Figure 6D) suggesting AMPK sensitivity was retained by this mutant. The data suggest that translocation of signaling cascades in close proximity to CFTR might be required for graded regulation of CFTR. This notion was supported by injection of oocytes with AMP to reach a final cytosolic concentration of 1 mM which did not inhibit CFTR. Although diffusion barriers for AMP could exist within the oocyte, the result suggests that global cytosolic AMP increase in unlikely to influence the membrane compartment. Furthermore, the inhibitors of the CFTR-specific PDE4 and PDE5 rolipram and dipyridamole also augmented CFTR whole cell conductance suggesting that CFTR-localized phosphodiesterase activity may generate environmental AMP that enhances AMPK activity in close proximity of CFTR (Figure 6E).

DISCUSSION

Regulation of CFTR by AMPK: The present results demonstrate that baseline CFTR activity is tonically inhibited by high baseline AMPK activity but after PKA stimulation, AMPK only has a modest inhibitory effect on wild type CFTR. This might explain why data reported previously by Hallows and colleagues found only modest inhibition of pre-activated CFTR by post-activation of AMPK in *Xenopus* oocytes[38]. It is not widely appreciated outside the specialist kinase field that AMPK has a substantial and constitutive baseline activity in most tissues that is further stimulated by increments in the intracellular AMP/ATP ratio or pharmacological activation by drugs such as phenformin. Additionally, independent regulation by the calmodulin dependent kinase (CAMKK) has been shown previously[118,119]. It may be of relevance to our findings in oocytes that PKA was shown to inhibit AMPK activity by attenuating phosphorylation through upstream calcium/calmodulin dependent protein kinase[120]. Thus multisite phosphorylation controls AMPK activity and the cross-talk between PKA and AMPK may be relevant for the competitive regulation of CFTR by PKA and AMPK as demonstrated in the present paper.

AMPK phosphorylates the R-domain: We provide biochemical and electro-physiological data consistent with the hypothesis that AMPK-regulation involves two so called inhibitory PKA sites at S737 and S768 in the R-domain[53,55]. Correspondingly, but with the caveat that

phosphorylation of these serines happens at a biochemical level *in vitro*, we nevertheless observe a dramatic difference in the consequent pattern of phosphopeptides in the R domain when the rank order in which these kinases are applied is reversed (as shown in Figure 2) and note the differential effect of serine 768 in this respect. Binding of the α1-subunit of AMPK to the residues 1420–1457 in the C-terminus of CFTR seems to be essential for AMPK regulation of CFTR, as demonstrated by a failure of AMPK to inhibit CFTR in the absence of this binding motif[38]. The present results (Figure 3) also confirm those of previous studies in that they demonstrate inhibition of the open probability of CFTR by phosphorylation through AMPK, rather than effects on membrane expression[35]. AMPK's actions are complex as this kinase regulates a number of transport proteins involved in secretion or absorption of electrolytes in epithelia. Thus epithelial Na^+ channels[97,121,122], the renal NKCC2[123,124], CFTR[35,38,103] and probably the secretory $Na^+/2Cl^-/K^+$-cotransporter (NKCC1)[124] can all be controlled by AMPK. Regulation occurs either indirectly, as in the case of epithelial Na^+ channels (ENaC), or directly through AMPK-phosphorylation as for CFTR, NKCC2 and probably NKCC1. AMPK is inhibitory on the ion transport generated by ENaC, CFTR, and NKCC1, however, the functional impact of AMPK on NKCC2 still remains to be determined[124]. It is also of interest that AMPK activation reduces inflammation in airway epithelial cells of CF patients[36] and we note that AMPK and CFTR are co-localized at the apical membrane in such tissues.

CFTR is the only ABC transporter that has a regulatory (R) domain, containing multiple consensus sites for phosphorylation by PKA and PKC[13]. Phosphorylation of the R-domain by PKA is a prerequisite, but is not sufficient to gate the Cl^- channels. Additional binding of ATP to both nucleotide binding domains is required to open the channel[45,116]. Phosphorylation at individual PKA sites has additive effects on the open probability of CFTR, and so phosphorylation of the individual serines occurs independently. Moreover, none of the PKA sites is absolutely necessary for activation of CFTR[53]. Notably, two of the PKA consensus sites (S737 and S768) have been suggested to be inhibitory since elimination of these sites largely augments activation of CFTR[53,55]. Our present results suggest that it might be AMPK rather than PKA that is phosphorylating S737 and S768 under baseline conditions, i.e. in the absence of any stimulation by secretagogues. This novel idea is consistent with the inhibition of CFTR in the presence of D mutants at one of these sites, namely S768.

Physiological relevance: It has been assumed that the inhibitory effects mediated by S737 and S768 can be overcome by stimulation with super-maximal concentrations of IBMX[53]. The present data clearly demonstrate that this is not the case: Even maximal stimulation of wtCFTR

with a cocktail of 8-Br$^-$-cAMP, IBMX and forskolin does not produce the same high level of conductance as S737A-CFTR or S768A-CFTR. A detailed single channel analysis by the Gadsby group arrived at a related conclusion that early phosphorylation of Ser 768 in CFTR impairs subsequent phosphorylation of stimulatory R-domain serines. They further postulated that the observed reduced sensitivity to activation by PKA imparted by Ser 768 might ensure activation of CFTR during strong stimulation but attenuating responses to weak signals[55]. Further data will be needed to test this idea but our phosphorylation data concur to the extent that they demonstrate that a complex interaction occurs through this critical serine.

In the present study we found that AMPK phosphorylation of S737 and S768 does not appear to affect activation or deactivation kinetics of CFTR. However, our data strongly suggest that increasing PKA activity 'competes out' AMPK sensitivity, so that only PKA will control CFTR. An antagonistic regulation of CFTR by AMPK under control conditions will guarantee that the channel is kept closed in the absence of secretagogues. Overall our data are consistent with the notion that AMPK activity acts as a 'biological rheostat' towards CFTR. As some epithelial tissues such as the sweat duct epithelium and the submucosal gland epithelium show a high baseline CFTR activity[125,126], it will be interesting to examine if these tissues lack of AMPKα1-expression or have otherwise a reduced AMPK-activity or sensitivity towards CFTR.

CFTR localized AMPK: Around 40% of resting energy expenditure in humans is used to control ion gradients across cells and crosstalk between transport and metabolism has been proposed that would adjust transport activity to the cellular energy supply[96]. CFTR is ideally localized to perform such a sensing role because it lies in a macromolecular complex together with receptors, adenylate cyclase, kinases, scaffolding proteins and phosphodiesterases. Thus CFTR can integrate various proteins to a 'channelsome', a functional unit that operates independently of other cellular compartments[59]. Receptor mediated compartmental activation of CFTR is probably disconnected from the cytosol, i.e. intracellular signaling by cAMP is spatially restricted and compartmentalized (reviewed in[127,128]). In this model, regulation of CFTR is predicted to occur without any detectable changes in cytosolic second messenger concentration, because CFTR is intimately connected to signaling elicited by stimulation of A2B adenosine and ß2 adrenergic receptors[129,130]. Moreover, type 2 lysophosphatidic acid (LPA) receptors also form macromolecular complexes with CFTR that are mediated through a PDZ scaffolding protein (NHERF2). In contrast to adenosine and ß2 adrenergic stimulation, LPA inhibits CFTR through a LPA2 receptor mediated Gi pathway[131]. Thus compartmentalized receptor-CFTR coupling appears to be critical for a rapid and specific signal transduction from the receptor to the

channel. PDEs provide the means to avoid spreading of intracellular signals by degrading cAMP, and therefore play a vital role in shaping intracellular gradients of the second messenger[45]. Particularly PDE4D, which couples to CFTR via the PDZ adaptor Shank2, precludes cAMP/PKA signals[62]. However, not only PDEs but also other mechanisms contribute to compartmentalization such as the cAMP transporter MRP4. This multidrug resistance protein also belongs to the large family of ABC-transporters, and physically couples to CFTR via PDZK1[132]. By providing an efflux pathway for cAMP, it constitutes an additional way of regulating cAMP levels in a microdomain underneath the surface membrane. In the present study both the PDE4 inhibitor rolipram and the PDE5 inhibitor dipyridamole augmented CFTR activity, confirming that both PDE-subtypes degrade cAMP locally, i.e. in close proximity to CFTR. In analogy to the well anticipated localized cAMP signaling, we hypothesize that local generation of AMP, perhaps in some instances generated by the very same phosphodiesterases from cAMP, controls the activity of AMPK, rather than global cytosolic changes in the ATP/AMP ratio. Thus AMPK may serve as a local controller of CFTR activity rather than coupling global cellular metabolism to CFTR's transport activity[96]. The data show that enhanced CFTR activity can be observed by mutating critical serines that negate inhibition by AMPK, such as serine 768. Our failure to reverse the gating defects in two common disease causing mutants (G551D and F508delCFTR) suggests that their mechanism of dysfunction is not due to an overactive AMPK but further work will have to establish whether inhibition of AMPK might be a therapeutic option in other mutations.

ACKNOWLEDGEMENTS

Supported by DFG-SFB699 A6 and DFG KU756/8-1. The staff in the Mehta laboratory is supported by the Wellcome Trust (KJT and DC; 079965/z/06/z & 075237/z/04/z respectively). We acknowledge the expert technical assistance by Ms. E. Tartler and Ms. A. Paech. We thank D.G. Hardie (Dundee) and A. Woods/D. Carling (London) for reagents, antibodies and advice.

CHAPTER 4.

Metformin Treatment of Diabetes Mellitus Increases the Risk for Pancreatitis in Patients Bearing the CFTR-mutation S573C

ABSTRACT

Metformin use in diabetes can cause acidosis and might be linked to pancreatitis. Here, we mechanistically focus on this relationship via a point mutation in the cystic fibrosis transmembrane conductance regulator (CFTR; ABCC7). CFTR is an ATP-hydrolyzing, cAMP/PKA-activated anion channel regulating pancreatic bicarbonate/chloride secretion across duct-facing apical membranes in epithelia. CFTR has two nucleotide binding domains (NBD1/2) which clamp two ATP molecules across their opposed, inverted interfacial surfaces which generates anion-conductance after ATP hydrolysis. Notably, CFTR mutations not causal for classical cystic fibrosis segregate with unexplained pancreatitis and one of these lies in NBD1 near its ATP-clamp (S573C; close to the Walker B aspartate D572). We recently showed that after raising [cAMP], wt-CFTR chloride-conductance, when expressed in *Xenopus* oocytes, remains elevated despite the presence of metformin. Yet here, we find that S573C-CFTR manifests a metformin-inhibitable whole cell chloride-conductance after cAMP elevation. In the absence of metformin, cAMP-activated S573C-CFTR also displays a reduced anion-conductance relative to wt-CFTR. Furthermore, intra-oocyte acidification inhibited wt-CFTR and abolished S573C-CFTR conductance. We conclude that defective S573C-CFTR remains both poorly conducting and inhibited by metformin and intracellular acidosis. This might explain the propensity to pancreatitis with this rare CF mutation.

Key words: Cystic fibrosis transmembrane conductance regulator, CFTR, AMP, PKA, AMP-activated protein kinase, S573C, Pancreatitis, Metformin, Pancreas, Chloride secretion.

Published in: **Kongsuphol P**, Hug M, Ousingsawat J, Witzgall R, Schreiber R, Kunzelmann K. Metformin treatment of diabetes mellitus increases the risk for pancreatitis in patients bearing the CFTR-mutation S573C. *Cellular Physiology and Biochemistry* 25, 389-396(2010).

Own experimental contribution: All DEVC experiments and related work.

Own writing contribution: Methods, Results and parts of Introduction and Discussion.

INTRODUCTION

Pancreatitis arises from a complex amalgam of genetic and environmental factors. In one instance, pancreatitis has an onset during fetal life in the common inherited disease, cystic fibrosis (CF) that affects about 1 in 4000 babies in the developed world. CF is caused by mutations in a phosphorylation and ATP-regulated chloride channel, the cystic fibrosis transmembrane-conductance regulator (CFTR). Even before CFTR was cloned two decades ago, the classical form of CF was recognized as a disease associated with low volumes of pancreatic fluid secretion containing less bicarbonate compared to non-CF pancreatic juice. Although the role of CFTR in bicarbonate secretion by CFTR in the pancreatic duct is not fully elucidated[133], it is now almost axiomatic that CFTR can conduct anions other than chloride and HCO_3^- is foremost amongst such candidates (another is glutathione). Bicarbonate transport via CFTR recycles with luminal Cl^- across the apical membrane of pancreatic duct cells and is therefore functionally coupled to luminal anion exchangers in charge of net HCO_3^- flux.

In fact CFTR may physically interact with two bicarbonate exchangers SLC26A6 and DRA (down regulated in adenoma)[9,134,135]. Activation of CFTR by DRA is facilitated by the C terminus of CFTR where PDZ ligands can interact through a conserved motif whereas the binding of the SLC26T STAS domain occurs through the CFTR regulatory (R) domain. The R domain is unique to CFTR when compared to other members of the ABC family and is the site of multiple regulation by protein kinases such as PKA (cAMP-dependent), PKC (lipid and calcium regulated) and AMP-activated kinase (a metabolic regulator). Binding of the STAS and R domains was shown to be regulated by PKA-mediated phosphorylation of the R domain[136,137] which we and others find to dramatically alter its structure (as judged by NMR and gel shift analysis) after phosphorylation by PKA. Thus R domain plasticity, associated changes in CFTR function and regulated HCO_3^- secretion appear to be tightly coupled. These ideas underpin classical Cystic fibrosis (CF) which is also characterized by a chronic lung disease that follows airway obstruction and chronic infection by many bacteria but particularly *Pseudomonas aeruginosa*. Other features include elevated sweat electrolyte concentrations, male infertility and almost always in such cases, there exists a severe degree of pancreatic insufficiency that follows the precipitation of pancreatic enzymes within the pancreas itself. Precipitation and premature activation lead to pancreatic autodigestion due to a failure of duct fluid secretion[138]. Moreover, the variability in repeat numbers of certain intronic elements at exon-intron boundaries CFTR gene characterized by the 5T poly-pyrimidine tract genotype have also been

found to be associated with chronic pancreatitis[139-143]. This complexity is exacerbated by a wider spectrum of non-classical CF that is 'organ-confined' affecting one or more organs but sparing the lung. Thus an emerging view is that it is possible for a patient to have CF of the organs and not CF as a whole. Here we expand this theme by focusing on one mutation in CFTR that lies close to one of the Walker B motifs involved in ATP binding to the first nucleotide binding domains of CFTR.

CFTR is regulated by many protein kinases and the adenosine monophosphate activated protein kinase (AMPK) is known to inhibit CFTR function[38,111]. AMPK is a ubiquitous Ser/Thr kinase with a substantial basal constitutive activity independent of AMP (hence the designation as AMP-activated rather than the AMP-regulated) and is the downstream effector of a cascade that is sensitive towards cellular energy. Upon ATP depletion, the substantial basal activity of AMPK is further activated by a rise in AMP which then stimulates catabolic pathways that ultimately lead to restoration of ATP production. In AMP-activated mode, AMPK also downregulates anabolic pathways that consume ATP, by direct phosphorylation of metabolic enzymes or by regulating gene expression[39]. Biguanidine compounds such as metformin (or the more potent but clinically toxic phenformin) activate AMPK in part by inhibiting complex I of the respiratory chain but additional mechanisms may also be operant[41,107]. Metformin is widely used in the clinical treatment of type II diabetes. Phenformin and metformin both promote insulin-stimulated glucose uptake in muscle tissues and lower hepatic glucose output. Metformin slightly increases the risks of lactic acidosis (much more so with phenformin) and patients using this compound can develop pancreatitis secondary to metformin poisoning, or at therapeutic metformin doses when a patient presents with renal failure[43,44]. Phenformin has been withdrawn from clinical use due the high incidence of lactic acidosis and it was replaced by metformin, which still has some risk of lactic acidosis and pancreatitis[43,107].

CFTR binds two ATP molecules using a sandwich structure consisting of nucleotide binding domains (NBDs) 1 and 2. We investigated the S573C point mutation that lies within the first nucleotide binding domain of CFTR adjacent to the Walker B aspartate at D572. S573C appears not to induce classical CF but instead segregates with pancreatitis of unknown etiology[88,89]. We tested the hypothesis that this was merely a chance finding by investigating S573C-CFTR function. Should we find no obvious change in CFTR function compared to wild type then we would have to conclude that the mutation was just a polymorphic variant of no particular consequence with just a random association with pancreatic failure. We find that this is not the

case and present a testable hypothesis about the means by which this mutant CFTR might induce pancreatitis. We speculated that the presence of the *CFTR* gene variant S573C in non-CF diabetes patients renders them more susceptible towards the development of pancreatitis and tested the potential role of metformin. We therefore examined the function of S573C and other point mutants of CFTR at this site by overexpression in *Xenopus* oocytes. The data indicate a lower channel activity of S573C-CFTR with a higher sensitivity towards metformin-induced closure. Impaired Cl^- channel function and regulatory function of S573C-CFTR may explain the higher incidence of this mutant in patients with pancreatitis.

MATERIALS AND METHODS

cRNAs for CFTR and double electrode voltage clamp: Oocytes were injected with cRNA (10 ng, 47 nl double-distilled water) encoding wtCFTR, S573C-CFTR, and S573A-CFTR. All mutants were generated by PCR and correct sequences were confirmed by restriction digest and by sequencing. Water injected oocytes served as controls. 2 - 4 days after injection, oocytes were impaled with two electrodes (Clark Instruments Ltd, Salisbury, UK), which had a resistances of < 1 MΩ when filled with 2.7 mol/l KCl. Using two bath electrodes and a virtual-ground head stage, the voltage drop across the serial resistance was effectively zero. Membrane currents were measured by voltage clamping (oocyte clamp amplifier, Warner Instruments LLC, Hamden CT) in intervals from -60 to +40 mV, in steps of 10 mV, each 1 s. The bath was continuously perfused at a rate of 5 ml/min. All experiments were conducted at room temperature (22 °C).

Materials and statistical analysis: All compounds used were of highest available grade of purity and were purchase from SIGMA or Calbiochem. Student's t-test was used for statistical analysis. A p value of <0.05 was regarded as significant.

RESULTS

S573C attenuates CFTR whole cell Cl^- conductance and is inhibited by metformin: Because S573C-CFTR has been found in patients with pancreatitis, we examined the ability of both wtCFTR and S573C-CFTR to generate Cl^- currents by maximal stimulation of *Xenopus* oocytes with IBMX (1 mM) and forskolin (2 µM). Under these conditions which elevate cAMP and

activate PKA, whole cell currents and conductances produced by S573C-CFTR were reduced (~20%) when compared to those produced by wt-CFTR (Figure 1A-D). AMP-activated protein kinase (AMPK) has been shown previously to inhibit CFTR and to be relevant *in vivo*[35,38,111,144]. The biguanide metformin that is used for treatment of type 2 diabetes mellitus, is also known to activate AMPK and therefore may inhibit CFTR, similarly to the more potent phenformin. We examined the effect of 2 mM metformin on CFTR-conductance and compared the effect with that of phenformin (1 mM). Metformin only inhibited S573C-CFTR but not wt-CFTR (compare second bars in Figure 1 E,F), in contrast to the more potent activator of AMPK, phenformin that inhibited both S573C-CFTR and wt-CFTR (Figure 1E,F). Thus mutation of serine 573 to a close analogue merely bearing an S replacing an oxygen in an alcoholic side chain reduces CFTR-conductance and appears to enhance both sensitivity towards AMPK-induced closure and conversely, this mutant fails to almost double CFTR conductance after pharmacological inhibition of AMPK using compound C (Figure 1E,F).

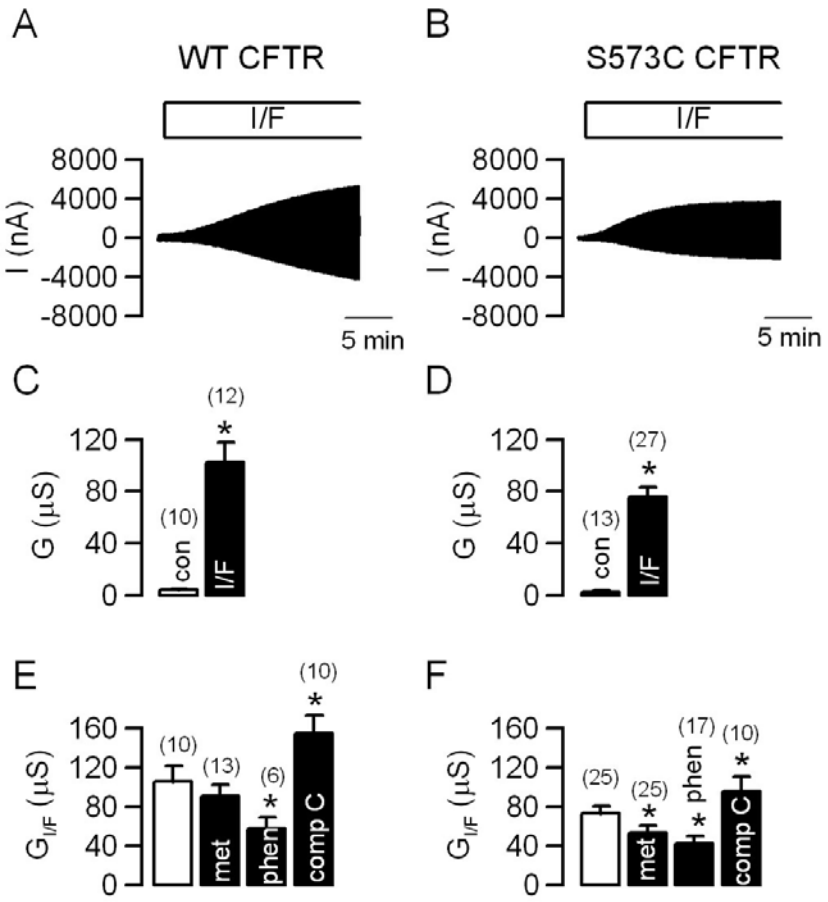

Figure 1: S573C attenuates CFTR whole cell Cl^- conductance. Current recordings obtained in *Xenopus* oocytes expressing wt-CFTR (**A**) or S573C-CFTR (**B**) showing activation of whole cell Cl^- currents by stimulation with IBMX (1 mM) and forskolin (2 µM). Summaries of whole cell conductances under control conditions and after stimulation with I/F (G) (**C,D**). Summaries of whole cell conductances activated by IBMX and forskolin ($G_{I/F}$) in ooctyes expressing wt-CFTR (**E**) and S573C-CFTR (**F**), and effects of the activators of AMPK metformin (Met; 2 mM) and phenformin (Phen; 1 mM) or the AMPK-inhibitor compound C (comp C; 80 µM). Mean ± SEM, (n) = number of cells measured. *significant increase in whole cell conductance (paired t-test).

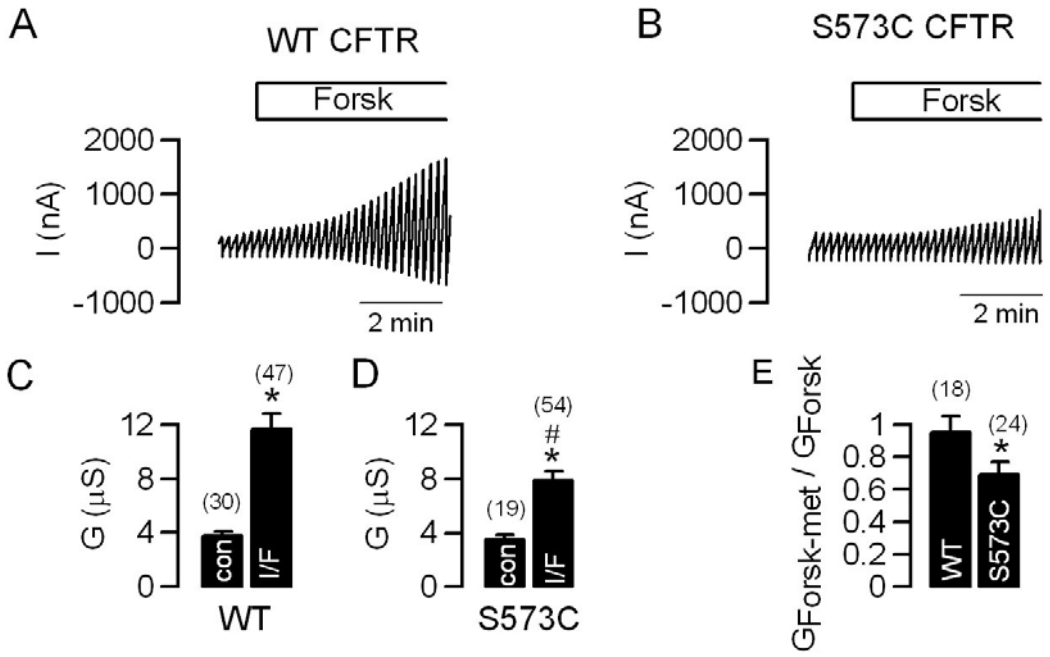

Figure 2: Metformin inhibits S573C-CFTR but not wt-CFTR. Current recordings obtained in a *Xenopus* oocyte expressing wt-CFTR (**A**) or S573C-CFTR (**B**) showing activation of whole cell Cl^- currents by stimulation with only forskolin (20 µM). Summaries of the whole cell conductances under control conditions and after stimulation with I/F (G) (**C,D**). Summaries of the ration of whole cell conductances activated in the presence of metformin (500 µM) or under control conditions in oocytes expressing wt-CFTR and S573C-CFTR (**E**). Mean ± SEM, (n) = number of cells measured. *significant increase in whole cell conductance (paired t-test). # significant difference when compared to wt-CFTR.

We demonstrated recently that CFTR is more susceptible towards inhibition by AMPK at lower cAMP-induced activation levels[111]. Therefore we stimulated oocytes with forskolin alone (20 µM), which caused about 10 % of maximal activation of CFTR (Figure 2A-D). Under these conditions it became quite obvious that S573C-CFTR has a reduced Cl^- conductance compared to wtCFTR (Figure 2C,D) and that 500 µM metformin did not inhibit wtCFTR and yet inhibited S573C-CFTR by about 25% (Figure 2E), thus confirming the enhanced sensitivity of S573C-CFTR for inhibition by metformin.

Phosphorylation of NBD1 is enhanced by the mutation S573A: Previously we found that two serines in the regulatory (R) domain of CFTR, S737 and S768, formerly identified as inhibitory PKA sites, are phosphorylated by AMPK and are the residues essential for inhibition by AMPK[111]. Since S573 is located in the first nucleotide binding domain (NBD1) of CFTR and because inhibition by the AMPK-activator metformin is enhanced for S573-CFTR, the present results suggest that NBD1 may contribute to regulation of CFTR by AMPK. We therefore

examined whether eliminating the serine at position 573, or a specific exchange of serine by cysteine is reducing CFTR activity. We investigated a S573A-CFTR mutant and found that it behaved like wild type and critically, has lost its inhibitory metformin sensitivity when stimulated by a rise in cAMP (Figure 3B). This suggested that CFTR is able to discriminate between a methylene group on the side chain at position S573 bearing either an OH (wild type) or the bulkier SH group. We interpreted this data to suggest that the S573C defect might lie downstream of AMPK due to perturbation of the milieu around either the adjacent Walker B aspartate (for example at D572) or near another local cysteine, as suggested by Chen *et al*. during their pH studies[145]. We were particularly interested in the role of pH in this process because Sheppard and colleagues had found that this very same region might be involved in pH sensing (Chen *et al*)[145]. To rule out a direct effect of AMPK on this serine, we examined phosphorylation of isolated NBD1 *in vitro* and found that AMPK indeed phosphorylated this domain of CFTR as expected (Figure 3A). We mutated serine 573 to an alanine and found that elimination of this potential phosphorylation site did not abolish phosphorylation of NBD1 by AMPK (Figure 3A). These results were consistent with existence of other phosphorylation sites for AMPK in NBD1, which are different from serine 573. Although *in vitro* phosphorylation data do not allow for quantitative assessment, it appears that AMPK-phosphorylation of the S573A mutant was somewhat augmented but this may equally be an artifact caused by slight differences relative amounts of protein. This was not taken further here because mutation at S573 did not have a dramatic effect on phosphorylation by AMPK compared to other CFTR sites (S768 for example) we had previously encountered leading us to concentrate on pH effects.

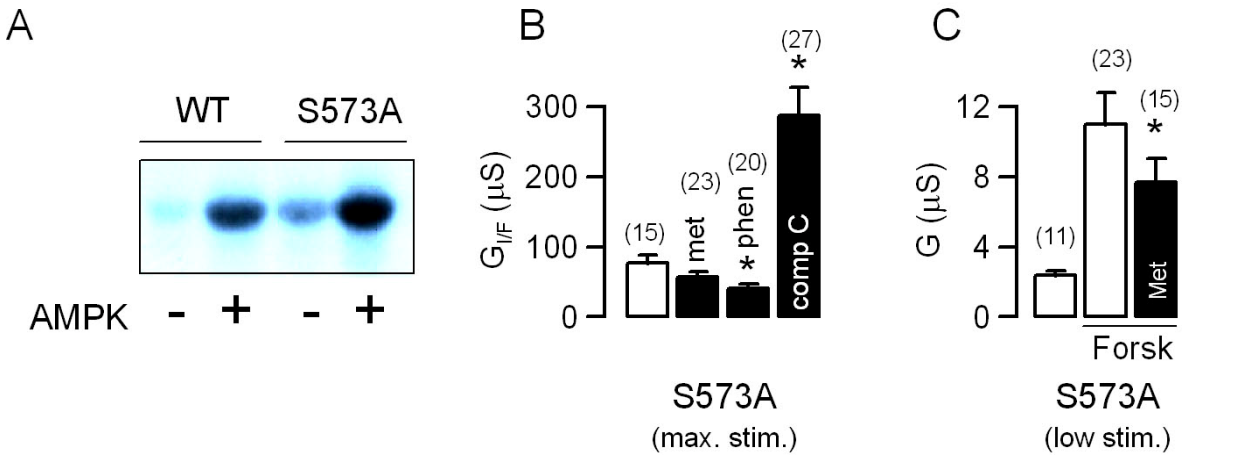

Figure 3: Phosphorylation and activation of S573A-CFTR. Phosphorylation by AMPK of the first nucleotide binding domains (NBD1) of wtCFTR and S573A-CFTR (**A**). Summary of the whole cell conductances activated by IBMX (1 mM) and forskolin (20 µM) ($G_{I/F}$) obtained in *Xenopus* oocyte expressing S573A-CFTR (**B**) or S573A-CFTR (**C**) and effects of activators (metformin, phenformin) or an inhibitor (compound C) of AMPK. Mean ± SEM, (n) = number of cells measured. *significant effects on whole cell conductance (paired t-test).

Acidification equally inhibits wtCFTR, S573A-CFTR and S573C-CFTR: Lactic acidosis with the consequence of pancreatitis may be caused by the use of antidiabetic biguanide drugs such as phenformin or metformin[43,44,146,147]. We wondered whether change in external pH and in particular acidosis may be an additional factor that inhibits the function of wild type CFTR. Also, the inhibitory effect of metformin may be potentiated in the presence of extracellular acidosis which could explain the pancreatitis observed upon treatment with biguanide drugs and lactic acidosis[43]. Water-injected control oocytes remained completely unaffected by extracellular acidification (Figure 4A,B). Moreover extracellular pH does not *per se* affect ion currents of *Xenopus* oocytes expressing CFTR (data not shown). Surprisingly, metformin (500 µM), when applied in the presence of acidic pH (pH 5.5), did not inhibit forskolin (20 µM) activated Cl^- currents, generated by wt-CFTR, S573C-CFTR, or S573A-CFTR (Figure 4C). Thus the S573C CFTR which had previously retained metformin-induced inhibition despite the presence of forskolin and IBMX, had now 'become wild type' just like S573A.

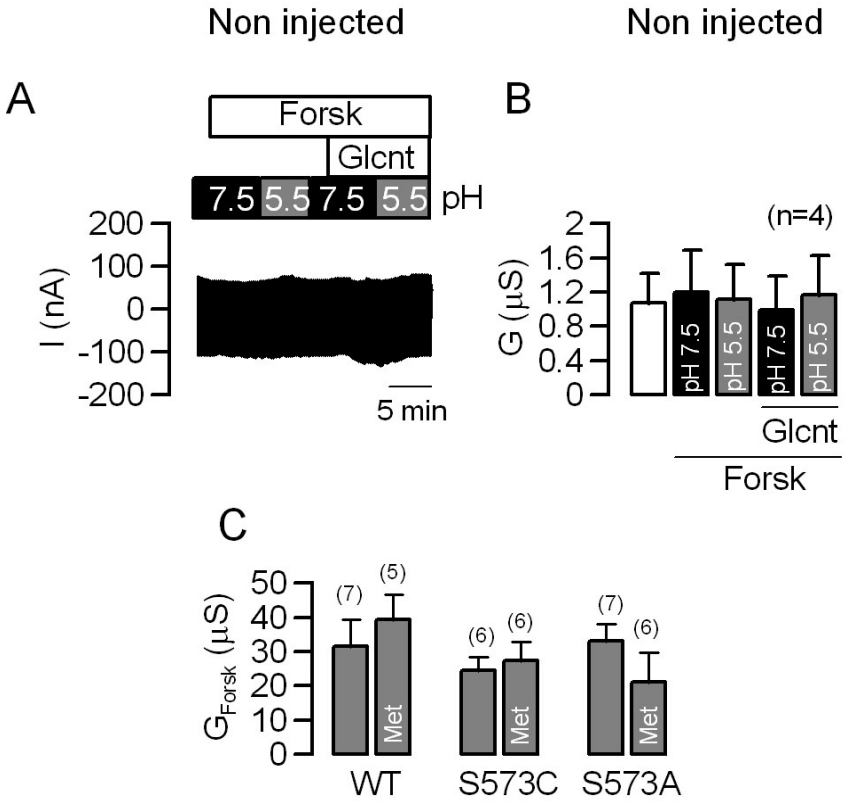

Figure 4: Extracellular acidification does not affect control oocytes. Current recording obtained in a non-injected *Xenopus* oocyte indicating lack of effects of forskolin or extracellular pH 5.5 (**A**). Summary of whole cell conductances obtained in control oocytes in the absence or presence of extracellular acidosis or stimulation with forskolin (**B**). Summary of the whole cell conductances generated by wt-CFTR, S573C-CFTR and S573A-CFTR when activated by forskolin (20 µM) in the presence of pH 5.5, and effects of metformin (500 µM) **(C)**. Mean ± SEM, (n) = number of cells measured.

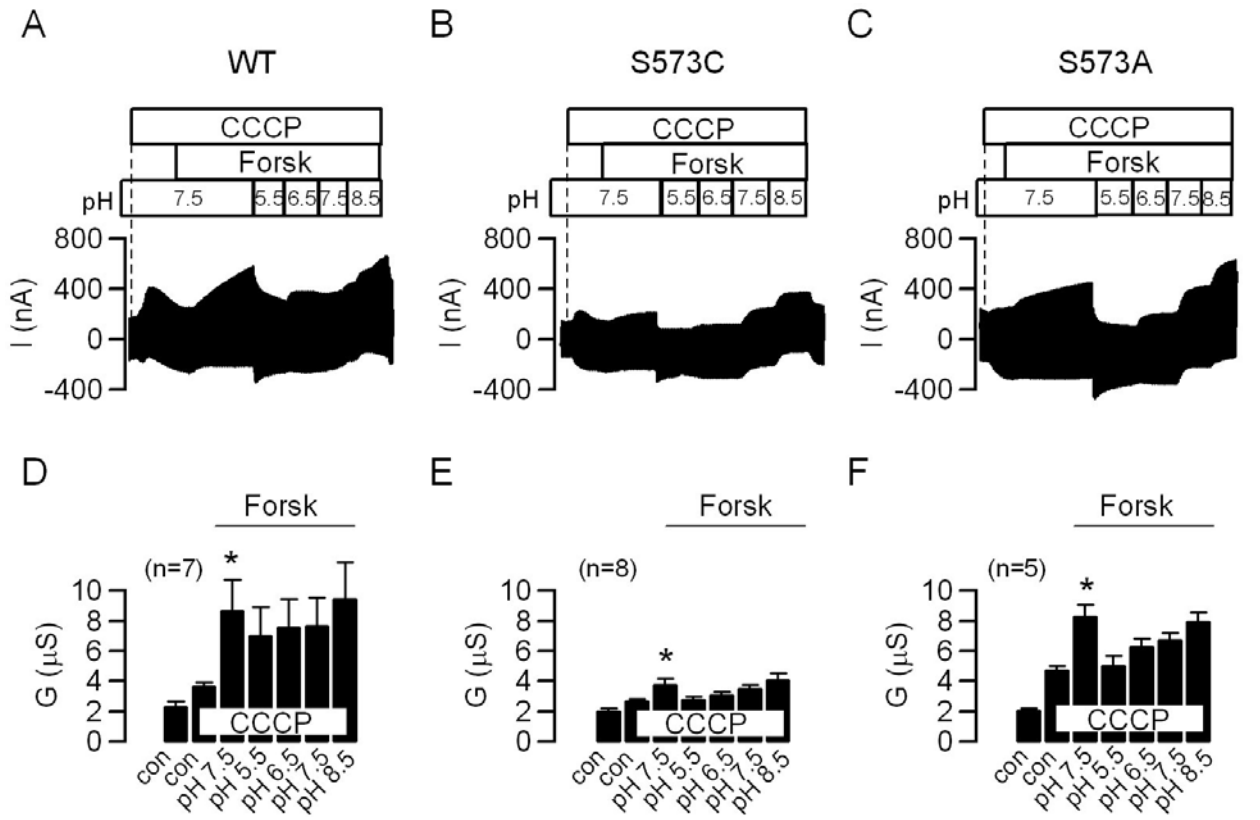

Figure 5: Inhibition of wtCFTR, S573C-CFTR, and S573A-CFTR by intracellular acidosis. Current recordings obtained in a *Xenopus* oocyte expressing wt-CFTR **(A**), S573C-CFTR (**B**), or S573A-CFTR (**C**). Summary of the whole cell conductances under control conditions and after stimulation with forskolin (20 µM), in the presence of CCCP at different extracellular pH. Acidification (pH 5.5, pH 6.5) but not alkalinization (pH 8.5) inhibits Cl^- currents generated by wt-CFTR, S573C-CFTR and S573A-CFTR. Acidosis completely inhibited currents produced by S573C-CFTR and S573A-CFTR. (**D-F**). Mean ± SEM, (n) = number of cells measured. *significant increase in whole cell conductance (paired t-test).

We further tested whether intracellular acidification differentially affects whole cell currents produced by wt-CFTR, S573C-CFTR, or S573A-CFTR. To that end oocytes were exposed to the protonophore carbonyl cyanide m-chloro phenylhydrazone (CCCP; 10 µM) at different extracellular pH ranging from pH 5.5 to pH 8.5. We observed regular activation of wt-CFTR and S573A-CFTR by forskolin (20 µM) in the presence of CCCP and at physiological pH (pH 7.5), while activation of S573C-CFTR was largely reduced (Figure 5A-C). Subsequent, acidification inhibited Cl^- currents produced by wt-CFTR, S573C-CFTR, or S573A-CFTR, while subsequent alkalinization did not affect whole cell Cl^- currents (Figure 5A-C). In fact acidosis completely inhibited S573C-CFTR currents. Taken together the present results are consistent with a model whereby the S573C-CFTR mutation predisposes to pancreatitis because it i) may sensitize

CFTR towards inappropriate regulation by AMPK, ii) is inhibited by the therapeutic biguanide metformin and iii) is inhibited potently by acidification.

DISCUSSION

Pancreatitis is not a common complication during metformin therapy; however, there are clinical cases of pancreatitis associated with metformin treatment[43]. CFTR has a clear role in alkalinizing the pH of the pancreatic juice, thereby solubilizing secreted enzymes, neutralizing acid chyme that is entering the duodenum and preventing premature activation of enzymes within the pancreatic duct[135,148]. Notably, fluid and HCO_3^- secretion occurs in a coordinated manner and it has been demonstrated recently that basolateral HCO_3^- entry via the Na^+/HCO_3^- cotransporter, pNBC1, and exit at the luminal side by the CFTR/SLC26 transporter-complex is coordinated by the protein IRBIT (inositol-1,4,5-trisphosphate receptors binding protein released with IP3)[136]. This is discussed further and in depth elsewhere[145] but our data suggest that near the site of ATP binding in NBD1, subtle alterations induced by and O-S group substitution next to the Walker B aspartate motif in NBD1 not only reduce the conductance of CFTR (as might be expected by a potential effect on ATP binding) but, unexpectedly, alter the ability of CFTR to respond to a step change in pH or metformin application. Moreover, functional CFTR is also essential for bicarbonate secretion in the small intestine[149] and CFTR-dependent duodenal HCO_3^- secretion in the mouse colon is strictly dependent on colocalization of ß2-adrenergic receptors and CFTR and the function of the Na^+/H^+ exchanger regulatory factor, NHERF1[150].

The team of Argent and Gray found inhibition of CFTR Cl^- currents in guinea pig pancreatic duct cells by extracellular HCO_3^- that may have clear implications for the current models of pancreatic ductal HCO_3^- secretion[151]. This may also be of clinical relevance, since a cystic fibrosis phenotype has been observed in a previously healthy adolescent presenting with metabolic alkalosis[152]. In contrast, CFTR does not seem to be regulated by extracellular pH, according to previous studies on isolated sweat ducts[153]. In contrast Reddy and coworkers found inhibition of phosphorylation by intracellular acidification, which might explain inhibition of CFTR currents by intracellular acidosis observed in the present study through interactions with other protein kinases[153]. The result of the present study pose challenging new questions with respect to the molecular mechanisms for predisposal of S573C towards AMPK-phosphorylation, the residues within NBD1 that are phosphorylated by AMPK, and how S573C within NBD1 affects pH

sensitivity of CFTR. Despite these open questions, the currents results suggest caution when biguanide drugs are used in a cystic fibrosis patient or patients carrying one of the CFTR variants predisposing to pancreatitis.

ACKNOWLEDGEMENTS

Supported by DFG SFB699 A6 and TargetScreen2 (EU-FP6-2005-LH-037365). We acknowledge the expert technical assistance by Ms. E. Tartler and Ms. P. Seeberger. We thank the Wellcome Trust (WT805639) for supporting DC in the Mehta laboratory.

CHAPTER 5.

CFTR Induces Acid Sensing and H^+ Activated Cl^- Transport

ABSTRACT

The cystic fibrosis transmembrane conductance regulator (CFTR) produces a cAMP-dependent Cl^- conductance of distinct properties that is essential for electrolyte secretion in human epithelial tissues. However, the functional consequences of CFTR-expression are multifaceted, encompassing much more than simply supplying a cellular cAMP-regulated Cl^- conductance. When we expressed CFTR in *Xenopus* oocytes, we found that extracellular acidic pH activates a Cl^- conductance that is, however, not due to CFTR Cl^- currents. The proton activated Cl^- conductance showed biophysical and pharmacological features of a Ca^{2+} dependent Cl^- conductance (CaCC) and was clearly Ca^{2+} dependent. In contrast to extracellular acidification, intracellular acid pH did not activate CaCC. Surprisingly, apart from fully functional wtCFTR, also the trafficking mutant F508del-CFTR was able to confer proton sensitivity, while the gating mutant G551D-CFTR did not generate a proton activated Ca^{2+} dependent Cl^- conductance. CFTR may induce or augment extracellular proton sensing by translocating proton receptors to the plasma membrane and by facilitating Ca^{2+} release from the endoplasmic reticulum. The data suggest the role of CFTR for pH sensing in bone cells and may provide a link to abnormal bone formation in cystic fibrosis.

Key words: Cystic fibrosis transmembrane conductance regulator, CFTR, acid, protons, F508del-CFTR, Ca^{2+} activated chloride secretion, CaCC, TMEM16A

Own experimental contribution: All of the experiments.

Own writing contribution: Methods, Results, Introduction and part of Discussion.

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-and protein kinase A (PKA)-regulated Cl^- channel and a regulator of other ion channels[58,59]. In normal, non-cystic fibrosis (CF) epithelial cells and in recombinant cells overexpressing CFTR, Cl^- currents with a linear current/voltage relationship are activated by an increase in intracellular cAMP that are not detected in epithelial cells derived from CF patients. CFTR is expressed in luminal membranes of both secretory and absorptive epithelia, where it not only generates a Cl^- conductance but also controls a number of other ion channels and membrane conductances[154]. Among the many membrane conductances and transport processes that are controlled by CFTR, there is the well described relationship between the cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+}-activated Cl^- channels (CaCCs). Enhanced Ca^{2+} activated Cl^- conductance has been detected in the airways of cystic fibrosis (CF) patients[155,156] as well as mouse airways and cultured airway epithelial cells[84,157]. Other studies have demonstrated that CFTR has an inhibitory effect on CaCCs[73,158].

The question how both Cl^- conductances are related has been reinforced by molecular identification of the novel Ca^{2+} dependent Cl^- channel TMEM16A (anoctamin 1, ANO1)[77,79,159]. TMEM16A is a member of a family of 10 homologous proteins that exist as various splice variants. In mouse, it is expressed particularly in epithelial cells and smooth muscle tissues[160,161]. By analyzing Cl^- transport in TMEM16A null mice, TMEM16A was identified as an essential component for Ca^{2+}-dependent Cl^- secretion in several epithelial tissues and mucociliary clearance of mouse airways[81,162].

These recent findings reinforce the concept that cAMP- and Ca^{2+} dependent Cl^- conductances are due to two different molecular entities and asks for the relationship of both proteins[163]. In the present study we report a novel finding that, once again, indicates a functional relationship between both currents. We found that endogenous Ca^{2+} dependent Cl^- currents present in *Xenopus* oocytes are activated by extracellular protons, however, only when CFTR is expressed. This is a novel finding that supports the role of CFTR as a conductance regulator, particularly of other Cl^- channels.

MATERIALS AND METHODS

cRNAs and double electrode voltage clamp: Oocytes obtained from female *Xenopus* laevis were defolliculated for 1 hr at 18°C with 1.5 mg/ml collanease type V (Sigma) in OR2 solution (in mmol/l): 82.5 NaCl, 2 KCl, 1 $MgCl_2$, and 5 HEPES pH 7.55. Oocytes were injected with 10 ng (47 nl double-distilled water) of cRNA encoding wt, ΔF508, or G551D CFTR. All mutants were constructed by PCR based site-direct mutagenesis techniques. Correct sequences were confirmed by restricted enzyme digestion and sequencing. Oocytes were maintained in ND97 solution (in mmol/l): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 2.5 Na-pyruvate, 0.5 Theopyllin, and 0.01 mg/ml Gentamycin, pH 7.55 at 18°C. 2-4 days after injection, membrane currents were measured by impaling an oocyte with two electrodes (Clark Instruments Ltd, Salisbury, UK) that filled with 2.7 mol/l KCl and a resistance of less than 1 MΩ. The bath was continuously perfused at a rate of 5 ml/min with ND96 solution (in mmol/l): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, and 2.5 Na-pyruvate, pH 7.55. The voltage drop across the serial resistance was optimized to zero using two bath electrodes and a virtual-ground head stage. Oocytes were voltage clamping (Oocyte clamp amplifier, Warner Instruments LLC, Hamden CT) from -60 to +40 mV interval, in steps of 10 mV, each 1 s. All experiments were conducted at room temperature (22 °C). Incase of drug preincubation before two electrode voltage clamp experiments, compounds were dissolved in ND96.

Materials: Reagents were purchased from the following sources; $CFTR_{inh}172$, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, 2Na (DIDS), 1,2-*bis*(*o*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM) and forsklolin (#344270) from Calbiochem; niflumic acid (NFA) carbonyl cyanide 3-chlorophenylhydrazone (CCCP), and 3-Isobutyl-1-methylxanthine (IBMX) from Sigma. Other chemicals used were with highly analytical graded.

Statistical analysis. Values were presented as mean ± SEM. Student *t*-test was used for statistical analysis. Significant difference was considered at $p \leq 0.05$.

RESULTS

Extracellular acidic pH activates Cl^- currents in CFTR expressing oocytes. In two electrode voltage clamp experiments we exposed *Xenopus* oocytes to extracellular acid pH of 5.5, which had no effects on the membrane conductance (Figure 1). In contrast when oocytes expressed

wtCFTR, acidic pH activated a whole cell conductance that showed a pronounced outward rectification and that was not inhibited by 10 µM of the $CFTR_{inh}172$. Expression of functional CFTR was demonstrated by activation of a CFTR-typical, linear Cl^- conductance by IBMX and Forskolin (1 mM/2 µM). Thus expression of wtCFTR suggests activation of a conductance by extracellular acidosis, which shows features of the endogenous Ca^{2+} activated Cl^- conductance in *Xenopus* oocytes. Interestingly extracellular acidic pH also activated a whole cell conductance in oocytes expressing the most common CFTR-mutant ΔF508-CFTR. In fact the H^+ activated conductance was even larger in ΔF508-CFTR expressing cells, although I/F activated CFTR conductance was low as expected. In contrast, expression of the non-functional mutant G551D-

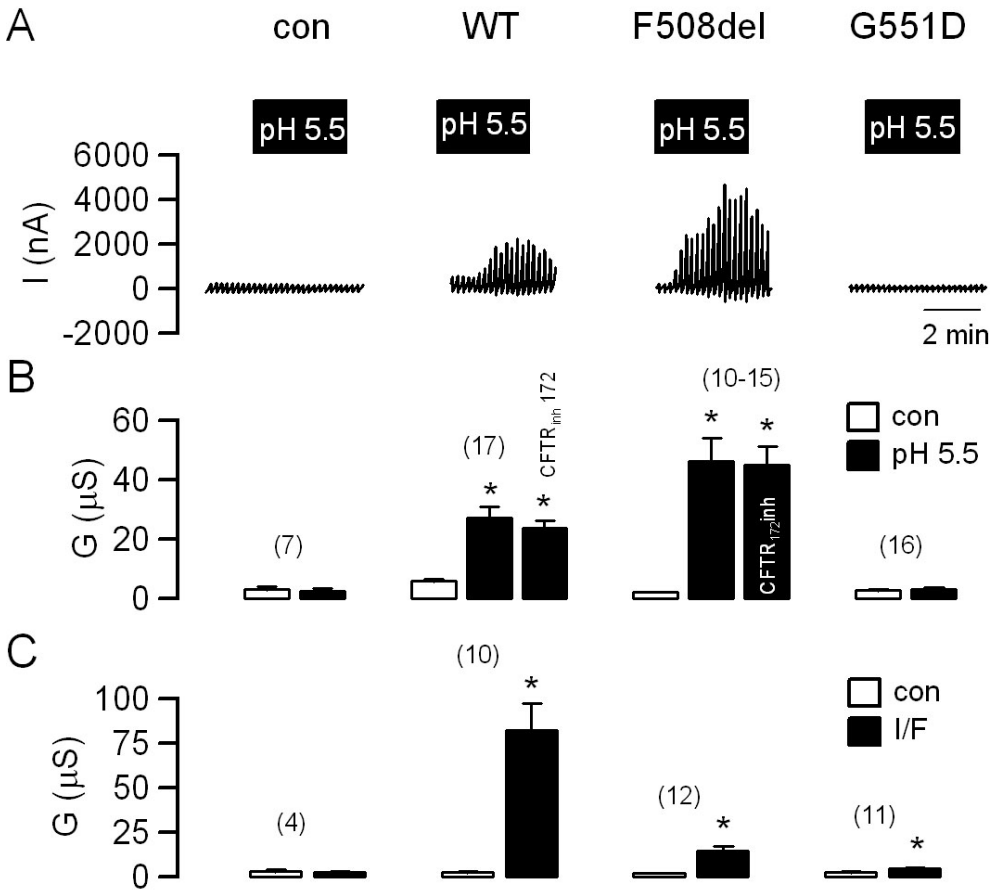

Figure 1. Extracellular acidosis induces currents in CFTR expressing oocytes. A) Original recordings from oocytes injected with water (control) or expressing wtCFTR, ΔF508-CFTR, or G551D-CFTR before and after exposure to acidic extracellular bath solution (pH 5.5). B) Summary of whole cell conductances obtained from oocytes expressing wtCFTR or different CFTR mutants in the presence of normal (pH 7.5) and acidic (pH 5.5), and effects of inhibition of CFTR by 10 µM $CFTR_{inh}172$ for 15-30 min before applying pH 5.5. C) Summary of whole cell conductances activated by 1 mM IBMX and 2 µM forskolin. Data represent mean ± SEM, (n) = number of experiments. * indicates significant difference when compared to control (paired t-test, $p \leq 0.05$).

CFTR did not produce any H^+ activated conductance (Figure 1). Taken together expression of wtCFTR and ΔF508-CFTR supply a H^+ sensitive whole cell conductance in *Xenopus* oocytes.

It has been previously reported that in pre-activated CFTR, cytosolic acidosis enhances CFTR activity[145]. We therefore examined whether extracellular acidification possible lowers intracellular pH and affects CFTR activity, which could be related to our findings. In order to induce intracellular acidification, the protonophore carbonyl cyanide m-chloro phenylhydrazone (CCCP), was applied in the presence of variable extracellular pH (Figure 2). Interestingly, intracellular acidification did neither activate a whole cell current in water injected control oocytes nor in wtCFTR expressing oocytes. This result suggests that activation of whole cell currents is due to extracellular but not intracellular acidification and that a H^+ gradient is required to activate the endogenous current.

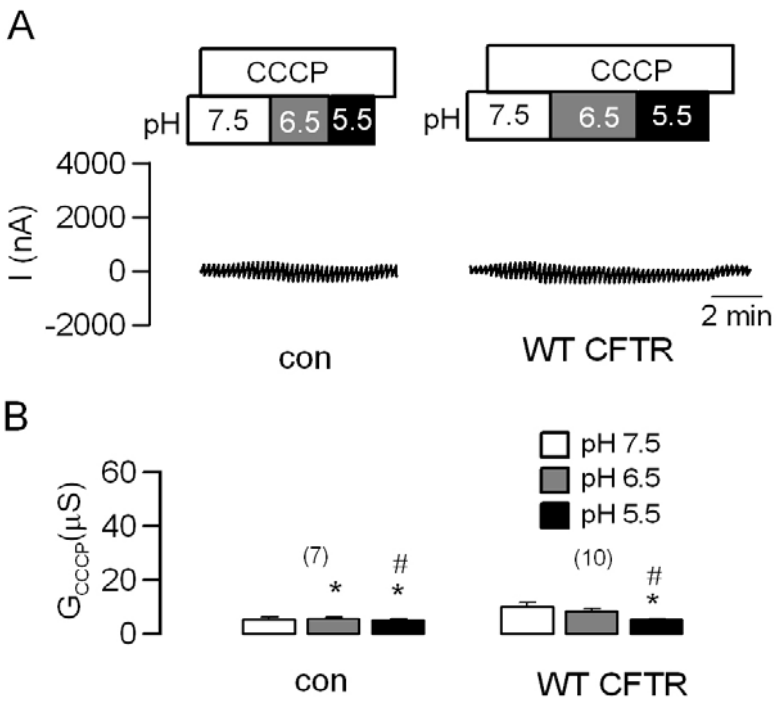

Figure 2. Intracellular acidification does not activate CaCC. A) Original recordings from a water injected control oocyte and a wtCFTR expressing oocyte, incubated with the protonophore CCCP (10 µM), and effects of acidification of the extracellular bath solution. B) Summary of whole cell conductances obtained from control oocytes and wtCFTR expressing oocytes when treated with CCCP and exposed to solutions of different extracellular pH. Data represent mean ± SEM, (n) = number of experiments. * indicates significant difference when compared to control (paired t-test, $p \leq 0.05$). # indicates significant difference when compared to pH 7.5 (unpaired t-test, $p \leq 0.05$).

Extracellular H^+ activate Ca^{2+} dependent Cl^- currents: We analyzed the whole cell current that was activated by extracellular H^+ in CFTR-expressing oocytes. Strong outward rectification of the current suggested activation of endogenous Ca^{2+} dependent Cl^- currents (Figure 3A,C). We therefore replaced extracellular Cl^- by $NO3^-$ or I^- and found that acid induced currents were augmented, by $NO3^-$ and I^- ($I^->NO3^->Cl^-$) indicating a halide permeability sequence typical for Ca^{2+} dependent Cl^- currents (CaCC) (Figure 3). We also applied the typical inhibitors of CaCC, di-isothiocyano-stilbene-2',2'-sulfonic acid (DIDS) and niflumic acid (NFA; both 100 µM) to the

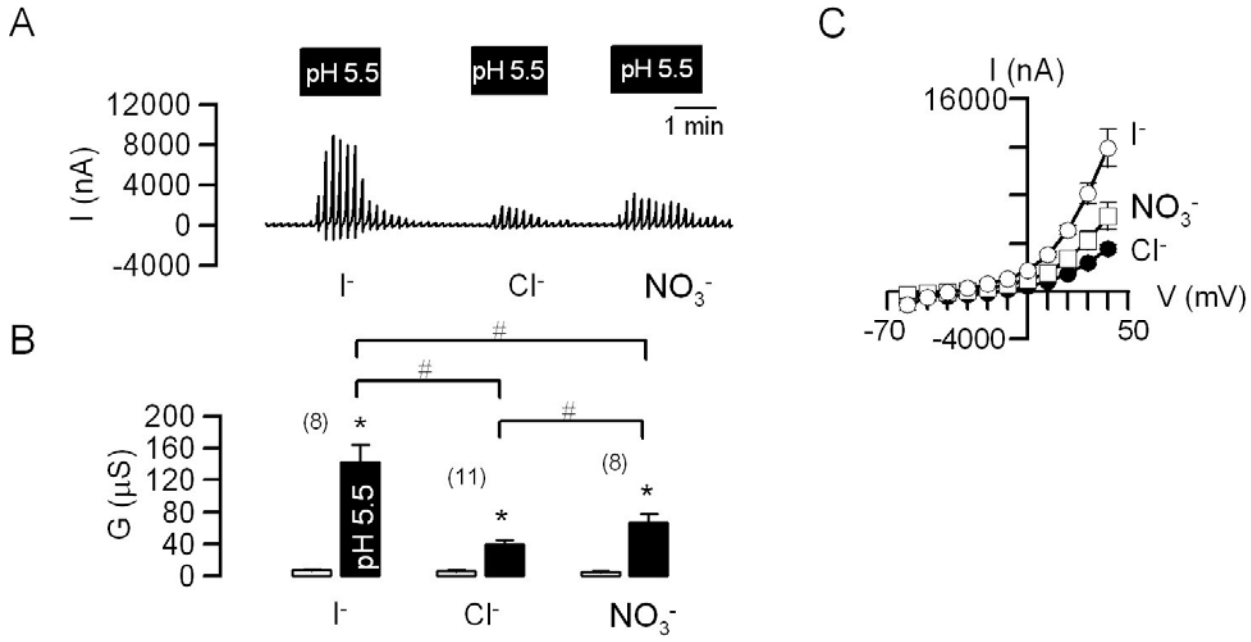

Figure 3. Ion selectivity of the H^+ activated currents indicates activation of CaCC. A) Original recordings from a CFTR-expressing oocyte exposed to extracellular pH 5.5 in the presence of different extracellular anions. B) Summary of whole cell conductances obtained from CFTR-expressing oocytes in the presence of different extracellular anions, before and after treatment with extracellular acidic pH 5.5. C) Current-voltage (IV) relationships of currents activated by extracellular acidic pH in wtCFTR expressing oocytes exposed to different extracellular anions. Data represent mean ± SEM, (n) = number of experiments. * indicates significant difference when compared to control (paired t-test, $p \leq 0.05$). # indicates significant difference in the presence of different extracellular halides (unpaired t-test, $p \leq 0.05$).

extracellular bath solution. We found that both inhibitors completely blocked H^+ activated whole cell conductances (Figure 4). Finally, we acidified the extracellular compartment in the absence of extracellular Ca^{2+} which, however, did not affect activation of CaCC (Figure 5). In contrast, when oocytes were preincubated with the Ca^{2+} chelator 1,2-Bis(2-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA; 50 µM), activation of CacC by extracellular H^+ was completely abolished. Similar was observed in the presence of BAPTA/extracellular Ca^{2+} free solution (Figure 5). These results indicate that extracellular acidification increases the intracellular Ca^{2+} concentration, which in turn activates CaCC, a process that is strictly dependent on the presence of CFTR.

CFTR may establish or translocate an unknown H^+ receptor to the plasma membrane: The results suggests that CFTR establishes a H^+ sensitive mechanism at the plasma membrane, that is coupled to an intracellular phoshoplipase $C/IP_3/Ca^{2+}$ - dependent signalling pathway. CFTR itself may also form a proton sensor. However, it has previously reported that CFTR is able to translocate functional receptor complexes to the plasma membrane, such as the

lysophosphatidic acid (LPA) receptor that inhibits cholera toxin-induced secretory diarrhea through CFTR-dependent protein interactions[131]. We found that oocytes also activate a Ca^{2+} dependent Cl^- current when exposed to 50 µM LPA (Figure 6). Thus oocytes express LPA-receptors that are coupled to intracellular Ca^{2+} signalling and activation of CaCC[164]. Notably, the

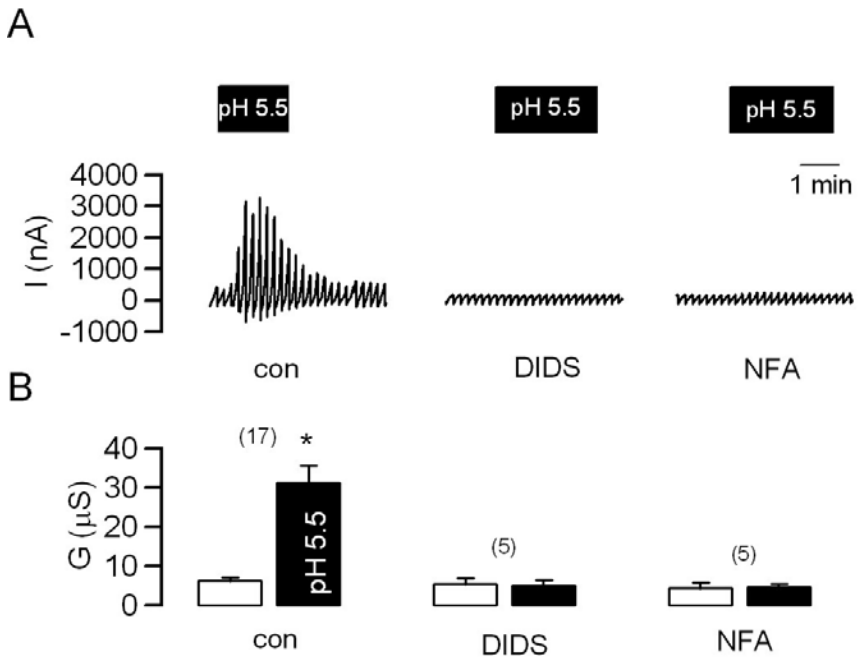

Figure 4. The H^+ activated current is blocked by inhibitors of CaCC. A) Original recording obtained from an oocyte expressing wtCFTR and exposed to extracellular pH 5.5 in the absence or presence of DIDS (100 µM) or NFA (100 µM). B) Summary of whole cell conductances obtained from wtCFTR expressing oocytes exposed to extracellular pH 5.5 in the absence or presence of DIDS (100 µM) or NFA (100 µM). Data represent mean ± SEM, (n) = number of experiments. * indicates significant difference when compared to control (paired t-test, $p \leq 0.05$).

LPA induced CaCC was augmented in CFTR expressing oocytes, suggesting that the presence of CFTR establishes a membrane localized signalling complex that augments Ca^{2+} signalling and activation of CaCC. When LPA was applied in the presence of extracellular acidic pH, the total current that was activated by LPA was larger, suggesting additive effects of CFTR on LPA and H^+ induced signal transduction and activation of CaCC. We therefore postulate that CFTR installs a H^+ sensitive mechanism at the plasma membrane by translocating a H^+ receptor to the membrane or by being itself H^+ sensitive. Exposure to H^+ increases intracellular Ca^{2+} and activates CaCC.

DISCUSSION

Extracellular acidic pH activates CaCC in CFTR expressing oocytes: We reported in the present study that expression of CFTR confers proton sensitivity to *Xenopus* oocytes. The data indicate that extracellular H^+/acidification activates the Ca^{2+} activated Cl^- conductance that is expressed

endogenously in *Xenopus* oocytes, but only when functional CFTR is present. Activation of CaCC is independent of stimulation of CFTR but is affected by CFTR-mutations. Notably, the most common CFTR mutation ΔF508-CFTR, which poorly traffics to the cell membrane in mammalian cells, but which is well expressed in *Xenopus* oocytes, confers an even enhanced H^+ sensitivity when compared to wtCFTR[4,165]. Once ΔF508-CFTR escapes from degradation and continues its way from the endoplasmic reticulum to the plasma membrane, the protein is actually able to form functional Cl^- channels with reduced open probability[166]. Due to the low temperature at which oocytes exist, maturation of ΔF508-CFTR is improved and ΔF508-CFTR-induced Cl^- currents are generally much higher than in mammalian cells[167,168].

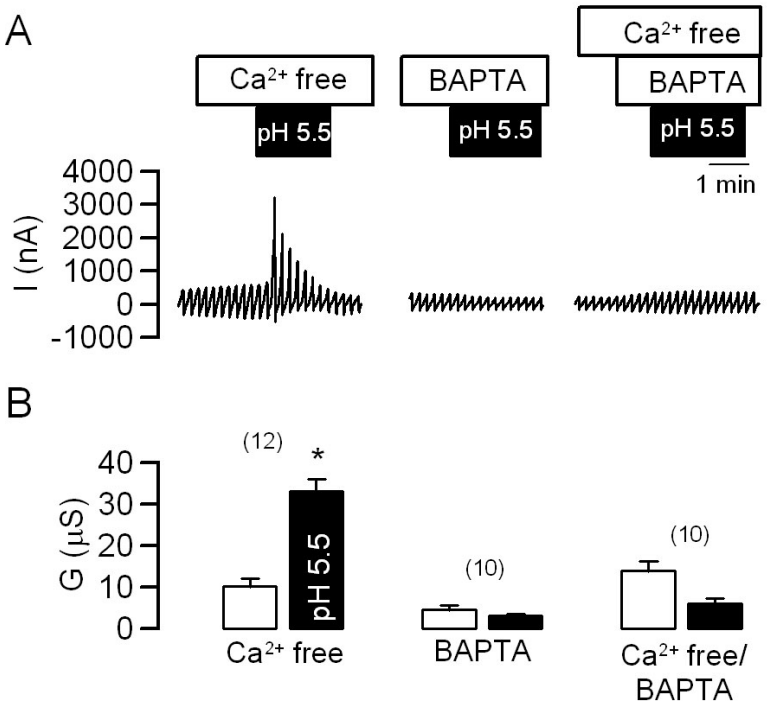

Figure 5. H^+ activation of CaCC requires intracellular Ca^{2+}. A) Original recording of a wtCFTR expressing oocyte exposed to extracellular pH 5.5 in the presence of a Ca^{2+} free bath solution, after incubation with BAPTA (50 µM) or both. B) Summary of whole cell conductances obtained from CFTR-expressing oocytes exposed to extracellular pH 5.5 in the presence of a Ca^{2+} free bath solution, after incubation with BAPTA (50 µM) or both. Data represent mean ± SEM, (n) = number of experiments. * indicates significant difference when compared to control (paired t-test, $p \leq 0.05$).

However, the surprising finding was that ΔF508-CFTR produced an even larger Ca^{2+} activated Cl^- current than wtCFTR, although expression of wtCFTR is expected to be more efficient than for ΔF508-CFTR. A possible answer to this question may give our recent study showing that ER-trapped ΔF508-CFTR can operate as a counter ion channel for Cl^- that facilitates release of Ca^{2+} from the endoplasmic reticulum similar to Bestrophin 1[169]. The present results from G551D-CFTR expressing oocytes indicate suggest that the ability of CFTR to conduct Cl^- is important to allow activation of CaCC by extracellular protons, although CFTR currents do not need to be activated to allow activation of CaCC. G551D-CFTR is a CFTR mutant which is properly expressed in cell membranes but which does not open upon stimulation with cAMP/PKA[170].

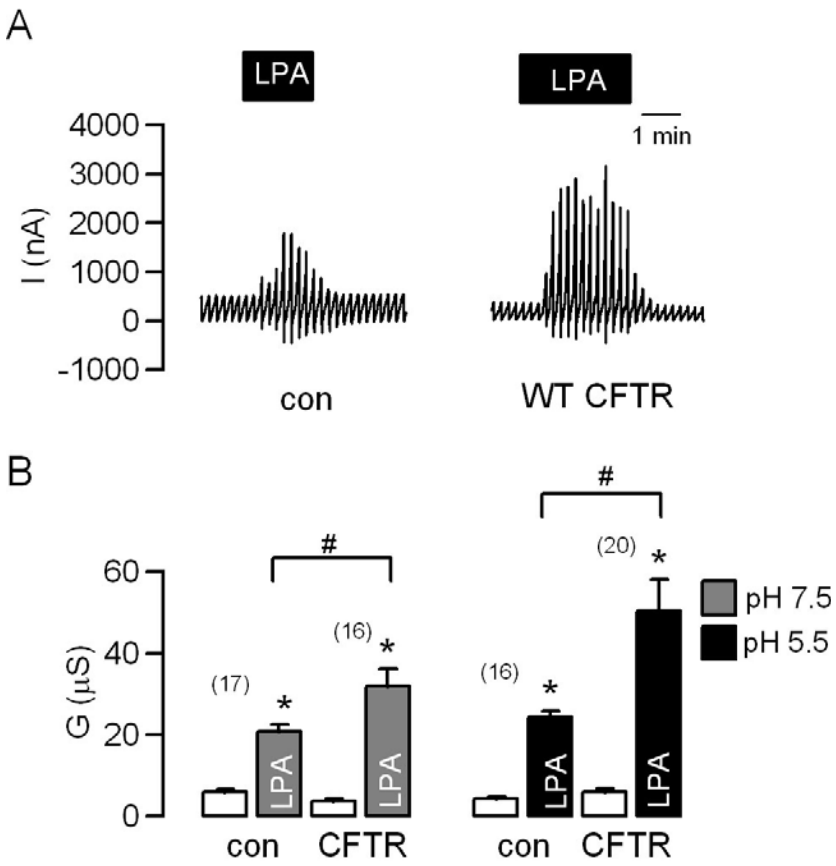

Figure 6. CFTR augments LPA-activation of CaCC. A) Original recordings of a control oocyte and a wtCFTR expressing oocyte exposed to lysophosphatidic acid (LPA, 50 µM). B) Summary of whole cell conductances obtained from control oocytes and from CFTR-expressing oocytes exposed to LPA in the presence of extracellular pH 7.5 or pH 5.5 Data represent mean ± SEM, (n) = number of experiments. * indicates significant difference when compared to control (paired t-test, $p \leq 0.05$). # indicates significant difference between the different groups (unpaired t-test, $p \leq 0.05$).

Membrane-translocation of a H^+ receptor, facilitating H^+ induced signalling, or CFTR as a H^+ receptor? The current data do not provide a definite answer as to how H^+ sensing is induced by CFTR. However, it is well known that CFTR translocates protein complexes to the apical membrane of epithelial cells by forming functional microdomains[154,171]. A spatiotemporal coupling of cAMP transporter to CFTR chloride channel function in gut epithelia has been demonstrated earlier[132]. Thus macromsolecular complexes consisting of receptors, CFTR and scaffolding proteins form functional units, coupling ß2-adrenergic and LPA receptors to CFTR[129,131]. It is also well esthablished that signaling pathways exist in Xenopus oocytes between LPA receptors, sphingosine-1-phosphate and phospholipase[164]. As suggested from the present experiments, CFTR may translocate LPA receptors to the cell membrane thus enhancing LPA-induced Cl^- currents (Figure 6). CFTR may also simply enhance coupling between LPA- and putative H^+-receptors and intracellular signalling molecules. However, since the ability of CFTR to produce H^+ sensitivity was depending on its ability to generate a Cl^- current, CFTR itself could also serve as H^+ receptor.

Possible physiological and clinical role of CFTR-dependent proton sensitivity: The results described in the present study would provide a mechanism how Cl^- secretion by Ca^{2+} activated Cl^- currents is coupled to extracellular pH, a process that may be of significant relevance in tissues such as airways, pancreas or duodenum. We may speculate that the ability of CFTR to confer proton sensitivity may be important for proper function of ameloblasts, odontoblasts and bone cells[172]. Abnormal bone formation, osteopenia and osteoporosis are well known problems in cystic fibrosis[173-176]. It is also known for long that both the functions of osteoblasts and osteoclasts is regulated by extracellular pH[177,178]. Subsequent studies should therefore elaborate the role of CFTR for pH sensing in bone cells and its possible role for abnormal bone formation in cystic fibrosis.

ACKNOWLEDGEMENTS

The continuous support by DFG SFB699A7 and TargetScreen2 (EU-FP6-2005-LH-037365) is gratefully acknowledged.

CHAPTER 6.

Summary

Cystic Fibrosis Transmembrane conductance Regulator (CFTR) is a cAMP dependent Cl^- channel that is expressed mainly on the apical membrane of epithelial cells. CFTR plays an essential role in electrolyte and water homeostasis. Defects in CFTR cause CF disease, which is the most common lethal genetic disorder among Caucasians. Besides functioning as a Cl^- channel, CFTR also acts as a regulator of other channels and transporters. Although CFTR has been known for more than 20 years, many questions regarding CFTR regulations and functions remain to be answered. In the present thesis, we performed various experiments in an attempt to understand CFTR in several aspects, including CFTR-regulation by AMPK *in vitro* and *in vivo*, the role of the S573C CFTR mutation for the risk to develop pancreatitis, and a novel role of CFTR in proton sensing and regulating CaCC.

Regulation of CFTR by AMPK *in vivo*

The present study has clarified the role of AMPK for epithelial Cl^- transport *in vivo* using AMPKα1-/- mice. The data demonstrate a significant role of AMPK in regulating CFTR. In real short circuit Ussing chamber experiment, an increase in CFTR Cl^- current is observed in the colon of AMPKα1-/- mice when exposed to cAMP. Unlike wild type littermates, the elevated cAMP-dependent Cl^- current in the AMPKα1-/- colon is insensitive to the AMPK activator phenformin. Furthermore, rectal potential difference (RPD) measurements indicate that AMPKα1-/- mice have an increased RPD both non-stimulated and after exposure to cAMP. These data suggested enhanced activity of CFTR in the large intestine of AMPKα1-/- mice. In correlation to previous *in vitro* observations, data from the present study demonstrate that AMPK inhibits CFTR *in vivo*.

Mechanistic insight into control of CFTR by AMPK

To identify the AMPK phosphorylation sites in CFTR and to find the mechanisms underlying AMPK regulation of CFTR, *Xenopus* oocytes were used to overexpress different CFTR mutations, and the whole cell currents of the overexpressed oocytes were measured using the double electrode voltage clamp (DEVC) technique. In contrast to previous observations, this study demonstrates that S737 and S768 are phosphorylated by AMPK rather than PKA. In *Xenopus* oocytes, single or multiple mutations of S737 and S768 to alanine increase CFTR whole cell currents around 4-fold. Moreover, in non-activated cells, S737A and S768A increase the basal CFTR Cl^- conductance around 5-fold. The elevated conductance is insensitive to the AMPK inhibitor phenformin and the AMPK activator compound C. Taken together, we demonstrate that AMPK inhibits PKA activation of CFTR. Moreover, AMPK indeed constitutively phosphorylates CFTR and keeps the channel shut at basal levels. We associated this finding to a spatiotemporal regulation of CFTR by cAMP. Data from the present study suggest a constitutive inhibition of CFTR by a tonic baseline AMPK activity. Because AMPK can also be activated without any detectable changes in the global AMP/ATP ratio, it is likely that AMPK regulation of CFTR is also controlled locally. The scenario can perhaps be described as AMP being generated by a Shank2/PDE complex bound to CFTR's PDZ domain. PDE degrades cAMP to AMP, increasing the AMP/ATP ratio close to CFTR, and thus activating AMPK. The activated AMPK therefore inhibits CFTR and keeps the channel close under non-stimulated conditions.

Metformin treatment of diabetes mellitus increases the risk of pancreatitis in patients bearing the CFTR-mutation S573C

Metformin is an AMPK activator and is a drug used for the treatment of diabetes type II. Lactic acidosis is a common secondary complication of metformin therapy, especially in patients with renal dysfunction, alcohol abuse, or liver disease. Patients with renal failure have been reported to develop the pancreatitis after metformin therapy. We examined the effects of metformin treatment on a pancreatitis-related S573C-CFTR mutation. The results demonstrate a slight but significant reduction of S573C-CFTR whole cell currents that are further inhibited by metformin. On the other hand, metformin treatment of wtCFTR does not affect whole cell current induced by cAMP. These data suggest that the S573C mutation increases the sensitivity towards AMPK, However *in vitro* phosphorylation experiments reveal the same pattern of AMPK phosphorylation

in wt and S573A-CFTR. Because lactic acidosis has been found during metformin therapy, and because CFTR is essential to control the pH balance in the pancreas, we challenged wt and S573C CFTR expressing oocytes by exposing them to acidic pH. The results demonstrate that intracellular acidificaiton reduces activation of wtCFTR, but almost completely abolishes Cl^- currents produced by S573C-CFTR.

Taken together these data imply that patients carrying the S573C mutation have only a slight defect in CFTR Cl^- currents. However, under metformin treatment, the Cl^- transport in patients carrying the S573C mutation is largely reduced. Thus S573C-carrier have a higher risk in developing a pancreatitis after metformin therapy.

CFTR induces acid sensing and H^+ activated Cl^- transport

In the last section of the present thesis we identified a novel role of CFTR for detection of the extracellular proton concentration and regulation of the Ca^{2+} activated Cl^- current. In wtCFTR expressing *Xenopus* oocytes, we observed a Ca^{2+} activated Cl^- current after exposure of the oocytes to extracellular acidic pH. This current was strongly outwardly rectifying, sensitive to DIDS and NPPB, and required intracellular Ca^{2+} for the activation. We hypothesize that CFTR translocate H^+-receptors to the plasma membrane and extracellular protons binding to these receptors induces an increase in intracellular [Ca^{2+}]. We speculate that CFTR-dependent H^+-sensing may be essential for bone metabolism, since CFTR is expressed in bone cells and because CF-patients suffer from osteoporosis.

REFERENCES

1. Marchiando, A.M., Graham, W.V. & Turner, J.R. Epithelial barriers in homeostasis and disease. *Annual Review of Pathology* 5, 119-44(2010).

2. Venkatasubramanian, J., Ao, M. & Rao, M.C. Ion transport in the small intestine. *Current Opinion in Gastroenterology* 26, 123-8(2010).

3. Kunzelmann, K. & Mall, M. Electrolyte transport in the mammalian colon: mechanisms and implications for disease. *Physiological Reviews* 82, 245-89(2002).

4. Amaral, M.D. & Kunzelmann, K. Molecular targeting of CFTR as a therapeutic approach to cystic fibrosis. *Trends in Pharmacological Sciences* 28, 334-41(2007).

5. Messick, J. A 21st-century approach to cystic fibrosis: optimizing outcomes across the disease spectrum. *Journal of Pediatric Gastroenterology and Nutrition* 51 Suppl 7, S1-7; quiz 3 p following S7(2010).

6. Quinton, P.M. Role of epithelial HCO_3^- transport in mucin secretion: lessons from cystic fibrosis. *American Journal of Physiology. Cell Physiology* 299, C1222-33(2010).

7. Strausbaugh, S.D. & Davis, P.B. Cystic fibrosis: a review of epidemiology and pathobiology. *Clinics in Chest Medicine* 28, 279-88(2007).

8. Choi, J.Y. *et al*. Cl^--dependent HCO_3^- transport by cystic fibrosis transmembrane conductance regulator. *Journal Of the Pancreas* 2, 243-6(2001).

9. Gray, M. a *et al*. Functional interactions of HCO_3^- with cystic fibrosis transmembrane conductance regulator. *Journal Of the Pancreas* 2, 207-11(2001).

10. Steward, M.C., Ishiguro, H. & Case, R.M. Mechanisms of bicarbonate secretion in the pancreatic duct. *Annual Review of Physiology* 67, 377-409(2005).

11. Krysa, J. & Steger, A. Pancreas and cystic fibrosis: the implications of increased survival in cystic fibrosis. *Pancreatology* 7, 447-50(2007).

12. Amaral, M.D. CFTR and chaperones, processing and degradation. *Journal of Molecular Neuroscience* 23, 41-48(2004).

13. Riordan, J.R. *et al*. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245, 1066-73(1989).

14. Ko, Y.H. & Pedersen, P.L. The first nucleotide binding fold of the cystic fibrosis regulator can function as an active ATPase. *Biochemistry* 22093-22096(1995).

15. Li, C. *et al*. ATPase activity of the cystic fibrosis transmembrane conductance regulator. *The Journal of Biological Chemistry* 271, 28463-8(1996).

16. Pasyk, E. a & Foskett, J.K. Cystic fibrosis transmembrane conductance regulator-associated ATP and adenosine 3’-phosphate 5'-phosphosulfate channels in endoplasmic reticulum and plasma membranes. *The Journal of Biological Chemistry* 272, 7746-51(1997).

17. Aleksandrov, A. a, Aleksandrov, L. a & Riordan, J.R. CFTR (ABCC7) is a hydrolyzable-ligand-gated channel. *Pflügers Archiv : European Journal of Physiology* 453, 693-702(2007).

18. Carson, M.R., Travis, S.M. & Welsh, M.J. The two nucleotide-binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) have distinct functions in controlling channel activity. *The Journal of Biological Chemistry* 270, 1711-1717(1995).

19. Gunderson, K.L. & Kopito, R.R. Conformational states of CFTR associated with channel gating: the role ATP binding and hydrolysis. *Cell* 82, 231-9(1995).

20. Gadsby, D.C. & Nairn, A.C. Regulation of CFTR channel gating. *Trends in Biochemistry Science* 19, 513-518(1994).

21. Vergani, P. *et al*. CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains. *Nature* 433, 876-80(2005).

22. Basso, C. *et al*. Prolonged nonhydrolytic interaction of nucleotide with CFTR’s NH2-terminal nucleotide binding domain and its role in channel gating. *The Journal of General Physiology* 122, 333-48(2003).

23. Aleksandrov, L. *et al*. The first nucleotide binding domain of cystic fibrosis transmembrane conductance regulator is a site of stable nucleotide interaction, whereas the second is a site of rapid turnover. *The Journal of Biological Chemistry* 277, 15419-25(2002).

24. Berger, A.L., Ikuma, M. & Welsh, M.J. Normal gating of CFTR requires ATP binding to both nucleotide-binding domains and hydrolysis at the second nucleotide-binding domain. *Proceedings of the National Academy of Sciences of the United States of America* 102, 455-60(2005).

25. Fischer, H. & Machen, T.E. The tyrosine kinase p60c-src regulates the fast gate of the cystic fibrosis transmembrane conductance regulator chloride channel. *Biophysical Journal* 71, 3073-82(1996).

26. Reddy, M.M. & Quinton, P.M. cAMP-independent phosphorylation activation of CFTR by G proteins in native human sweat duct cAMP-independent phosphorylation activation of CFTR by G proteins in native human sweat duct. *American Journal of Physiology. Cell Physiology* 280, 604-613(2001).

27. Golin-Bisello, F., Bradbury, N. & Ameen, N. STa and cGMP stimulate CFTR translocation to the surface of villus enterocytes in rat jejunum and is regulated by protein kinase G. *American Journal of Physiology. Cell Physiology* 289, C708-16(2005).

28. Picciotto, M.R. *et al*. Phosphorylation of the cystic fibrosis transmembrane conductance regulator. *The Journal of Biological Chemistry* 267, 12742-12752(1992).

29. Jia, Y., Mathews, C.J. & Hanrahan, J.W. Phosphorylation by protein kinase C is required for acute activation of cystic fibrosis transmembrane conductance regulator by protein kinase A. *The Journal of Biological Chemistry* 272, 4978-84(1997).

30. Townsend, R.R. *et al*. Identification of protein kinase A phosphoryation sites on NBD1 and R domains of CFTR using electrospray mass spectrometry with selective phosphate ion monitoring. *Cardiovascular Research* 1865- 1873(1996).

31. Cheng, S.H. *et al*. Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell* 66, 1027-36(1991).

32. Winter, M.C. *et al*. Effect of ATP concentration on CFTR Cl- channels: a kinetic analysis of channel regulation. *Biophysical Journal* 66, 1398-403(1994).

33. Winter, M.C. & Welsh, M.J. letters to nature Stimulation of CFTR activity by its phosphorylated R domain. *Nature* 389, 18-20(1997).

34. Hallows, K.R. *et al*. Physiological modulation of CFTR activity by AMP-activated protein kinase in polarized T84 cells. *American Journal of Physiology. Cell physiology* 284, C1297-308(2003).

35. Hallows, K.R. *et al*. Regulation of channel gating by AMP-activated protein kinase modulates cystic fibrosis transmembrane conductance regulator activity in lung submucosal cells. *The Journal of Biological Chemistry* 278, 998-1004(2003).

36. Hallows, K.R. *et al*. Up-regulation of AMP-activated kinase by dysfunctional cystic fibrosis transmembrane conductance regulator in cystic fibrosis airway epithelial cells mitigates excessive inflammation. *The Journal of Biological Chemistry* 281, 4231-41(2006).

37. King, J.D. *et al*. AMP-activated protein kinase phosphorylation of the R domain inhibits PKA stimulation of CFTR. *American Journal of Physiology. Cell Physiology* 297, C94-101(2009).

38. Hallows, K.R. *et al*. Inhibition of cystic fibrosis transmembrane conductance regulator by novel interaction with the metabolic sensor AMP-activated protein kinase. *The Journal of Clinical Investigation* 105, 1711-21(2000).

39. Hardie, D.G., Hawley, S. a & Scott, J.W. AMP-activated protein kinase--development of the energy sensor concept. *The Journal of Physiology* 574, 7-15(2006).

40. Mehta, A. The cystic fibrosis transmembrane recruiter the alter ego of CFTR as a multi-kinase anchor. *Pflugers Archiv: European Journal of Physiology* 455, 215-221(2007).

41. Hawley, S. a *et al*. The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* 51, 2420-5(2002).

42. Zhou, G. *et al*. Role of AMP-activated protein kinase in mechanism of metformin action. *Commentary* 108, 1167-1174(2001).

43. Mallick, S. Metformin induced acute pancreatitis precipitated by renal failure. *Postgraduate Medical Journal* 80, 239-240(2004).

44. Fimognari, F.L. *et al*. Metformin-induced pancreatitis: a possible adverse drug effect during acute renal failure. *Diabetes Care* 29, 1183(2006).

45. Sheppard, D.N. & Welsh, M.J. Structure and function of the CFTR chloride channel. *Physiological Reviews* 79, S23-45(1999).

46. Dahan, D. *et al*. Regulation of the CFTR channel by phosphorylation. *Pflügers Archiv : European Journal of Physiology* 443 Suppl, S92-6(2001).

47. Ostedgaard, L.S., Baldursson, O. & Welsh, M.J. Regulation of the cystic fibrosis transmembrane conductance regulator Cl^- channel by its R domain. *The Journal of Biological Chemistry* 276, 7689-92(2001).

48. Rich, D.P. *et al*. Effect of deleting the R domain on CFTR-generated chloride channels. *Science* 253, 205-7(1991).

49. Ma, J. *et al*. Function of the R domain in the cystic fibrosis transmembrane conductance regulator chloride channel. *The Journal of Biological Chemistry* 272, 28133-41(1997).

50. Hoshi, T., Zagotra, W.N. & Aldrich, R.W. Shaker potassium channel inactivation. *Science* 250, 533-538(1990).

51. Csanády, L. *et al*. Severed channels probe regulation of gating of cystic fibrosis transmembrane conductance regulator by its cytoplasmic domains. *The Journal of General Physiology* 116, 477-500(2000).

52. Chang, X.B. *et al*. Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *The Journal of Biological Chemistry* 268, 11304-11(1993).

53. Wilkinson, D.J. *et al*. CFTR activation: additive effects of stimulatory and inhibitory phosphorylation sites in the R domain. *The American Journal of Physiology* 273, L127-33(1997).

54. Seiber, F.S. *et al*. cAMP-dependent protein kinase-mediated phosphorylation of cystic fibrosis transmembrane conductance regulator residue Ser-753 and Its role in channel activation. *The Journal of Biological Chemistry* 270, 2158-2162(1995).

55. Csanády, L. *et al*. Preferential phosphorylation of R-domain Serine 768 dampens activation of CFTR channels by PKA. *The Journal of General Physiology* 125, 171-86(2005).

56. Li, H. *et al*. The cystic fibrosis transmembrane conductance regulator Cl^- channel: a versatile engine for transepithelial ion transport. *Sheng li xue bao : [Acta Physiologica Sinica]* 59, 416-430(2007).

57. Hegedus, T. *et al*. Role of individual R domain phosphorylation sites in CFTR regulation by protein kinase A. *Biochimica et Biophysica Acta* 1788, 1341-9(2009).

58. Kunzelmann, K. CFTR : Interacting with everything *? News Physiology Science* 16, 167-170(2001).

59. Li, C. & Naren, A.P. Macromolecular complexes of cystic fibrosis transmembrane conductance regulator and its interacting partners. *Pharmacology & Therapeutics* 108, 208-23(2005).

60. Li, C. & Naren, A.P. Macromolecular complexes of cystic fibrosis transmembrane conductance regulator and its interacting partners. Pharmacology & Therapeutics 108, 208-23(2005).

61. Jarnaess, E. & Taskén, K. Spatiotemporal control of cAMP signalling processes by anchored signalling complexes. *Biochemical Society Transactions* 35, 931-7(2007).

62. Lee, J.H. *et al*. Dynamic regulation of cystic fibrosis transmembrane conductance regulator by competitive interactions of molecular adaptors. *The Journal of Biological Chemistry* 282, 10414-22(2007).

63. Bachhuber, T. *et al*. Cl^- interference with the epithelial Na^+ channel ENaC. *The Journal of Biological Chemistry* 280, 31587-94(2005).

64. Kunzelmann, K., Schreiber, R. & Boucherot, a Mechanisms of the inhibition of epithelial Na^+ channels by CFTR and purinergic stimulation. *Kidney International* 60, 455-61(2001).

65. Ho, K. The ROMK-cystic fibrosis transmembrane conductance regulator connection: new insights into the relationship between ROMK and cystic fibrosis transmembrane comductance regulator channels. *Current Opinion in Nephrology and Hypertension* 7, 49-58(1998).

66. Wang, W. Regulation of the ROMK channel: interaction of the ROMK with associated proteins. *American Journal of Physiology. Renal Physiology* 277, F826-F831(1999).

67. Kim, D. & Steward, M.C. The role of CFTR in bicarbonate secretion by pancreatic duct and airway epithelia. *The Journal of Medical Investigation* 56 Suppl, 336-42(2009).

68. Steward, M.C. & Ishiguro, H. Molecular and cellular regulation of pancreatic duct cell function. *Current Opinion in Gastroenterology* 25, 447-53(2009).

69. Song, Y. *et al*. Effects of Slc26a6 deletion and CFTR inhibition on HCO_3^- secretion by mouse pancreatic duct. *The Journal of Medical Investigation* 56 Suppl, 332-5(2009).

70. Levin, M.H. & Verkman, a S. Aquaporins and CFTR in ocular epithelial fluid transport. *The Journal of Membrane Biology* 210, 105-15(2006).

71. Guggino, W.B. Outwardly rectifying chloride channels and CF: a divorce and remarriage. *Journal of Bioenergetics and Biomembranes* 25, 27-35(1993).

72. Hryciw, D.H. & Guggino, W.B. Cystic fibrosis transmembrane conductance regulator and the outwardly rectifying chloride channel: A relationship between two chloride channels expressed in epithelial cells. *Clinical and Experimental Pharmacology and Physiology* 892-895(2000).

73. Kunzelmann, K. *et al*. The cystic fibrosis transmembrane conductance regulator attenuates the endogenous Ca^{2+} activated Cl^- conductance of Xenopus oocytes. *Pflügers Archiv : European Journal of Physiology* 435, 178-81(1997).

74. Wei, L. *et al*. The C-terminal part of the R-domain, but not the PDZ binding motif, of CFTR is involved in interaction with Ca^{2+} -activated Cl^- channels. *Pflügers Archiv : European Journal of Physiology* 442, 280-285(2001).

75. Eggermont, J. Calcium-activated chloride channels: (un)known, (un)loved? *Proceedings of the American Thoracic Society* 1, 22-7(2004).

76. Hartzell, H.C. *et al*. Anoctamin/TMEM16 family members are Ca^{2+}-activated Cl^- channels. *The Journal of Physiology* 587, 2127-39(2009).

77. Yang, Y.D. *et al*. TMEM16A confers receptor-activated calcium-dependent chloride conductance. *Nature* 455, 1210-5(2008).

78. Schroeder, B.C. *et al*. Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. *Cell* 134, 1019-29(2008).

79. Caputo, A. *et al*. TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. *Science* 322, 590-4(2008).

80. Rock, J.R., Futtner, C.R. & Harfe, B.D. The transmembrane protein TMEM16A is required for normal development of the murine trachea. *Developmental Biology* 321, 141-9(2008).

81. Ousingsawat, J. *et al*. Loss of TMEM16A causes a defect in epithelial Ca^{2+}-dependent chloride transport. *The Journal of Biological Chemistry* 284, 28698-703(2009).

82. West, R.B. *et al*. The novel marker, DOG1, is expressed ubiquitously in gastrointestinal stromal tumors irrespective of KIT or PDGFRA mutation status. *The American Journal of Pathology* 165, 107-13(2004).

83. Mall, M. *et al*. CFTR-mediated inhibition of epithelial Na^+ conductance in human colon is defective in cystic fibrosis. *The American Journal of Physiology* 277, G709-16(1999).

84. Grubb, B.R., Vick, R.N. & Boucher, R.C. Hyperabsorption of Na^+ and raised Ca^{2+}-mediated Cl^- secretion in nasal epithelia of CF mice. *The American Journal of Physiology* 266, C1478-83(1994).

85. Ribeiro, C.M.P. *et al*. Chronic airway infection/inflammation induces a Ca^{2+}_i-dependent hyperinflammatory response in human cystic fibrosis airway epithelia. *The Journal of Biological Chemistry* 280, 17798-806(2005).

86. Tarran, R. *et al*. Normal and cystic fibrosis airway aurface liquid homeostasis. The effects of phasic shear stress and viral infections. *The Journal of Biological Chemistry of Biological Chemistry* 280, 35751-35759(2005).

87. Ribeiro, C.M.P. *et al*. Cystic fibrosis airway epithelial $Ca^{2+}{}_i$ signaling: the mechanism for the larger agonist-mediated $Ca^{2+}{}_i$ signals in human cystic fibrosis airway epithelia. *The Journal of Biological Chemistry* 280, 10202-9(2005).

88. Keiles, S. & Kammesheidt, A. Identification of CFTR, PRSS1, and SPINK1 mutations in 381 pateints with pancreatitis. *Pancreas* 33, 221-227(2006).

89. Schrijver, I. *et al*. Diagnostic testing by CFTR gene mutation analysis in a large group of Hispanics: novel mutations and assessment of a population-specific mutation spectrum. *The Journal of Molecular Diagnostics* 7, 289-99(2005).

90. Riordan, J.R. Assembly of functional CFTR chloride channels. *Annual Review of Physiology* 67, 701-18(2005).

91. Gadsby, D.C., Vergani, P. & Csanády, L. The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature* 440, 477-83(2006).

92. Mense, M. *et al*. In vivo phosphorylation of CFTR promotes formation of a nucleotide-binding domain heterodimer. *The EMBO journal* 25, 4728-39(2006).

93. Serohijos, A.W.R. *et al*. Phenylalanine-508 mediates a cytoplasmic-membrane domain contact in the CFTR 3D structure crucial to assembly and channel function. *Proceedings of the National Academy of Sciences of the United States of America* 105, 3256-61(2008).

94. Motoshima, H. *et al*. AMPK and cell proliferation--AMPK as a therapeutic target for atherosclerosis and cancer. *The Journal of Physiology* 574, 63-71(2006).

95. Hardie, D.G. The AMP-activated protein kinase pathway--new players upstream and downstream. *Journal of Cell Science* 117, 5479-87(2004).

96. Hallows, K.R. Emerging role of AMP-activated protein kinase in coupling membrane transport to cellular metabolism. *Current Opinion in Nephrology and Hypertension* 14, 464-71(2005).

97. Carattino, M.D. *et al*. Epithelial sodium channel inhibition by AMP-activated protein kinase in oocytes and polarized renal epithelial cells. *The Journal of Biological Chemistry* 280, 17608-16(2005).

98. Jørgensen, S.B. *et al*. Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *The Journal of Biological Chemistry* 279, 1070-9(2004).

99. Jørgensen, S.B. *et al*. Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. *The FASEB Journal :* 19, 1146-8(2005).

100. Jensen, T.E. *et al*. AMPK alpha1 activation is required for stimulation of glucose uptake by twitch contraction, but not by H_2O_2, in mouse skeletal muscle. *PloS One* 3, e2102(2008).

101. Viollet, B. *et al*. Physiological role of AMP-activated protein kinase (AMPK): insights from knockout mouse models. *Biochemical Society Transactions* 31, 216-9(2003).

102. Lazo, Y. *et al*. Renal phenotype in AMPK alpha-1 for physiologic asciences. *Acta Physiologica* 190, Suppl 655; P96(2007).

103. Walker, J. *et al*. Activation of AMP-activated protein kinase reduces cAMP-mediated epithelial chloride secretion. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 285, G850-60(2003).

104. Grubb, B.R. *et al*. Ion transport across the normal and CF neonatal murine intestine Ion transport across the normal and CF neonatal murine intestine. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 277, G167-G174(1999).

105. Randak, C. *et al*. Regulates gating of the ABC transporter CFTR. *Cell* 115, 837-850(2003).

106. Grubb, B.R. & Boucher, R.C. Pathophysiology of gene-targeted mouse models for cystic fibrosis. *Physiological Reviews* 79, S193-214(1999).

107. Woollhead, a M. *et al*. Pharmacological activators of AMP-activated protein kinase have different effects on Na+ transport processes across human lung epithelial cells. *British Journal of Pharmacology* 151, 1204-15(2007).

108. Sausbier, M. *et al*. Distal colonic K^+ secretion occurs via BK channels. *Journal of the American Society of Nephrology* 17, 1275-82(2006).

109. Mayer, A. *et al*. AMP-activated protein kinase regulates lymphocyte responses to metabolic stress but is largely dispensable for immune cell development and function. *European Journal of Immunology* 38, 948-56(2008).

110. Harris, S.I. *et al*. Mitochondrial respiratory capacity and Na^+- and K^+-dependent adenosine triphosphatase-mediated ion transport in the intact renal cell. *The Journal of Biological Chemistry* 256, 10319-28(1981).

111. Kongsuphol, P. *et al*. Mechanistic insight into control of CFTR by AMPK. *The Journal of Biological Chemistry* 284, 5645-53(2009).

112. Guerra, L. *et al*. Na^+/H^+ exchanger regulatory factor isoform 1 overexpression modulates cystic fibrosis transmembrane conductance regulator (CFTR) expression and activity in human airway 16HBE14O- cells and rescues deltaF508 CFTR functional expression in cystic fibrosis ce. *The Journal of Biological Chemistry* 280, 40925-33(2005).

113. Barnes, A.P. *et al*. Phosphodiesterase 4D forms a cAMP diffusion barrier at the apical membrane of the airway epithelium. *The Journal of Biological Chemistry* 280, 7997-8003(2005).

114. Treharne, K.J. *et al*. Nucleoside diphosphate kinase--a component of the [Na^+]- and [Cl^-]-sensitive phosphorylation cascade in human and murine airway epithelium. *Pflügers Archiv : European Journal of Physiology* 443 Suppl , S97-102(2001).

115. Corton, J.M. *et al*. A specific method for activating AMP-activated protein kinase in intact cells ? *European Journal of Biochemistry* 565, 558-565(1995).

116. Gadsby, D.C. & Nairn, a C. Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. *Physiological Reviews* 79, S77-S107(1999).

117. Sun, F. *et al*. E3KARP mediates the association of ezrin and protein kinase A with the cystic fibrosis transmembrane conductance regulator in airway cells. *The Journal of Biological Chemistry* 275, 29539-46(2000).

118. Guo, X. *et al*. Membrane biology pharmacological evidence that calcium is not required for P2 -receptor-stimulated Cl^- -secretion in HT29-Cl.16E. *Journal of Membrane Biology* 246, 239-246(1997).

119. McAlroy, H.L. *et al*. Multiple P2Y receptor subtypes in the apical membranes of polarized epithelial cells. *British Journal of Pharmacology* 131, 1651-8(2000).

120. Hurley, R.L. *et al*. Regulation of AMP-activated protein kinase by multisite phosphorylation in response to agents that elevate cellular cAMP. *The Journal of Biological Chemistry* 281, 36662-72(2006).

121. Woollhead, A.M. *et al*. Phenformin and 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) activation of AMP-activated protein kinase inhibits transepithelial Na^+ transport across H441 lung cells. *The Journal of Physiology* 566, 781-92(2005).

122. Bhalla, V. *et al*. AMP-activated kinase inhibits the epithelial Na^+ channel through functional regulation of the ubiquitin ligase Nedd4-2. *The Journal of Biological Chemistry* 281, 26159-69(2006).

123. Fraser, S. *et al*. Regulation of the energy sensor AMP-activated protein kinase in the kidney by dietary salt intake and osmolality. *American Journal of Physiology. Renal physiology* 288, F578-86(2005).

124. Fraser, S. a *et al*. Regulation of the renal-specific Na^+-K^+-$2Cl^-$ co-transporter NKCC2 by AMP-activated protein kinase (AMPK). *The Biochemical Journal* 405, 85-93(2007).

125. Wine, J.J. Parasympathetic control of airway submucosal glands: central reflexes and the airway intrinsic nervous system. *Autonomic Neuroscience : Basic & Clinical* 133, 35-54(2007).

126. Quinton, P.M. Cystic fibrosis: lessons from the sweat gland. *Physiology* 22, 212-25(2007).

127. Cooper, D.M.F. Compartmentalization of adenylate cyclase and cAMP signalling. *Biochemical Society Transactions* 33, part 6, 1319-1322(2005).

128. Steinberg, S.F. & Brunton, L.L. Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. *Annual Review of Pharmacology and Toxicology* 41, 751-73(2001).

129. Naren, A.P. *et al*. A macromolecular complex of beta 2 adrenergic receptor, CFTR, and ezrin/radixin/moesin-binding phosphoprotein 50 is regulated by PKA. *Proceedings of the National Academy of Sciences of the United States of America* 100, 342-6(2003).

130. Huang, P. *et al*. Compartmentalized autocrine signaling to cystic fibrosis transmembrane conductance regulator at the apical membrane of airway epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 98, 14120-5(2001).

131. Li, C. *et al*. Lysophosphatidic acid inhibits cholera toxin-induced secretory diarrhea through CFTR-dependent protein interactions. *The Journal of Experimental Medicine* 202, 975-86(2005).

132. Li, C. *et al*. Spatiotemporal coupling of cAMP transporter to CFTR chloride channel function in the gut epithelia. *Cell* 131, 940-51(2007).

133. Sohma, Y. *et al*. 150 mM HCO_3^- - How does the pancreas do it ? Clues from computer modelling of the duct cell acini. *Journal Of the Pancreas* 2, 198-202(2001).

134. Choi, J.Y. *et al*. Aberrant CFTR-dependent HCO_3^- transport in mutations associated with ystic fibrosis. *Nature* 410, 94-97(2001).

135. Novak, I. Keeping up with bicarbonate. *The Journal of Physiology* 528, 235(2000).

136. Yang, D. *et al*. IRBIT coordinates epithelial fluid and HCO_3^- secretion by stimulating the transporters pNBC1 and CFTR in the murine pancreatic duct. *Journal of Clinical Investigation* 119, 193-202(2009).

137. Wang, Y. *et al*. Slc26a6 regulates CFTR activity in vivo to determine pancreatic duct HCO_3^- secretion: relevance to cystic fibrosis. *The EMBO Journal* 25, 5049-57(2006).

138. Rosenstein, B.J. & Cutting, G.R. The diagnosis of cystic fibrosis: a consensus statement. Cystic Fibrosis Foundation Consensus Panel. *The Journal of Pediatrics* 132, 589-95(1998).

139. Cohn, J. a, Mitchell, R.M. & Jowell, P.S. The role of cystic fibrosis gene mutations in determining susceptibility to chronic pancreatitis. *Gastroenterology* 33, 817-37, vii(2004).

140. Cohn, J.A. *et al*. Relation between mutat ions of the cysticfibrosis gene and idiopathic pancreatitis. *The New England Journal of Medicine* 339, 653-658(1998).

141. Chen, J.M. & Ferec, C. Molecular basis of hereditary pancreatitis. *European Journal of Human Genetics* 8, 473-9(2000).

142. Sharer, N. *et al*. Mutations of the cystic fibrosis gene in patients with chronic pancratitis. *The New England Journal of Medicine* 339, 645-652(1998).

143. Ren, C.L. Mutation of the cystic fibrosis gene and pancratitis. *The New England Journal of Medicine* 340, 238-239(1999).

144. Kongsuphol, P. *et al*. Regulation of Cl^- secretion by AMPK in vivo. *Pflügers Archiv : European Journal of Physiology* 457, 1071-8(2009).

145. Chen, J.-H., Cai, Z. & Sheppard, D.N. Direct sensing of intracellular pH by the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. *The Journal of Biological Chemistry* 284, 35495-506(2009).

146. Chase, H.J. & Mogan, G. Phenformin-associated pancreatitis. *Annual Review of Internation Medicine* 87, 314-5(1977).

147. Paterson, K., BJ, P. & Lawson, D. Undesired effects of biguanide therapy. *Adverse Drug Reaction and Acute Poinsoning Reviews* 3, 173-82(1984).

148. Hegyi, P. *et al*. Controversies in the role of SLC26 anion exchangers in pancratic ductal bicarbonate secretion. *Pancreas* 37, 232-233(2008).

149. Seidler, U. *et al*. A functional CFTR protein is required for mouse intestinal cAMP- , cGMP- and Ca^{2+} -dependent HCO_3^- secretion. *Physiology* 505, 411-423(1997).

150. Singh, A.K. *et al*. Differential roles of NHERF1 , NHERF2 , and PDZK1 in regulating CFTR-mediated intestinal anion secretion in mice. *The Journal of Clinical Investigation* 119, 540-50(2009).

151. O'Reilly, C.M. *et al*. Cystic fibrosis transmembrane conductance regulator currents in guinea pig panreatic duct cells: inhibition by bicarbonate ions. *Gastroenterology* 118, 1258-61(2000).

152. Bates, C.M., Baum, M. & Quigley, R. Cystic fibrosis presenting alkalosis in a previously with hypokalemia healthy adolescent and metabolic was prescribed. *Journal of American Society of Nephrology* 8, 352-355(1997).

153. Reddy, M.M. *et al*. Cytosolic pH regulates G Cl^- through control of phosphorylation states of CFTR Cytosolic pH regulates G Cl^- through control of phosphorylation states of CFTR. *American Journal of Physiology. Cell Physiology* 275, C1040-C1047(1998).

154. Kunzelmann, K., Schreiber, R. & Cook, D. Mechanisms for the inhibition of amiloride-sensitive Na^+ absorption by extracellular nucleotides in mouse trachea. *Pflügers Archiv : European Journal of Physiology* 444, 220-6(2002).

155. Mall, M. *et al*. Modulation of Ca^{2+}-activated Cl^- secretion by basolateral K^+ channels in human normal and cystic fibrosis airway epithelia. *Pediatric Research* 53, 608-18(2003).

156. Knowles, M.R., Clarke, L.L. & Boucher, R.C. Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. *The New England Journal of Medicine* 325, 533-538(1991).

157. Paradiso, a M., Ribeiro, C.M. & Boucher, R.C. Polarized signaling via purinoceptors in normal and cystic fibrosis airway epithelia. *The Journal of General Physiology* 117, 53-67(2001).

158. Wei, L. *et al*. Interaction between calcium-activated chloride channels and the cystic fibrosis transmembrane conductance regulator. *Pflügers Archiv : European Journal of Physiology* 438, 635-41(1999).

159. Kunzelmann, K. *et al*. Bestrophin and TMEM16-Ca^{2+} activated Cl^- channels with different functions. *Cell Calcium* 46, 233-41(2009).

160. Ferrera, L. *et al*. Regulation of TMEM16A chloride channel properties by alternative splicing. *The Journal of Biological Chemistry* 284, 33360-8(2009).

161. Schreiber, R. *et al*. Expression and function of epithelial anoctamins. *The Journal of Biological Chemistry* 285, 7838-45(2010).

162. Rock, J.R. *et al*. Transmembrane protein 16A (TMEM16A) is a Ca^{2+}-regulated Cl^- secretory channel in mouse airways. *The Journal of Biological Chemistry* 284, 14875-80(2009).

163. Anderson, M.P. & Welsh, M.J. Calcium and cAMP activate different chloride channels in the apical membrane of normal and cystic fibrosis epithelia. *Proceedings of the National Academy of Sciences of the United States of America* 88, 6003-7(1991).

164. Noh, S.J. *et al*. Different signaling pathway between sphingosine-1-phosphate and lysophosphatidic acid in Xenopus oocytes: functional coupling of the sphingosine-1-phosphate receptor to PLC-xbeta in Xenopus oocytes. *Journal of Cellular Physiology* 176, 412-23(1998).

165. Drumm, M.L. *et al*. Chloride conductance expressed by delta F508 and other mutant CFTRs in Xenopus oocytes. *Science* 254, 1797-9(1991).

166. Dalemans, W. *et al*. Altered chloride ion channel kinetics associated with the F508del cystic fibrosis mutation. *Nature* 354, 526-528(1991).

167. Denning, G.M. *et al*. Processing of mutant cystic fibrosis transmembrane conductance regulator is temerpature-sensitive. *Nature* 358, 761-764(1992).

168. Treharne, K.J. *et al*. Cellular physiology biochemistry and biochemistry inhibition of protein kinase CK2 closes the CFTR Cl^- channel, but has no effect on the cystic fibrosis mutant delta F508-CFTR. *Cellular Physiology and Biochemistry* 347-360(2009).

169. Aldehni, F. *et al*. ER localized and PAK2 phosphorylated bestrophin1 confers receptor activation of epithelial Ca^{2+} dependent ion channels. *Acta Physiologica* 195, Suppl 669:P472(2009).

170. Oceandy, D. *et al*. Gene complementation of airway epithelium in the cystic fibrosis mouse is necessary and sufficient to correct the pathogen clearance and inflammatory abnormalities. *Human Molecular Genetics* 11, 1059-67(2002).

171. Penmatsa, H. *et al*. Compartmentalized cyclic adenosine 3,5-monophosphate at the plasma membrane clusters PDE3A and cystic fibrosis transmembrane conductance regulator into microdomains. *Molecular Biology of the Cell* 21, 1097-1110(2010).

172. Bronckers, A. *et al*. The cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in maturation stage ameloblasts, odontoblasts and bone cells. *Bone* 46, 1188-96(2010).

173. Dif, F. *et al*. Severe osteopenia in CFTR-null mice. *Bone* 35, 595-603(2004).

174. Döring, G. & Conway, S.P. Osteoporosis in cystic fibrosis. *Jornal de Pediatria* 84, 18-25(2008).

175. Haston, C.K. *et al*. Persistent osteopenia in adult cystic fibrosis transmembrane conductance regulator-deficient mice. *American Journal of Respiratory and Critical Care Medicine* 177, 309-15(2008).

176. Pashuck, T.D. *et al*. Murine model for cystic fibrosis bone disease demonstrates osteopenia and sex-related differences in bone formation. *Pediatric Research* 65, 311-6(2009).

177. Henriksen, K. *et al*. Osteoclast activity and subtypes as a function of physiology and pathology--implications for future treatments of osteoporosis. *Endocrine Reviews* (2010).

178. Arnett, T. Regulation of bone cell by acid-base balance. *Proceedings of the Nutrition Society* 62, 511-20(2003).

179. Shumaker, H. *et al*. CFTR drives Na^{+} -nHCO_3^- cotransport in pancreatic duct cells : a basis for defective HCO_3^- secretion in CF. *American Journal of Physiology. Cell Physiology* 276, 16-25(1999).

ACKNOWLEDGEMENTS

For all the success of my thesis work, I would like to gratefully thank my supervisor Prof. Dr. Karl Kunzelmann for advising me throughout my Ph.D. course. I would also like to acknowledge PD. Dr. Rainer Schreiber who always gave me great suggestions.

I would like to express my deepest appreciation to all the members of the committees Prof. Dr. Ralph Witzgall, Prof. Dr. Richard Warth, Prof. Dr. Herbert Tschochner and Prof. Dr. Michael Thomm. The work on my thesis was continuously supported by SFB699 A6/A7, and TargetScreen2 (EU-FP6-2005-LH-037365).

I sincerely thank everybody in the lab Ji, Raquell, Diana, and Tina who have always been my lovely companies and who have always been there for me. Special thanks go to Brigitte, Tini, Julia, Patricia, and Agnes for their great assistances.

Finally, I am deeply thankful to my beloved family, especially to my mom who has always gave me love and sweet words. Special thanks to ‘M’ who has always been beside me and gave me all the love.

Patthara Kongsuphol

CURRICULUM VITAE

PERSONAL INFORMATION

Name	Miss Patthara Kongsuphol
Date of birth	March 24th, 1981
Nationality	Thai
Current address	Ludwig-Thomastrasse 43, Regensburg, 93051, Germany
Email	pattharak@gmail.com

EDUCATIONS

2007-present	Ph.D., Faculty of Natural Sciences III: Biology and Preclinical medicine, University of Regensburg, Germany.
2003-2006	M.Sc. (Physiology) Department of Physiology, Faculty of Science, Mahidol University, Thailand.
1999-2003	B.Sc. (Physical Therapy) Faculty of Siriraj Hospital Medicine, Mahidol University, Thailand.

RESEARCH EXPERIENCES

2006-2007	Predoctoral fellow, Center of Neuroscience, Faculty of Science, Mahidol University, Thailand.
2004-2006	Visiting student, Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan. (Japanese Government Scholarship, Monbukagakusho)

PUBLICATIONS

1. Kunzelmann K, **Kongsuphol P**, Chootip K, Toledo C, Martins JR, Almaca J, Tian Y, Witzgall R, Ousingsawat J, Schreiber R. Role of the Ca^{2+}-activated Cl^- channels

bestrophin and acoctamin in epithelial cells. *Biological Chemistry* 392(1-2):125-34 (2011).

2. Tian Y, **Kongsuphol P**, Hug M, Ousingsawat J, Witzgall R, Schreiber R, Kunzelmann K. Calmodulin-dependent activation of the epithelial calcium-dependent chloride channel TMEM16A. *The FASEB Journal*, inpress(2010).

3. **Kongsuphol P**, Hug M, Ousingsawat J, Witzgall R, Schreiber R, Kunzelmann K. Metformin treatment of diabetes mellitus increases the risk for pancreatitis in patients bearing the CFTR-mutation S573C. *Cellular Physiology and Biochemistry* 25, 389-396(2010).

4. Carota I, Theilig F, Oppermann M, **Kongsuphol P**, Rosenauer A, Schreiber R, Jensen BL, Walter S, Kunzelmann K, Castrop H. Localization and functional characterization of the human NKCC2 isoforms. *Acta Physiologica (Oxford, England)* 199, 327-38(2010).

5. Schreiber R, Uliyakina I, **Kongsuphol P**, Warth R, Mirza M, Martins JR, Kunzelmann K. Expression and function of epithelial anoctamins. *The Journal of Biological Chemistry* 285, 7838-45(2010).

6. Preston P, Wartosch L, Günzel D, Fromm M, **Kongsuphol P**, Ousingsawat J, Kunzelmann K, Barhanin J, Warth R, Jentsch TJ. Disruption of the K^+ channel beta-subunit KCNE3 reveals an important role in intestinal and tracheal Cl^- transport. *The Journal of Biological Chemistry* 285, 7165-75(2010).

7. Kunzelmann K, **Kongsuphol P**, Aldehni F, Tian Y, Ousingsawat J, Warth R, Schreiber R. Bestrophin and TMEM16-Ca^{2+} activated Cl^- channels with different functions. *Cell Calcium* 46, 233-41(2009).

8. Almaça J, Tian Y, Aldehni F, Ousingsawat J, **Kongsuphol P**, Rock JR, Harfe BD, Schreiber R, Kunzelmann K. TMEM16 proteins produce volume-regulated chloride currents that are reduced in mice lacking TMEM16A. *The Journal of Biological Chemistry* 284, 28571-8(2009).

9. Almaça J, **Kongsuphol P**, Hieke B, Ousingsawat J, Viollet B, Schreiber R, Amaral MD, Kunzelmann K. AMPK controls epithelial Na^+ channels through Nedd4-2 and causes an epithelial phenotype when mutated. *Pflügers Archiv : European Journal of Physiology* 458, 713-21(2009).

10. **Kongsuphol P**, Cassidy D, Hieke B, Treharne KJ, Schreiber R, Mehta A, Kunzelmann K. Mechanistic insight into control of CFTR by AMPK. *The Journal of Biological Chemistry* 284, 5645-53(2009).

11. **Kongsuphol P**, Mukda S, Nopparat C, Villarroel A, Govitrapong P. Melatonin attenuates methamphetamine-induced deactivation of the mammalian target of rapamycin signaling to induce autophagy in SK-N-SH cells. *Journal of Pineal Research* 46, 199-206(2009).

12. **Kongsuphol P**, Hieke B, Ousingsawat J, Almaca J, Viollet B, Schreiber R, Kunzelmann K. Regulation of Cl- secretion by AMPK in vivo. *Pflügers Archiv : European Journal of Physiology* 457, 1071-8(2009).

PRESENTATIONS

1. Poster presentation. Functional CFTR is required for extracellular acidosis inducing CaCC. Europen Cystic Fibrosis Society. 2010, Carcavelos (Portugal).
2. Oral presentation. The Ca^{2+}-activated Cl^- channels Bestrophin and Anoctamin have different functions in epithelial cells. International symposium “An Integrated Approach to the Physiology of Organic Cation Transporters” and “Göttinger Transporttage”. 2010, Göttingen (Germany).
3. Oral presentation. CFTR activates Calcium activated Chloride Channels in Xenopus oocytes through extracellular acidosis. Doktorandentreffen der SFB’s 423 und 699. 2010, Hersbruck (Germany).
4. Oral presentation. Functional study of S573C CFTR: Pancreatitis Relevance? Doktorandentreffen der SFB’s 423 und 699. 2009, Neumarkt (Germany).
5. Oral presentation. Compartmentalized control of CFTR by AMPK. Doktorandentreffen der SFB’s 423 und 699. 2008, Erlangen (Germany).