Chapter 2

ROLE OF CFTR AND OTHER ION CHANNELS IN CYSTIC FIBROSIS

Karl Kunzelmann[1], Tanja Bachhuber[1], Gabriele Adam[1], Thilo Voelcker[1], Bettina Mürle[1], Marcus Mall[2], and Rainer Schreiber[1]

[1]*Institut für Physiologie, Universität Regensburg, Universitätstraße 31, D-93053 Regensburg, Germany. Tel: +49 (0) 941 943 4302, Fax: +49 (0) 941 943 4315, uqkkunze@mailbox.uq.edu.au.*

[2]*Cystic Fibrosis/Pulmonary Research and Treatment Center, School of Medicine. The University of North Carolina at Chapel Hill. 7011 Thurston Bowles Building, Chapel Hill, NC 27599-7248, USA.*

1. INTRODUCTION

Cystic fibrosis is the most common severe inherited disease among the Caucasian population. It is caused by mutations in the CFTR (cystic fibrosis transmembrane conductance regulator) protein, a Cl^- channel that plays a central role in the process of ion secretion and absorption in epithelial tissues. CFTR is expressed in polarized epithelial cells together with a number of other ion channels, carriers and pumps. Mutations in the CFTR gene lead to a defect in Cl^- secretion in these epithelial tissues. This has been proposed to be the cause for clinical symptoms observed in cystic fibrosis.[1,2] However, some of the transport defects observed in *in vivo* measurements in cystic fibrosis patients, in tissues from CF patients, or in transgenic mice, carrying CFTR mutations, cannot be reconciled with the concept of a defective Cl^- conductance as the single resaon for the transport defects observed in CF (Figure 1).[2-4] Because CFTR is regulated by second messengers, cytosolic factors and membrane receptors[5] and also regulates other membrane conductances, epithelial transport properties are largely dependent on CFTR function (Figure 1). In this short review, we will discuss the role of CFTR during secretion and absorption of electrolytes and will elucidate the contribution of basolateral K^+ channels to epithelial transport.

We will then describe the correlation between the epithelial Na^+ channel ENaC, CFTR and stimulation by purinergic agonists. New aspects of pharmacotherapy of cystic fibrosis and pharmacological manipulation of ion channel activity will be reviewed. Thus, this review will focus on the role of CFTR in the airway epithelium.

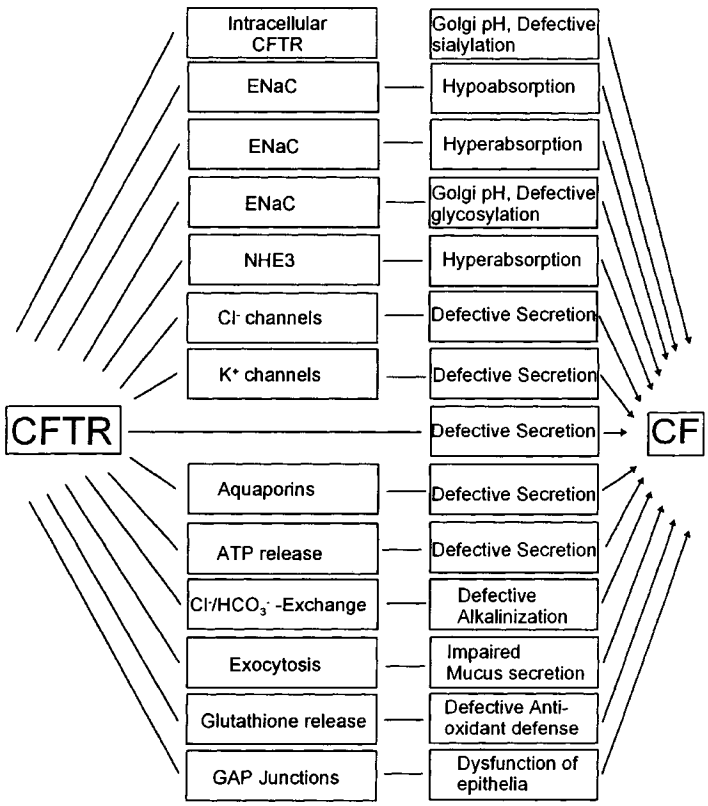

Figure 1. Summary of the effects of CFTR on membrane transport proteins in vitro and in vivo and putative impact on pathophysiology and phenotype in cystic fibrosis.

2. CFTR, CYSTIC FIBROSIS AND DEFECTIVE EPITHELIAL ION TRANSPORT

The CFTR protein is located in both secretory as well as absorptive epithelial cells. In the airways, CFTR is located predominantly in the luminal membrane of cells forming serous end pieces of submucosal glands (Figure 2). Thus, most Cl^- secretion takes place in the serous part of the

submucosal glands and is in charge of flushing the submucosal glands, thereby removing mucus (Figure 2). It is easily conceivable that a defect in CFTR mediated Cl^- secretion will lead to accumulation of mucus within the glands, occlusion of the ducts and inflammation. Apart from expression of CFTR in the submucosal glands, expression is also found in the superficial epithelium, although to a lesser degree. Here, CFTR is forming an absorptive pathway for Cl^- ions, similar to the function of CFTR in the sweat duct. As shown in Figure 3, ion transport in either secretory or absorptive direction requires additional transport proteins. Thus, Cl^- secretion requires accumulation of Cl^- inside the cell by the basolateral $Na^+/2Cl^-/K^+$-cotransporter (NKCC1). Basolateral K^+ channels serve as a recycling mechanism for K^+ ions and hyperpolarize the cell membranes, which facilitates and maintains electrolyte secreting. The basolateral shunt pathway allows secretion of Na^+ to the luminal side, which is driven by the lumen negative transepithelial voltage. In the absorptive superficial epithelium, CFTR colocalizes with epithelial Na^+ channels (ENaC) and due to the depolarizing effect of Na^+ absorption, Cl^- is driven in the absorptive direction. Cl^- ions are released to the basolateral compartment via anion exchangers, KCl cotransporter or basolaterally located Cl^- channels. As indicated for the Cl^- secretion, absorption of Cl^- ions does require a basolateral K^+ conductance. The molecular nature of these basolateral K^+ channels will be discussed in the next chapter. Ultimately, both secretion and absorption are driven by the ATP consuming Na^+/K^+ - ATPase.

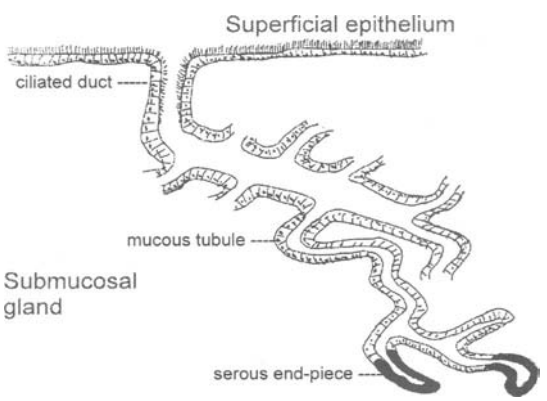

Figure 2. Model of a submucosal gland in human airways.

Cystic fibrosis is characterized by inadequate ion secretion in conjunction with enhanced NaCl absorption in airways and colon. Hypoabsorption of NaCl has been observed for other tissues such as the sweat duct epithelium.

This leads to the well described phenomenon of enhanced salt excretion with the sweat.[6] However, apart from this and the gastrointestinal symptoms observed in CF, the progressive lung disease is the single most important and life limiting factor in CF. According to the so called isotonic volume hypothesis[7], at least two mechanisms contribute to the CF lung disease: i) enhanced absorption of Na^+, and consequently, hyperabsorption of fluid and electrolytes by the airway surface epithelium, and ii) impaired cAMP dependent Cl^- secretion. Both transport defects have been detected initially in short circuit measurements in excised CF airways and cultured CF airway epithelial cells.[3,8] Patch clamp analysis on CF airway epithelial cells identified enhanced amiloride sensitive whole cell currents and an increase in the activity of the Na^+ channel.[9,10] These results are confirmed by microelectrode measurements and Ussing chamber recordings on freshly isolated CF respiratory tissues.[10] Moreover, the nasal potential difference (PD) *in vivo* in CF patients showed enhanced amiloride induced PD changes and hyperabsorption of Na^+ along with defective Cl^- secretion. The so called isotonic volume transport theory[7,11] predicts an isotonic contraction of the airway surface liquid (ASL), the thin watery layer that covers the ciliated airway epithelial cells. Dehydration of the ASL leads to impaired mucociliary clearance as a key factor in innate lung defense, and results in mucus impactions on airway surfaces. In contrast, an alternative low salt or defensin theory predicts a low salt or hypotonic ASL under normal conditions, and an increase in ASL tonicity in CF airways. Accordingly, it has been proposed that hypertonic ASL inhibits the activity of antimicrobial defensin like molecules.[12,13] However, subsequent measurements of the salt

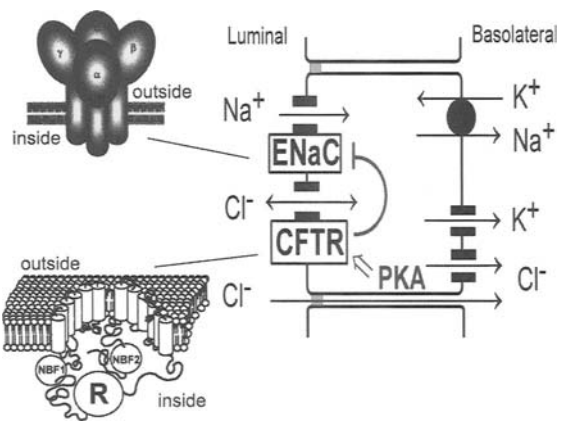

Figure 3. Model for regulation of epithelial Na^+ channels (ENaC) by CFTR. In airway epithelial cells.

concentration and properties of the ASL using more sophisticated methods did not reveal an increased salt concentration in the ASL of CF patients.[14-16] While an increase in ASL tonicity is unlikely to occur in CF, airway mucus accumulation, occlusion of small airways and a defective HCO_3^- secretion are very likely to be major determinants of the CF airway disease.[17,18]

3. CONTRIBUTION OF BASOLATERAL POTASSIUM CHANNELS IN EPITHELIA

Basolateral K^+ channels are essential to maintain a hyperpolarized membrane voltage and the electrical driving force that is required for Cl^- secretion and Na^+ absorption (Figure 4). Similar to intestinal epithelia, also airways posses at least two different types of K^+ channels, which are activated either by increases in intracellular Ca^{2+} or camp/[19,20] Ca^{2+} activated K^+ channels maintain the negative membrane voltage in resting epithelial cells and supply the driving force during Ca^{2+} mediated stimulation of secretion.[21] However, when intracellular cAMP is enhanced and luminal Cl^- channels are activated, the cells depolarize. Under these conditions, Ca^{2+} influx into the cell is limited and Ca^{2+} dependent K^+ channels become less active.[22-24] Loss of activity of Ca^{2+} activated K^+ channels is compensated by parallel activation of cAMP dependent K^+ channels, repolarizing the membrane voltage.[25] Apart from these two types of K^+ channels, other K^+ conductances may also participate in maintaining the negative membrane voltage, but are currently only poorly characterized.[26,27] Thus, it is not clear to what degree a large conductance Ca^{2+} dependent K^+ channel contributes to the basolateral K^+ conductance in airway epithelial cells. The cAMP activated K^+ conductance can be blocked specifically by the chromanol compound 293B.[20,28] The channel was initially cloned from heart muscle and was named K_VLQT1 (KCNQ1), indicating its role in the long QT-syndrome. K_VLQT1 is the α subunit in a K^+ channel complex together with the small regulatory β subunit, KCNE3.[20,29,30] KCNE3 has a large impact on K^+ channel properties and pharmacology, similar to those of minK (KCNE1, IsK).[29,31,32]

The open probability of the Ca^{2+} activated K^+ channel is largely enhanced by increase in intracellular Ca^{2+}.[22,33,34] Moreover, evidence exists that the channel activity is modulated by phosphorylation.[24] This channel has been isolated initially from human brain cells (hSK4), pancreas (hIK1) and T cells (hKCa4; KCNN4).[35-38] The channel is also activated by 1-ethyl-2-benzimidazolone (1-EBIO).[39-42] Patch clamp studies showed that the channel is blocked by low concentrations of the antifungal antibiotic clotrimazole and the imidazole compounds clotrimazole.[41]

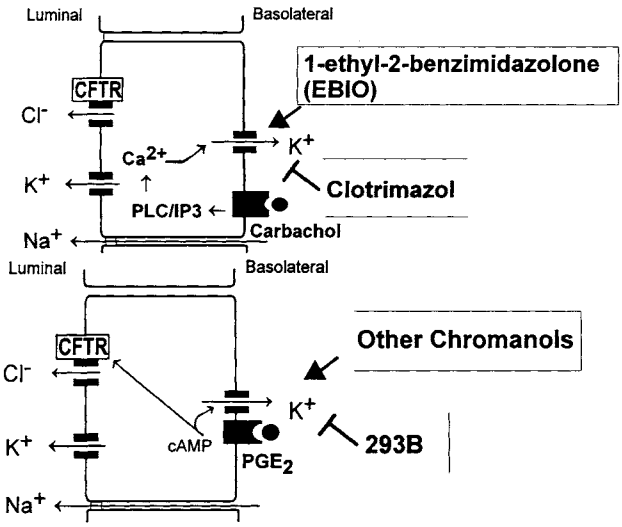

Figure 4. Model of secretion by airway epithelial cells. Contribution of basolateral K^+ channels activated by increase of intracellular Ca^{2+} (upper model) or cAMP (lower model).

4. INHIBITION OF THE EPITHELIAL SODIUM CHANNEL ENAC BY CFTR

Regulation of the epithelial Na^+ channel ENaC by CFTR Cl^- channels was first detected in an epithelial cell line and in fibroblasts transfected with ENaC and CFTR.[43] Amiloride sensitive short circuit currents, as detected in Ussing chamber recordings, and amiloride sensitive whole cell currents were found to be inhibited by cAMP in cells co-transfected with CFTR. These initial results were confirmed by a subsequent study, in which CFTR and ENaC were coexpressed in oocytes of *Xenopus laevis*. Amiloride sensitive whole cell currents were measured using the double electrode voltage clamp method.[44] ENaC currents were inhibited by activation of wtCFTR Cl^- currents, but not by mutant ΔF508-CFTR. Several subsequent studies used basically identical techniques and arrived at similar conclusions. Notably, a CFTR inhibition of amiloride sensitive Na^+ absorption was observed in Ussing chamber studies on both human airways and colon. This inhibition was not seen in tissues from CF patients.[10,45] Moreover, Ussing chamber studies on the mouse colon revealed cAMP dependent inhibition of amiloride sensitive short circuit currents in normal, but not in transgenic G551D-CFTR mice.[46] These results were further supported by other techniques such as microelectrodes and whole cell patch clamp recordings,

which were applied to native human and rat epithelial cells.[10,47] Finally, the open probability (Po) of ENaC channels reconstituted into planar lipid bilayers was determined and a decrease in the single channel Po was found by activation of co-reconstituted CFTR.[48] The results from the different studies suggest a twofold effect of CFTR on amiloride sensitive transport: i) The baseline Na^+ conductance in the non-stimulated tissue is reduced without activation of CFTR. ii) After stimulation of wtCFTR by cAMP, amiloride sensitive Na^+ absorption is further inhibited. The situation is very different in the sweat duct, where activation of CFTR does not inhibit but increases Na^+ absorption.[6,49] Accordingly, the defect in NaCl absorption in the CF sweat duct is due to both defective Na^+ and Cl^- channel function. The reason for the different impact of CFTR on ENaC in airways and sweat duct remains obscure. Numerous studies have shown a decrease of ENaC currents during activation of CFTR in *Xenopus* oocytes.[44,50-54] CFTR's ability to downregulate ENaC largely depends on the direction and the magnitude of the Cl^- current through CFTR Cl^- channels.[55] Thus, Cl^- flux through CFTR and/or changes in the intracellular Cl^- concentration could serve as the signal for inhibition of ENaC (Figure 5). The inhibitory effects of CFTR on ENaC are clearly suppressed in the presence of a low extracellular Cl^- concentration, suggesting that Cl^- influx and eventually accumulation in the cytosol is essential for the inhibition of EnaC.[52,55] Such a mechanism exists in the mouse salivary duct epithelium.[56,57] For this feedback regulation of ENaC, $G_{\alpha i2}$ subunits of Cl^- sensitive trimeric GTP binding proteins play a central role. However, recent experiments exclude a role of G proteins in the downregulation of ENaC by CFTR in *Xenopus* oocytes.[53]

The results challenge the question whether Cl^- currents generated by other Cl^- channels, are also able to inhibit ENaC. In fact, inhibition of ENaC is not unique to CFTR, but is also caused by other Cl^- conductances and consecutive increase in the intracellular Cl^- concentration. Thus, ENaC is inhibited in amphotericin B permeabilized oocytes by increasing the bath Cl^- concentration from 5 to 50 mmol/l.[52] Furthermore, another type of Cl^- channel, the ClC-0 channel, was coexpressed together with ENaC which also inhibited EnaC.[52] This result has been confirmed recently by experiments coexpressing CFTR with ClC-2 Cl^- channels.[58]The question is, after all, how do Cl^- ions actually inhibit ENaC? This is currently under examination, yet, preliminary experiments suggest a direct interference of Cl^- ions with the ENaC channel. Previous experiments in *Xenopus* oocytes excluded the contribution of Cl^- sensitive proteins, such as GTP binding proteins and the nucleoside diphosphate kinase (NDPK).[51,53] A current working hypothesis proposes a model in which Cl^- ions interfere with positive charges, located in close proximity to the inner mouth of ENaC channels, thereby inhibiting

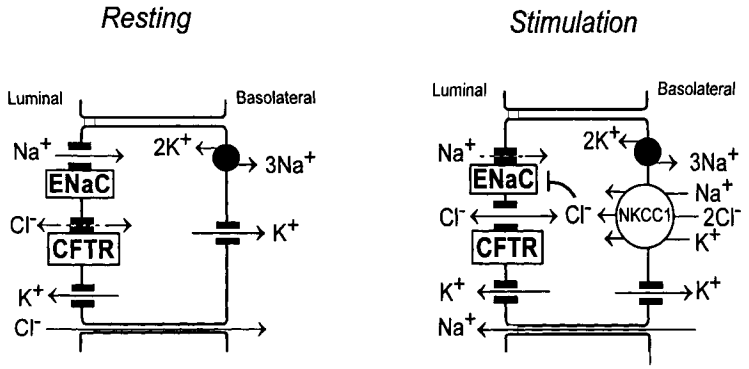

Figure 5. Epithelial cells under resting conditions and after stimulation with secretagogues.

permeation of Na^+ ions through EnaC.[59] In good agreement with such a model are results from recent patch clamp experiments which show reduced Na^+ channel currents in the presence of a high cytosolic Cl^- concentration.[59] Using different techniques such as Western blots, double electrode voltage clamp and patch clamp experiments, binding sides for phosphatidylinositol 4,5-bisphosphate (PIP_2) have been identified in the N termini of β,γ-ENaC.[60,61] Negatively charged PIP_2 regulates ENaC at the level of the inner plasma membrane through a mechanism that is independent of ENaC trafficking. It remains to be shown, whether a similar scenario applies to the CFTR mediated inhibition of ENaC.

How do the results obtained in *Xenopus* oocytes compare to the situation in epithelial tissues? Apart from a few early studies on the intracellular Na^+ and Cl^- concentration in airway epithelial cells, not much is known about changes in the intracellular Cl^- concentration during acute stimulation of airway and colonic epithelial cells.[62-66] Possibly only the Cl^- concentration close to the cell membrane and in close proximity to ENaC may be relevant for Na^+ channel inhibition. Opening of luminal Cl^- channels may increase cellular uptake and absorption of both Cl^- and Na^+ and thereby inhibit ENaC. Increase in intracellular Cl^- activity may occur initially by an influx of Cl^- through apically located CFTR Cl^- channels. However, secretagogues also activate the basolateral $Na^+/2Cl^-/K^+$ cotransporter (NKCC1), which leads to an uptake of Cl^- into the cell. Recent experiments were performed in our laboratory on mouse kidney collecting duct cells, which have been transfected with a construct expressing the Cl^- sensitive yellow fluorescent protein YFP. M1 cells express ENaC along with CFTR and NKCC1 and exhibit quenching of the fluorescence during activation of CFTR by secretagogues.[67-69] This indicated an increase in $[Cl^-]_i$ concentration by about 17 mmol/l. Replacing the extracellular Ringer solution by a low (5 mmol/l)

Cl^- solution induced an efflux of Cl^- and a dequenching of the fluorescence signal. The efflux was more pronounced, and thus the slope of the fluorescence increase was steeper after stimulation of additional CFTR Cl^- channels (Figure 6). These data demonstrate that stimulation of M1 cells and thus activation of CFTR Cl^- channels causes an uptake of Cl^- and increase in $[Cl^-]_i$.

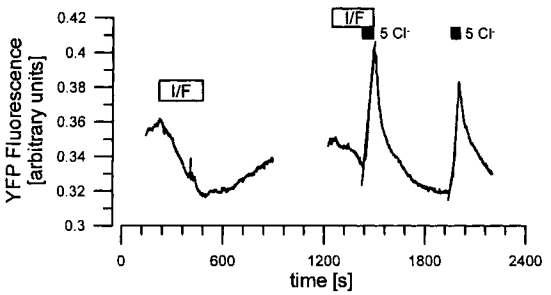

Figure 6. Change of YFP fluorescence in mouse collecting duct cells upon stimulation with IBMX and forskolin and in the presence of high (145 mmol/l) or low (5 mmol/l) extracellular Cl^- concentration.

5. PURINERGIC REGULATION OF ENAC AND CL^- CONDUCTANCE

Activation of electrolyte secretion by stimulation with extracellular nucleotides in both normal and CF airways is well described.[70-73] It has been demonstrated that basolateral application of ATP to human airway epithelial cells activates Cl^- secretion indirectly, by activating K^+ channels, while luminal application of ATP and UTP activates alternative Ca^{2+}-dependent Cl^- channels, in murine and human normal and CF respiratory epithelia via binding to purinergic $P2Y_2$ receptors.[19,71,74-77] $P2Y_2$ receptors couple to intracellular protein lipase C (PLC) and G-proteins, and thereby increase intracellular Ca^{2+} upon activation.[74,78] $P2Y_2$ receptors are co-localized with $P2Y_6$ and probably $P2Y_4$ receptors on the mucosal side of the epithelium.[79-82] The luminal $P2Y_2$ receptor is activated by ATP or UTP, while $P2Y_6$ receptors are stimulated through metabolic break down products of UTP, such as UDP.[83] ATP and UTP are released constitutively and in response to mechanical stress *in vitro* and *in vivo* during coughing.[84,85] ATP release has been demonstrated to be CFTR independent and to occur during membrane stretch.[80,86] Luminal ATP/UTP activates Ca^{2+} dependent Cl^- channels of unknown molecular identity.[87-89] A family of apparently Ca^{2+} / calmodulin activated Cl^- channels (hCaCC-1, hCaCC-2, hCaCC-3) have been found to

be expressed in the digestive and respiratory mucosa and are thus candidate proteins for the luminal Ca^{2+} activated Cl^- channel.[19,90,91] The recently identified family of bestrophins are other potential candidates.[92,93] As mentioned above, activation of luminal Cl^- channels is paralleled by activation of basolateral Ca^{2+} dependent K^+ channels.[19,94]

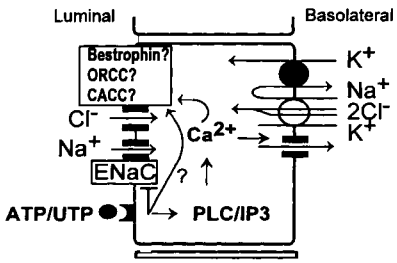

Figure 7. Regulation of ion transport by purinergic stimulation.

Activation of mucosal purinergic receptors does not only stimulate Cl^- secretion but also modulates Na^+ absorption. This has been shown meanwhile for various epithelia, including kidney and airway epithelial cells.[77,95-97] In lung epithelia, inhibition of Na^+ transport by extracellular nucleotides has been reported for human and rabbit trachea, rat distal airway cells, porcine bronchi and cultured human bronchial epithelial cells.[77,95,98-100] In native human airway tissues, the fractional inhibition of the amiloride sensitive Na^+ transport was similar in normal and CF tissues.[77] However, due to enhanced basal Na^+ transport in CF, the absolute magnitude of nucleotide-mediated inhibition of Na^+ absorption is significantly increased in CF compared to normal tissues.[77] Although several transport proteins participate in transepithelial Na^+ absorption, such as luminal epithelial Na^+ channels (ENaC), the basolateral Na^+/K^+ ATPase and basolateral K^+ channels,[46] it is likely that purinergic stimulation inhibits ENaC directly.[60,101] The mechanism of ATP or UTP mediated inhibition of Na^+ absorption is currently under investigation. Ca^{2+} itself or Ca^{2+} dependent protein kinase C (PKC) are not in charge of the inhibition of amiloride sensitive Na^+ transport in the airways.[77,78] It has been shown that amiloride sensitive transport is inhibited by a G protein sensitive mechanism, that it requires the function of phospholipase C and also involves Cl^- transport over the luminal membrane.[78] Recent papers show activation of ENaC channels by phosphatidylinositols, probably by interaction with putative PIP_2 binding domains in the N-termini of the β and γ subunits of heterotetrameric ENaC channels. Hydrolysis of PIP_2 by phospholipase C to produce 1,4,5-inositol

trisphosphate (IP_3) and diacylglycerol (DAG) leads to deactivation of ENaC channels.[60,61] Thus anionic phospholipids like PIP_2, abundantly present in the inner leaflet of eukaryotic plasma membranes, are not just precursors for intracellular second messengers but are in itself regulatory molecules.[102] According to this, cleavage of PIP_2 by activation of PLC through stimulation of purinergic receptors is probably the mechanism by which ENaC and thus amiloride sensitive Na^+ absorption is inhibited.[101]

6. ION CHANNEL THERAPY IN CYSTIC FIBROSIS

Because defective Cl^- secretion and enhanced Na^+ absorption are crucial to the pathophysiology of the CF lung disease, normalizing the transport abnormalities are the major therapeutical target. Several strategies have been developed to overcome the reduced Cl^- secretion caused by defective CFTR Cl^- channel function. Accordingly, strategies aim at correcting the maturation and activity of mutant CFTR. These strategies have been outlined in previous reports.[103,104] As an alternative approach, transepithelial electrolyte secretion may be promoted by enhancing the driving force for luminal Cl^- exit. This can be achieved by activation of K^+ channels located on the basolateral side of the epithelium. While activators for the cAMP regulated KCNQ1 K^+ channel are not yet readily available, specific openers for the Ca^{2+} activated K^+ channel have been identified meanwhile. Among these, the benzimidazolone compounds 1-EBIO or chlorzoxazone have been found to hyperpolarize the basolateral membrane of epithelial cells (Figure 4).[40,105] Other efforts focus on inhibition of epithelial Na^+ channels to counteract the enhanced Na^+ absorption.[106] Early clinical trials using aerosolized amiloride showed some improvement in mucociliary clearance in the airways of CF patients.[107,108] The limited evidence for a positive long-term benefit[109,110] may be due to the rapid clearance of amiloride from the airways.

As outlined above, topical application of aerosolized ATP or UTP could have a dual therapeutic effect by counteracting increased Na^+ absorption and promoting Ca^{2+} dependent Cl^- secretion in airways from CF patients. *In vivo*, however, both UTP and ATP are rapidly degraded (< 1 min) from the airways by ecto-nucleotidases, limiting the therapeutic effects of both nucleotides. Thus, efforts are put into the development of more stable and longer lasting ligands of purinergic receptors. In that respect, it has been suggested to combine stable analogues of UTP with long acting Na^+ channel blockers, such as loperamide.[111] Recently, synthetic ligands of purinergic receptors have been developed, with a higher biological and chemical stability compared to UTP or ATP.[112-114] Novel $P2Y_2$ receptor agonist were

developed for the treatment of CF. Initial results from clinical studies in normal volunteers demonstrate the safety of these compounds.[115] Recent, randomized, double-blinded, phase I studies with patients suffering from mild to moderate cystic fibrosis show promising results.[116] Thus, synthetic ligands of purinergic $P2Y_2$ receptors have a potential for future use in the treatment of cystic fibrosis.

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