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Selective Activation of Cystic Fibrosis Transmembrane Conductance Regulator Cl^- and HCO_3^- Conductances

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Summary

While cystic fibrosis transmembrane conductance regulator (CFTR) is well known to function as a Cl^- channel, some mutations in the channel protein causing cystic fibrosis (CF) disrupt another vital physiological function, HCO_3^- transport. Pathological implications of derailed HCO_3^- transport are clearly demonstrated by the pancreatic destruction that accompany certain mutations in CF. Despite the crucial role of HCO_3^- in buffering pH, little is known about the relationship between cause of CF pathology and the molecular defects arising from specific mutations. Using electrophysiological techniques on basolaterally permeabilized preparations of microperfused native sweat ducts, we investigated whether: a) CFTR can act as a HCO_3^- conductive channel, b) different conditions for stimulating CFTR can alter its selectivity to HCO_3^- and, c) pancreatic insufficiency correlate with HCO_3^- conductance in different CFTR mutations. We show that under some conditions stimulating CFTR can conduct HCO_3^-. HCO_3^- conductance in the apical plasma membranes of sweat duct appears to be mediated by CFTR and not by any other Cl^- channel because HCO_3^- conductance is abolished when CFTR is: a) deactivated by removing cAMP and ATP, b) blocked by 1 mM DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid) in the cytoplasmic bath and, c) absent in the plasma membranes of ΔF508 CF ducts. Further, the HCO_3^-/Cl^- selectivity of CFTR appears to be dependent on the conditions of stimulating CFTR. That is, CFTR activated by cAMP + ATP appears to conduct both HCO_3^- and Cl^- (with an estimated selectivity ratio of 0.2 to 0.5). However, we found that in the apparent complete absence of cAMP and ATP, cytoplasmic glutamate activates CFTR Cl- conductance without any HCO_3^- conductance. Glutamate activated CFTR can be induced to conduct HCO_3^- by the addition of ATP without cAMP. The non-hydrolysable AMP-PNP (5'-adenylyl imidodiphosphate) cannot substitute for ATP in activating HCO_3^- conductance. We also found that a heterozygous R117H/ΔF508 CFTR sweat duct retained significant HCO_3^- conductance while a homozygous ΔF508 CFTR duct showed virtually no HCO_3^- conductance. While we suspect that the conditions described here are not optimal for selectively activating CFTR Cl^- and HCO_3^- conductances, we surmise that CFTR may be subject to dramatic alterations in its conductance, at least to these two anions under distinctly different physiological conditions which require distinctly different physiological functions. That is

physiologically, CFTR may exhibit Cl^- conductance with and/or without HCO_3^- conductance. We also surmise that the severity of the pathogenesis in CF is closely related to the phenotypic ability of a mutant CFTR to express a HCO_3^- conductance.

It is well established that CFTR is a cAMP and ATP activated Cl^- channel [1, 2]. However, evidence particularly from the studies on pancreatic function indicates that HCO_3^- ion transport is significantly affected in CF [3, 4, 5]. Now the question becomes how this Cl^- channel affects the transport of another major physiological anion HCO_3^-. A clear answer to this question seems essential to understand the pathogenesis of CF and to develop appropriate therapies to cure it. Possible role(s) of CFTR in HCO_3^- secretion include 1) CFTR may control another anion channel permeable to HCO_3^-, 2) CFTR may work in concert with a Cl^- / HCO_3^- exchanger, and/or 3) CFTR it self may be a HCO_3^- selective channel under specific intracellular regulatory conditions. Recently modelling of pancreatic ducts [5] indicates that at least in the distal portions of the pancreatic duct very high concentrations of luminal HCO_3^- (140 mM) can be achieved through a HCO_3^- selective anion channel located in the apical plasma membrane. However, relatively smaller HCO_3^-/Cl selectivity (0.2) of CFTR precludes secretion of predominantly HCO_3^- rich fluid into the lumen unless the intracellular Cl^- concentration remains negligible. A potential solution of this problem could be physiologically increasing the selectivity of CFTR to HCO_3^- over Cl^-. Even though we do not have other examples in which an ion channel undergoes a physiological change in selectivity, we present evidence here which demonstrates that CFTR can show high selectivity to both HCO_3^- and Cl^-. More significantly, the Cl^-/HCO_3^- selectivity of CFTR can be changed depending up on the nature of conditions of activation.

CFTR HCO_3^- permeability: In an earlier study using physiological concentrations of HCO_3^- (25 mM) we were unable to detect HCO_3^- permeability in the sweat duct [6]. However, several studies from other laboratories using much higher iso-osmotic concentration of HCO_3^- (≥ 135 mM) reported a relatively small CFTR HCO_3^- conductance [7, 8, 9]. We have therefore investigated whether CFTR in the apical membranes of the native sweat duct is permeable to HCO_3^- under these conditions. We now have

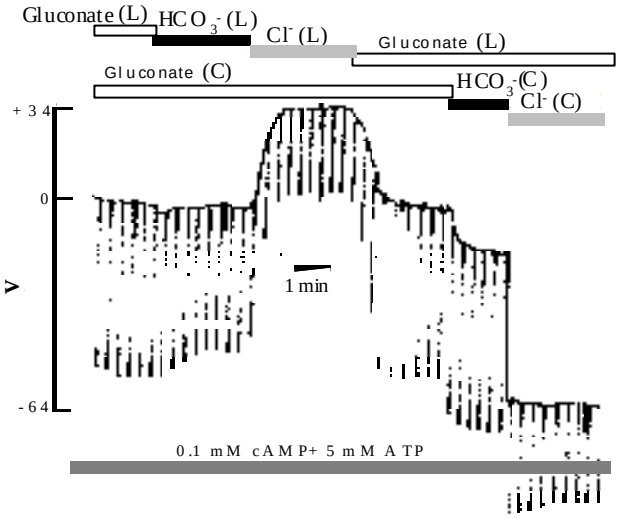

Figure 1. cAMP + ATP activated CFTR conducts HCO_3^-This experiment was designed to test whether CFTR is permeable to HCO_3^- from lumen to cell and *vice versa* by following HCO_3^- diffusion potentials and conductance. HCO_3^- diffusion potentials were generated by perfusing with 140 mM HCO_3^- on one side and 140 mM gluconate on the contralateral side of the epithelium. Notice that CFTR was impermeable to HCO_3^- from lumen to cytosol when cytosolic bath was perfused with gluconate. In contrast, HCO_3^- was clearly permeable from cell to lumen when the lumen was perfused with gluconate. CFTR Cl^- conductance was measured as Cl^- diffusion potentials (generated by 140 mM Cl^- in the cell and 140 mM gluconate in the lumen) and conductance to obtain HCO_3^-/Cl^- selectivity of CFTR. Cl^- and HCO_3^- permeability was indicated by an increase in the respective anion diffusion potentials and a corresponding decrease in the magnitude of negative voltage deflections in response to 50 nA/500 mS transepithelial constant current pulses.

evidence which suggest that activating CFTR with cAMP and ATP stimulate both Cl^- and HCO_3^- permeability (Figure 1) with a Cl^-/HCO_3^- selectivity of about 0.5 or better. Furthermore, the evidence that HCO_3^- permeability is abolished after inhibiting CFTR either by removing ATP and cAMP or by adding the channel blocker DIDS (1 mM) to the cytoplasmic bath indicate that HCO_3^- permeability in the apical membrane is due to CFTR and not due to any other Cl^- channel (results not shown). The fact that HCO_3^- selectivity of CFTR was detectable at relatively high HCO_3^- concentration [4, 6] may raise the question of whether changes in the concentration of HCO_3^- or other organic anions in the cytosol influence the HCO_3^- selectivity of CFTR?

Altered Cl^-/HCO_3^- selectivity: Early studies revealed that CFTR appears to conduct HCO_3^- from the cell into the lumen irrespective of the ion composition in the lumen (Cl^-, gluconate and HCO_3^-). However, CFTR HCO_3^- conductance from lumen to cytosol is dependent on the cytosolic ion-composition, i.e. CFTR conducts HCO_3^- when Cl^- but not gluconate (Glu-) is present in the cytoplasm (Figure 1 and reference [4]). These results suggested that the relative HCO_3^- selectivity of CFTR is not fixed but may alter as a function of cytosolic ion composition. In addition, the following evidence suggest that the Cl^-/HCO_3^- selectivity of CFTR can be altered by changing the conditions of stimulating CFTR. Recently we discovered that cytosolic glutamate stimulates CFTR Cl^- conductance in the complete absence of cAMP and ATP. Figure 2 is a representative example of an electrophysiological experiment showing that glutamate alone activates CFTR Cl^- conductance. In this experiment application of 140 mM glutamate to the cytoplasmic bath resulted in a large increase in Cl^- diffusion potential and conductance. However, we were intrigued by the fact that glutamate activated Cl^- but not HCO_3^- conductance through CFTR as indicated by the lack of HCO_3^- diffusion potentials when impermeant anion gluconate was substituted by equimolar concentration of HCO_3^- in the lumen (Figure 2). These results indicated that cytosolic glutamate activated CFTR selectively permeable to Cl^- but not to HCO_3^-. However, we and others have reported that hydrolytic and non-

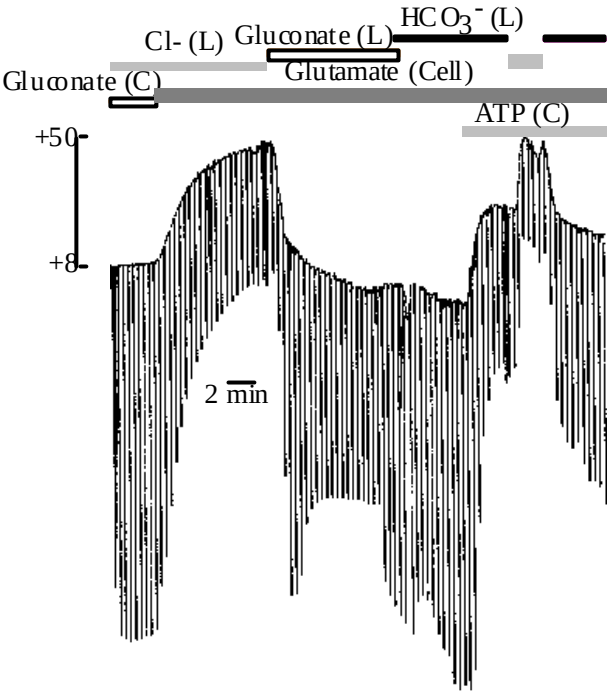

Figure 2. The effect of glutamate on CFTR Cl^- and HCO_3^- conductances.
In this experiment substituting glutamate for gluconate in the cytoplasmic bath resulted in a large increase in Cl^- diffusion potential (generated by 140 mM Cl^- in the lumen and 140 mM glutamate in the cytoplasmic bath) and conductance in the complete absence of cAMP and ATP. Notice that substituting luminal Cl^- with impermeant anion gluconate reversed the anion diffusion potential to the junction potential values indicating that glutamate, in fact, activated Cl^- conductance. In contrast, substituting HCO_3^- for gluconate (in the lumen) in the presence of glutamate in the cytoplasmic bath had little effect on the anion diffusion potentials or conductance suggesting that glutamate alone did not activate CFTR HCO_3^- conductance. However, addition of 5mM ATP to the cytoplasmic bath in the presence of glutamate resulted in a large increase in both HCO_3^- and Cl^- conductances as indicated by changes in the respective ion diffusion potentials and electrical conductances.

hydrolytic ATP binding play a significant role in CFTR channel function [10, 11].

We therefore asked what role, if any, ATP plays in determining the anion selectivity of CFTR channel. Surprisingly, we found that adding ATP in the presence of glutamate apparently induced a HCO_3^- conductance in CFTR as shown in Figure 2. Still, as in the case of cAMP + ATP stimulated CFTR, glutamate + ATP activated CFTR was more selective to Cl^- than HCO_3^-. The glutamate + ATP induced HCO_3^- selectivity appeared to involve ATP hydrolysis because, the non-hydrolyzable ATP analog, AMP-PNP failed to activate CFTR HCO_3^- conductance. Preliminary results indicated that the non-specific kinase inhibitor staurosporine (10^{-6} M) did not block glutamate activated CFTR Cl^- and HCO_3^- conductances suggesting that phosphorylation of CFTR [11] may not be involved in the activation process (results not shown). Even though, we are not yet certain of the physiological conditions under which CFTR functions as a HCO_3^- selective channel, these results may suggest that the anion selectivity of CFTR may be physiologically altered to function either as an exclusively Cl^- or HCO_3^- channel. We suspect that glutamate may act on a cytoplasmic receptor associated with CFTR or that it may mimic a messanger or ligand that physiologically activate CFTR. The physiological implications of such a possibility is significant. For example, CFTR may function either as a HCO_3^- selective channel in tissues which predominantly secrete HCO_3^- (e.g. pancreatic ducts) or act as a Cl^- channel in tissues which predominantly secrete or absorb Cl^- (e.g. sweat glands).

Correlation between HCO_3^- Selectivity of Mutant CFTR and Severity of Pancreatic Disease in CF

The observation that CFTR is permeable to HCO_3^- seems to provide a physiological basis for abnormal HCO_3^- secretions and the resultant pancreatic pathology in CF. However, it is well known that about 10% of CF patients retain pancreatic insufficiency [12]. While CF patients expressing certain mutant forms of CFTR (e.g. ΔF508, GFF1D CFTR) are generally pancreatic insufficient, others (e.g., R117H CFTR) appear to

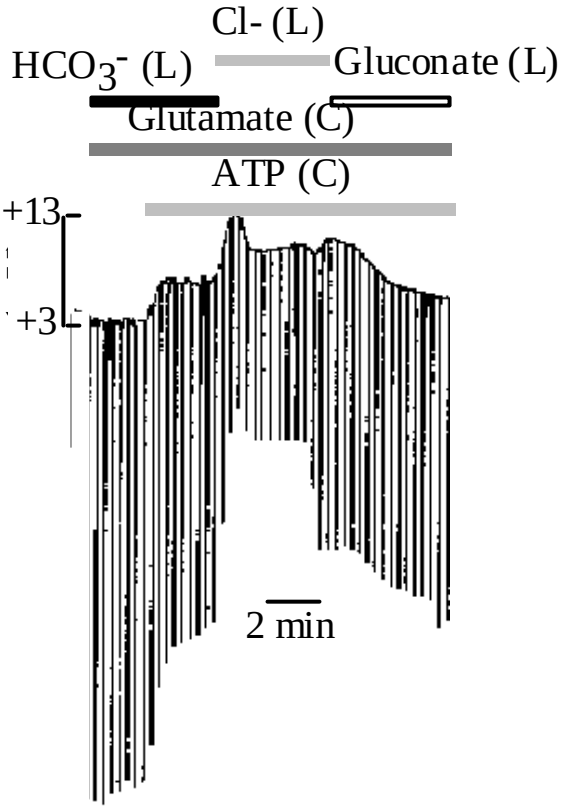

Figure 3. The effect of activating R117H CFTR with glutamate on HCO_3^- and Cl^- conductances.
Notice that stimulating CFTR with cytosolic glutamate alone did not activate HCO_3^- conductance in this duct. However, addition of 5 mM ATP activated both HCO_3^- and Cl^- conductances which is indicated by an increase in HCO_3^- and Cl^- diffusion potentials from lumen to cell generated by either 140 mM HCO_3^- or 140 mM Cl^- against glutamate in the cytoplasmic bath. Also notice that the increases in the respective anion diffusion potentials were followed by increases in electrical conductances as shown by a decrease in the negative voltage delections in response to 50 nA/500 mS transepithelial current pulses. This experiment also shows that in this CF duct both HCO_3^- and Cl^- conductances are relatively smaller as compared to the wild type CFTR from normal ducts as shown in Figures 1 and 2 which is indicated by relatively smaller changes in HCO_3^- and Cl^- diffusion potentials and conductances.

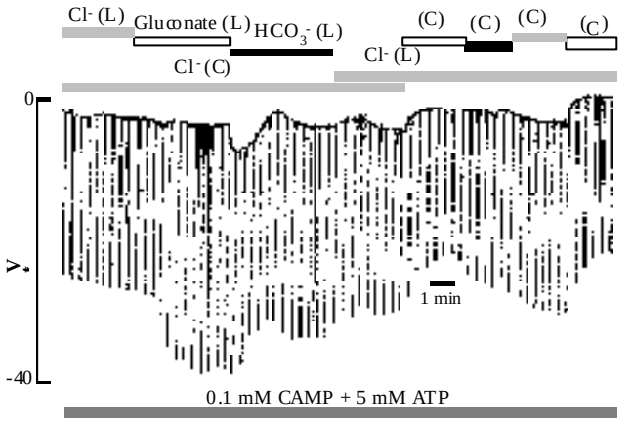

Figure 4. Lack of HCO_3^- and Cl^- conductances in a ΔF508 CF duct.
In this experiment we tested the effect of cAMP + ATP on both CFTR HCO_3^- and Cl^- conductances. As shown, HCO_3^- and Cl^- gradients were established either from lumen to cell or *vice versa* while perfusing the contralateral side with the impermeant anion gluconate. Stimulating CFTR with cAMP + ATP had little effect on either HCO_3^- or Cl^- diffusion potentials and conductance suggesting that the HCO_3^- and Cl^- conductances in wild type (normal) and R117H ΔF508 CF ducts are most likely due to activation of CFTR and not likely due to activation of any other Cl^- conductance.

be pancreatic sufficient. We therefore sought to determine whether there is a correlation between the pancreatic sufficiency and the ability of mutant CFTR to conduct HCO_3^-. In other words, if CFTR HCO_3^- conductance has a physiological role in pancreatic HCO_3^- secretion and pancreatic pathology in CF, R117H CFTR should retain some degree of HCO_3^- conductance, while the ΔF508 CFTR expressing ducts should not conduct HCO_3^-. Figure 3 is a representative example showing significant HCO_3^- and Cl^- conductances in the apical plasmamembranes of sweat ducts from an R117H/ΔF508 CF subject, even though the magnitude of these conductances appear to be smaller as compared to that of wild type CFTR (Figure 2). Since ΔF508 CFTR is absent in the plasma membrane of these ducts, we attribute the HCO_3^- permeability in these ducts primarily to the R117H CFTR mutant. In contrast, the apical membranes from a pancreatic insufficient CF subject (homozygous for ΔF508 CFTR) were impermeable to both Cl^- and HCO_3^- when stimulated with cAMP and ATP as shown in Figure 4. These results indicate that there is a strong correlation between the pancreatic sufficiency, and the HCO_3^- conductance of the CFTR genotype. These observations emphasize the potential role of CFTR in regulating HCO_3^- concentration (hence control of pH) in normal exocrine secretions and in the pathogenesis of CF.

Conclusion

We showed that CFTR conducts both Cl^- and HCO_3^-. The HCO_3^-/Cl^- selectivity of CFTR appears to be a function of intracellular ion composition and mode of activation of CFTR. Furthermore, the correlation between the HCO_3^- conductance of different mutants of CFTR and the severity of pancreatic disease in CF suggest a significant role for CFTR in managing pH and epithelial HCO_3^- transport in normal and disease conditions.

Methods

We isolated single sweat ducts from fresh biopsies of human skin. Segments of ducts greater than 1,000 μm were microperfused with a double barrel luminal micropipette that served to perfuse and record transepithelial voltage on one side and to pass constant current pulses on the other. This arrangement allowed estimation of the specific membrane conductance from the cable equation [13]. After confirming the integrity of the perfused tubule, we applied 1,000-5,000 units/mL of alpha-toxin from *Staphylococcus aureus* to the bath solution in order to selectively permeabilize the basolateral membrane [14]. This procedure leaves the epithelium with an intact and functional apical membrane and a non-selective basal membrane permeable to molecules of up to about 5,000 mwu. Since activation of CFTR is exquisitely sensitive to

ATP and cAMP, its activity can be readily controlled in this preparation by controlling the presence of either of these nucleotides in the cytosolic bathing solution. That is, addition of 10 μM cAMP plus 5 mM ATP activates CFTR in seconds whereas removal of either cAMP or ATP from the cytosolic bath deactivates the channel. We then determined the permeability of the apical membrane to HCO_3^- and Cl^- relative to the impermeant gluconate anion by measuring the transapical membrane diffusion potential generated by chemical gradients for these anions and the simultaneous changes in membrane conductances.

Our general experimental protocol was to first inactivate CFTR and perfuse the cytosol with K-gluconate or K-glutamate while changing the composition of the luminal perfusate from gluconate to Cl^- to HCO_3^-, not necessarily in that order (we used the K^+ salt, the duct apical membrane is impermeable to K^+). We then activated CFTR and repeated the luminal perfusate changes. Next, we deactivated CFTR by withdrawing ATP and/or cAMP and continued to perfuse the lumen of the tubule with 150 mM K-gluconate while we proceeded to change the composition of the cytosolic bath from gluconate to Cl^- to HCO_3^- as before. These maneuvers were repeated again after activating CFTR a second time and the transepithelial diffusion potential differences and conductances were recorded after each change on either side of the membrane. The fact that there were no significant changes in electrical parameters until cAMP and ATP were added provides strong evidence that CFTR is the only significant, activatable ionic conductance in the membrane under these conditions. After establishing that there were no significant changes in specific conductance or diffusion potential through the apical membrane so long as CFTR remained inactivated, we omitted these steps in the protocol in order to increase experimental efficiency.

The luminal perfusion Ringer's solutions contained (in mM) NaCl (150), K (5), PO_4 (3.5), $MgSO_4$ (1.2), Ca^{2+} (1), and amiloride (0.01), pH. 7.4. Cl^- free luminal Ringer's solution was prepared by complete substitution of Cl^- with impermeant anion gluconate. The cytoplasmic/bath solution contained K (145), gluconate (140), PO_4 (3.5), $MgSO_4$ (1.2), and 260 μM Ca^{2+} buffered with 2.0 mM (EGTA: Sigma, St. Luis, USA) to 80 nM free Ca^{2+}, pH 6.8. Glutamate (140 mM), K^+ ATP (5), and cAMP (0.1), were added to the cytoplasmic bath as needed. After permeabilization of the basolateral membrane of the duct, we have perfused lumen either with KCl, K-gluconate or $KHCO_3$ (140 mM each).

Key words Adenosine Triphosphate; Bicarbonates; Chlorides; Cyclic AMP; Cystic Fibrosis; Cystic Fibrosis Transmembrane Conductance Regulator; Epithelial Cells; Glutamic Acid; Ion Transport; Permeability; Sweat Glands

Abbreviations AMP-PNP: 5'-adenylyl imidodiphosphate; CF: cystic fibrosis; CFTR: cystic fibrosis transmembrane conductance regulator; DIDS: 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid; Glu: gluconate

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