Genetic Disorders of Membrane Transport II. Regulation of CFTR by small molecules including HCO_3^-*

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Illek, Beate, Horst Fischer, and Terry E. Machen. Genetic Disorders of Membrane Transport. II. Regulation of CFTR by small molecules including HCO_3^-. *Am. J. Physiol.* 275 (*Gastrointest. Liver Physiol.* 38): G1221–G1226, 1998.—Cystic fibrosis (CF) affects a number of epithelial tissues, including those in the gastrointestinal tract. The goal of this review is to summarize data related to regulation of the protein product of the CF gene, CF transmembrane conductance regulator (CFTR), by a variety of small molecules. There has been a surge of interest in discovering small molecules that could be exogenously added to cells and tissues to regulate CFTR and could potentially be used alone or in combination with genetic approaches for therapy in CF. We will discuss the apparent mechanisms of action of genistein, milrinone, 8-cyclopentyl-1,3-dipropylxanthine, IBMX, and NS-004; several of which appear to interact directly with one or both nucleotide binding domains of CFTR. We also discuss how HCO_3^- interacts with CFTR as both a permeating anion and a potential regulator of Cl^- permeation through the CFTR ion channel. It is likely that there are complicated interactions between Cl^- and HCO_3^- in the secretion of both ions through the CFTR and the anion exchanger in intestinal cells, and these may yield a role of CFTR in regulation of intestinal HCO_3^- secretion as well as of intra- and extracellular pH.

cystic fibrosis; cystic fibrosis transmembrane conductance regulator; pharmacology; epithelial transport; chloride secretion

CYSTIC FIBROSIS (CF) is the most common fatal genetic disease of the Caucasian population, with an incidence of 1 in 2,500 live births and a carrier frequency of ~1 in 25. The disease is caused by mutations in the CF transmembrane conductance regulator (CFTR) (30), which functions as a cAMP-activated Cl^- channel. At the cellular level, CFTR dysfunction results in defective cAMP-regulated Cl^- conductance, primarily in cells of epithelial origin (27). Although lung disease is the primary cause of mortality in CF patients, a significant proportion of the morbidity can be directly attributed to gastrointestinal complications. The duodenum, jejunum, ileum, and colon express high levels of CFTR mRNA (30). Immunocytochemical analysis also demonstrated high CFTR protein expression at the luminal surfaces along the intestine (7). The small intestine of CF patients exhibits decreased Cl^- and fluid secretion that results in meconium ileus (lower water content and higher viscosity compared with non-CF patients) in ~10% of all CF newborns and accumulation of mucus and intestinal obstructions [primarily in ileocecum and large intestine in >20% of adult patients (see Ref. 13)]. Intestinal pathophysiology appears to be the hallmark of recently developed transgenic CF mouse models. The ileocecum and large intestine appear to be the most common sites of intestinal blockade, whereas jejunal obstructions occur less frequently (13).

Thus CFTR plays an important role in intestinal secretion. This secretion is controlled by multiple hormones and nerve cell transmitters, which will couple to the activation of CFTR as well as the other ion channels and transporters that are required to generate transepithelial secretion. Phosphorylation of CFTR by protein kinases (PK) and dephosphorylation by protein phosphatases (PP) is considered the major way by which CFTR Cl^- channel activity is physiologically regulated. In addition, the normal gating cycle (both opening and closing) of CFTR requires ATP binding and hydrolysis at the two nucleotide binding domains (NBDs) of CFTR (see Ref. 11). ATP concentration is likely maintained constant in cells and is therefore not a significant physiological regulator.

In addition to phosphorylation/dephosphorylation and ATP binding and hydrolysis, investigators have searched for exogenous compounds that are potential therapeutic activators of the mutant CFTR protein found in CF patients. Figure 1 shows the chemical structures of several such small molecules that will be discussed in this review. Some of these small molecules appear to bind directly to one or both of the NBDs of CFTR and, if CFTR has been prephosphorylated, increase the open probability of the channel. In the first portion of this review, PHARMACOLOGICAL REGULATION OF CFTR BY SMALL MOLECULES, we will discuss work showing that genistein, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), IBMX and other xanthines, and NS-004 all seem to operate this way. In addition, we will discuss the phosphodiesterase (PDE) inhibitor milrinone, which also stimulates CFTR but through a mechanism that is less well characterized.

***Second in a series of invited articles on Genetic Disorders of Membrane Transport**

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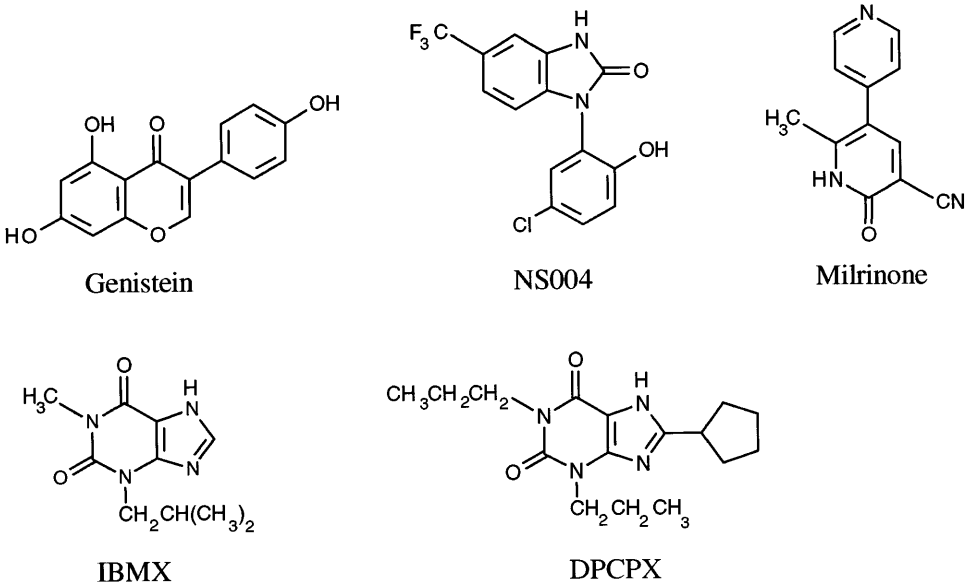

Fig. 1. Chemical structures of compounds discussed in text. DPCPX, 8-cyclopentyl-1,3-dipropylxanthine.

In the second portion of this review, HCO_3^- PERMEATION AND POSSIBLE REGULATION OF CFTR-MEDIATED Cl^- CURRENTS, we will discuss the question of HCO_3^- permeation and potential regulation of CFTR. This has been a controversial area of research. Most secretory epithelia secrete both Cl^- and HCO_3^-, and CFTR seems to be involved in a critical way that is still poorly understood. We will discuss data relevant to the question of whether the key role of CFTR is to conduct HCO_3^- itself or to play some sort of permissive role in HCO_3^- secretion through an adjacent anion exchanger that requires Cl^- at the cell's luminal aspect to exchange for cellular HCO_3^-. Alternatively, CFTR could also be required to keep cellular Cl^- concentration low enough that Cl^- enters the cell on the exchanger and exchanges for cellular HCO_3^-. We will also discuss the possibility that HCO_3^- may regulate the activity of CFTR by altering the permeation of Cl^- due to the multi-ion pore behavior of CFTR.

PHARMACOLOGICAL REGULATION OF CFTR BY SMALL MOLECULES

Genistein. Genistein is a member of the large class of naturally occurring flavonoids. Some flavonoids are more potent than genistein in activating CFTR (19), but we will focus on genistein because it has been investigated the most thoroughly. Genistein is a potent activator of CFTR (e.g., Ref. 20, 22), but the activating mechanism has been debated. Genistein did not seem to operate through activation of protein kinase A (PKA), PKC, or PKG, because there were no increases in intracellular cAMP, Ca^{2+}, or cGMP concentrations (21, 32). Genistein is a well-known tyrosine kinase (PTK) inhibitor, and two other PTK inhibitors (tyrphostin 47 and tyrphostin B42) also stimulated CFTR, and genistein-stimulated channel activity was blocked by the tyrosine phosphatase inhibitor vanadate (21, 32). However, several other PTK inhibitors (tyrphostin A23, tyrphostin A51, erbstatin analog, and herbimycin) failed to stimulate CFTR (20). Genistein also stimulated CFTR equally well when GTP (a poor substrate for tyrosine kinases) was used as a replacement for ATP (10). Some results suggested that genistein activated CFTR by blocking a PP: inactivation rates of CFTR currents on cAMP removal were markedly decreased by genistein (20), and CFTR was phosphorylated on the same amino acids by both genistein (which did not raise cAMP concentration) and forskolin (which did) (29).

More recent data have indicated that the most likely explanation for the stimulatory effect of genistein was through direct binding to an NBD of phosphorylated CFTR. Genistein did not directly open CFTR channels, because genistein did not stimulate CFTR in ATP- or cAMP-depleted monolayers (22). Similarly, genistein activation of CFTR in excised patches required both ATP and prior phosphorylation by PKA (10). In addition, genistein stimulated phosphatase-resistant, thiophosphorylated CFTR, even in the presence of PKI (to block PKA) or VO_4 (to block PP) (10, 39). Kinetic analysis indicated that a genistein concentration of <35 μM induced prolonged openings of CFTR (similar to the effect of a nonhydrolyzable ATP analog), whereas a genistein concentration of >35 μM caused prolonged closed times (38).

An attractive model for stimulatory and inhibitory effects of genistein on CFTR is that of Gadsby et al. (11) for CFTR gating. This model (11) proposes that ATP binding and hydrolysis at NBD1 control channel opening and closing at low phosphorylation levels, and once ATP hydrolysis at NBD1 has opened a highly phosphorylated CFTR channel, ATP binding to NBD2 can stabilize the open state. Hydrolysis of the nucleotide at NBD2 terminates the stabilization. Agents that interfere with ATP hydrolysis at NBD2 [e.g., 5′-adenylylimidodiphosphate (AMP-PNP) or pyrophosphate] induce prolonged burst opening. Genistein is a competitive inhibitor with ATP for binding to PTK (inhibitor constant, 13.7 μM) and to other ATP-binding proteins, and

crystal structure analysis of the Src family tyrosine kinase Hck revealed that the structurally related flavone and CFTR activator quercetin (19) localized to the binding site for the adenine ring of AMP-PNP or ATP (34). When considering the sequence homology shared by Walker A-type-binding motifs of PTK and NBDs of CFTR, it therefore seems possible that genistein competes for ATP-binding sites on CFTR, possibly at NBD2 (10), thereby slowing down ATP hydrolysis and preventing CFTR channel closure. It is unlikely that genistein activates CFTR by binding to a Src kinase, because this would require activation of a tyrosine kinase and genistein blocks tyrosine kinases. It might also be proposed that higher concentrations of genistein inhibit CFTR by competing with ATP at NBD1 (which may have a lower affinity for genistein binding) and prevent CFTR from opening.

NS-004. The substituted benzimidazolone NS-004 has effects that are quite similar to those exhibited by genistein. NS-004 activated CFTR (at 0.1 to 1 μM) and ΔF508-CFTR (at >10 μM) without raising cAMP concentration and also in the presence of a PKA inhibitor (12), but it was necessary to pretreat cells with forskolin before patch excision to observe CFTR channel activation by NS-004. In addition, the drug caused active CFTR channels to increase their activity, but it had no effect when added after channel rundown, indicating that NS-004 required phosphorylated CFTR. NS-004 did not inhibit in vitro PP activity. Thus, although NS-004 was thought to operate by directly opening CFTR (12), the drug may use a mechanism similar to that of genistein to increase open probabilities of phosphorylated, active channels without affecting either PK or PP.

Milrinone. The cardiotonic drug milrinone is an inhibitor of cGMP-inhibited PDE (a class III PDE) and was selected from several drugs inhibiting PDE isozymes (23). Because PDE inhibitors raise cellular cAMP concentration and CFTR is activated by increases in cAMP concentration, it has been assumed that milrinone (and also IBMX, see below) stimulate CFTR by raising cAMP. However, the exact mechanisms have not been determined. Milrinone activated both normal and ΔF508-CFTR in transformed nasal epithelial cells (24), and a combination of forskolin and milrinone (but not milrinone alone) increased the potential difference across nasal epithelium of ΔF508-CFTR mice in vivo but not in transgenic mice lacking CFTR (24). Milrinone activated CFTR even when cells had been maximally stimulated with forskolin or some other cAMP agonist, indicating that the drug likely had other effects than to stimulate production of cAMP.

IBMX, DPCPX, and other xanthine derivatives. IBMX is an inhibitor of PDE with broad specificity. Despite its broad use as a PDE inhibitor, it seems unlikely that IBMX works solely via this mechanism. IBMX activated CFTR even when cells had been maximally stimulated with forskolin or some other cAMP agonist (23). Although other substituted xanthine derivatives activated CFTR, there was no correlation between effects on CFTR and cellular cAMP and ATP concentrations (4). We have also found, through use of a fluorescence assay for PKA activity (as opposed to measurements of cAMP concentration using RIA), that forskolin caused fibroblasts to raise cAMP concentration to such a high level that PKA activity was saturated (by maximally binding cAMP), and IBMX (which indeed raised cAMP concentration) had no further effect to increase PKA activity (B. Eckert and T. E. Machen, unpublished observations). It therefore seems likely that the stimulatory effects of IBMX on CFTR are due to combined effects to raise cAMP (when PKA has not been saturated) and also to block PP or directly bind to CFTR.

The xanthine derivative DPCPX is an adenosine A_1 antagonist that may have some selectivity for ΔF508-CFTR compared with effects on wild-type CFTR. DPCPX stimulates ΔF508-CFTR channels in a variety of epithelial and fibroblast cells (8, 15). In some cells, correction of the CF defect by transfection with the CFTR gene rendered the transfected cells insensitive to DPCPX. In contrast, DPCPX stimulated recombinant CFTR channels from HEK cell microsomal membranes incorporated into planar lipid layers (2). The effects of DPCPX seemed not to be due to an effect on A_1 receptors, because Northern analysis showed that a DPCPX-sensitive cell line (CFPAC-1) had no A_1 receptor mRNA, and another xanthine analog 1,3-diallyl-8-cyclohexylxanthine (DAX) was a potent CFTR activator but a poor A_1 adenosine receptor antagonist. Using a rapid membrane filtration assay, Cohen et al. (6) reported that DPCPX bound to NBD1 of ΔF508-CFTR [dissociation constant (K_d) = 1.0 nM] with an affinity 17 times higher than that to NBD1 of wild-type CFTR (K_d = 17.0 nM) with a rank order among different xanthines of DAX > DPCPX > caffeine > DA-DPCPX > adenosine >> IBMX > 2-thio-DPCPX. These results were consistent with a unique binding site for DPCPX and related xanthines on NBD1 of CFTR in the vicinity of F508 (2). DPCPX was ~25-fold more potent than IBMX in potentiating ΔF508-CFTR by a mechanism other than elevation of intracellular cAMP concentration (15). Stimulation of CFTR by DPCPX or DAX required prephosphorylated CFTR (2). Thus DPCPX (and likely all the other xanthines, including IBMX), genistein, and NS-004 all seemed to activate prephosphorylated CFTR by binding directly to CFTR. DPCPX appeared to bind to NBD1 rather than to NBD2, and it also seemed to be more selective for ΔF508-CFTR than wild-type CFTR.

Pharmacological stimulation of mutant CFTR and therapeutic potential. The most common mutation in CF, ΔF508-CFTR, leads to a trafficking-impaired protein that gets degraded in the cell (40). Some mutations lead to trafficking-competent, but misfunctional, proteins (e.g., G551D-CFTR; Ref. 40). It appears that mutants with different misfunctions will need selective treatments. For example, genistein failed to activate ΔF508-CFTR in CF bronchial epithelial cells (22), but addition of genistein and cAMP agonists to NIH/3T3 cells overexpressing ΔF508-CFTR caused a strong, synergistic activation (18). Alternatively, when CF nasal epithelial cells were treated with 4-phenylbutyrate

or low temperature to increase expression of ΔF508-CFTR in the membrane (31, 40), genistein was a potent stimulant (9). In addition, the trafficking-competent G551D-CFTR mutant expressed in fibroblasts was stimulated by the combination of genistein and forskolin when forskolin alone was ineffective (9). These data indicate that genistein targets CFTR mutants present in the plasma membrane. Both NS-004 and, especially, DPCPX have also been shown to activate ΔF508-CFTR. IBMX produced a small, CFTR-related secretory response in jejunum, cecum, and rectum from G551D mice but had no effect in the nasal epithelium (36). NS-004 restored near normal channel activity from P574H-CFTR (a mild, trafficking-impaired mutation in NBD1) (3). These results suggest that, in addition to their direct effects on CFTR, these drugs may also have other effects that increase the number of channel proteins found in the membrane. It is also important to remember that there may be tissue-specific effects of the drugs, because epithelial cells will require concomitant activity of basolateral K^+ channels to secrete fluid efficiently.

HCO_3^- PERMEATION AND POSSIBLE REGULATION OF CFTR-MEDIATED Cl^- CURRENTS

Background: Role of CFTR in HCO_3^- secretion across intestine. HCO_3^- secretion is a key function that occurs in the stomach, pancreas, and small and large intestine. HCO_3^- secretion is particularly important in the duodenum to protect the intestinal mucosa against damage from large amounts of acid from the stomach (1). Transepithelial secretion of HCO_3^- likely involves the concerted activities of an apical anion channel, Cl^-/HCO_3^- exchange, HCO_3^- uptake across the basolateral membrane, and/or production of HCO_3^- by intracellular carbonic anhydrase activity. The paracellular pathway may also contribute, since a transepithelial gradient for HCO_3^- favoring secretion occurs. Electrogenic HCO_3^- secretion is activated by secretagogues that increase intracellular cAMP, cGMP, or Ca^{2+} concentrations (1), and this HCO_3^- secretion required the presence of CFTR in all segments of the small intestine in mice (16, 17, 33). However, the specific mechanism(s) involved remains a mystery. With the use of the pH stat method in combination with inhibitors of anion exchange and CFTR, recent work (5) with duodenum from normal and CFTR knockout mice shows that CFTR may have contributed to HCO_3^- secretion in two ways: directly by conducting HCO_3^- into the lumen and also indirectly by conducting Cl^- into the lumen, which could be recycled back into the cell via anion exchange for HCO_3^-. Because CFTR is expressed at highest levels in the crypts (7), electrogenic HCO_3^- secretion is predicted to occur primarily in this region. In contrast, CFTR is expressed at lower levels and carbonic anhydrase activity is expressed at higher levels in the villus epithelium, so CFTR-coupled Cl^-/HCO_3^- exchange activity may be primarily localized to the villus.

Does CFTR conduct HCO_3^-? Patch-clamp studies showed that HCO_3^- was conducted through CFTR in NIH/3T3 cells recombinantly expressing wild-type CFTR (26), with a permeability ratio of Cl^- to HCO_3^- of 0.25. Similar measurements have also been presented recently by Linsdell et al. (25), who found a permeability ratio of Cl^- to HCO_3^- of 0.14. We also measured CFTR-mediated permeation of HCO_3^- across the apical membrane of Calu-3 monolayers, and conductance ratios of cAMP-stimulated HCO_3^- and Cl^- currents of 0.1–0.27 were obtained (22). Thus HCO_3^--to-Cl^- permeability or conductance ratios of CFTR ranged from 0.10 to 0.27. A role of CFTR's function as a HCO_3^- conductor has also been suggested in CF models using a variety of methods comparing wild-type CFTR-expressing cells with ΔF508-CFTR-expressing CF cells (22, 35) or wild-type with CFTR knockout mice (CFTR −/−) (5, 13, 17, 33). These reports suggested the possibility that a defect in HCO_3^- secretion through CFTR may contribute to the pathophysiology of CF pulmonary disease (22, 35). In contrast to these experiments that showed that CFTR conducts HCO_3^-, Quinton and Reddy (28) (P. Quinton, personal communication) showed using microelectrode methods that in intact sweat ducts HCO_3^- was equally impermeant through CFTR as gluconate. This contradiction remains unresolved.

Does CFTR contribute to cytosolic and/or extracellular pH regulation? If CFTR is permeant to HCO_3^-, it is expected that it should contribute to movements of HCO_3^- into and out of the cell and thereby alter pH of the cell or extracellular fluids. In NIH/3T3 cells expressing exogenous CFTR, we showed forskolin-stimulated changes of pH that were absent in cells expressing ΔF508-CFTR (26). Data have also been presented for biliary epithelial cells showing that CFTR may regulate the activity of the anion exchanger and alter cytosolic pH regulatory ability in these cells (14) In contrast, we have recently compared cytosolic pH regulation in CF and CFTR-corrected nasal cells and found no apparent differences (L. Lu, E. Wunderlich, and T. E. Machen, unpublished results). A possible explanation was that these cells expressed Na^+/H^+ and Cl^-/HCO_3^- exchange and Na^+-HCO_3^- cotransport, and these mechanisms were much more prominent than CFTR in regulating cytosolic pH. Thus, in cells that express prominent activity of transporters that regulate cytosolic pH, the conductive pathway for HCO_3^- across CFTR might be of minor importance for cytosolic pH regulation. However, CFTR might still contribute importantly to the accumulation of HCO_3^- in the poorly buffered apical/luminal fluid.

Do anions regulate Cl^- permeation through CFTR? It has been well described that thiocyanate (SCN) is highly permeant across CFTR, but when mixtures of Cl^- and SCN are used, the conductance of CFTR decreases (37). This effect has been interpreted in terms of multi-ion pore behavior of CFTR, i.e., interactions of anions within the pore can lead to anomalous effects. Also, the halide anion I^- both permeated and blocked CFTR. We have similarly shown that both F^- and HCO_3^- added to the apical surface reduced Cl^- conductance of CFTR in Calu-3 cells (Illek and Machen,

unpublished observations). In vivo both Cl^- and HCO_3^- are present in the cytosol at similar concentrations, and both are driven by the membrane potential into the pore of CFTR, suggesting that both ions may compete for common binding sites in the permeation path, and thus affect the permeation of each other. This could be particularly important for tissues that secrete large amounts of HCO_3^-.

Summary: Role of CFTR in HCO_3^- secretion. The exact role of CFTR in HCO_3^- permeation under physiological conditions likely depends on the relative expression of CFTR and other HCO_3^- channels, HCO_3^- transporters and paracellular permeability of HCO_3^-. In addition, the cell-to-lumen electrochemical driving forces for Cl^- and HCO_3^- will determine ion movements across the luminal cell membrane and tight junctions. CFTR appears to be important both as a HCO_3^- conductance and also in some way as a direct or indirect regulator of adjacent anion exchange, e.g., as a source of luminal Cl^- that may exchange for cellular HCO_3^- and/or as a means of keeping cellular Cl^- concentration low enough that the anion exchanger operates as an HCO_3^- secretion mechanism. Many CFTR-expressing epithelia, including the intestine, pancreas, and liver, exhibit different gradients of Cl^- and HCO_3^- along the length of the organ, so these relationships will also be affected by the anatomic structures.

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