Schultz, B. D., A. K. Singh, D. C. Devor, and R. J. Bridges. Pharmacology of CFTR Chloride Channel Activity. Physiol. Rev. 79, Suppl.: S109–S144, 1999.—The pharmacology of cystic fibrosis transmembrane conductance regulator (CFTR) is at an early stage of development. Here we attempt to review the status of those compounds that modulate the Cl⁻ channel activity of CFTR. Three classes of compounds, the sulfonylureas, the disulfonic stilbenes, and the arylaminobenzoates, have been shown to directly interact with CFTR to cause channel blockade. Kinetic analysis has revealed the sulfonylureas and arylaminobenzoates interact with the open state of CFTR to cause blockade. Suggestive evidence indicates the disulfonic stilbenes act by a similar mechanism but only from the intracellular side of CFTR. Site-directed mutagenesis studies indicate the involvement of specific amino acid residues in the proposed transmembrane segment 6 for disulfonic stilbene blockade and segments 6 and 12 for arylaminobenzoate blockade. Unfortunately, these compounds (sulfonylureas, disulfonic stilbenes, arylaminobenzoate) also act at a number of other cellular sites that can indirectly alter the activity of CFTR or the transepithelial secretion of Cl⁻. The nonspecificity of these compounds has complicated the interpretation of results from cellular-based experiments. Compounds that increase the activity of CFTR include the alkylxanthines, phosphodiesterase inhibitors, phosphatase inhibitors, isoflavones and flavones (genistein), benzimidazolones, and psoralens. Channel activation can arise from the stimulation of the cAMP signal transduction cascade, the inhibition of inactivating enzymes (phosphodiesterases, phosphatases), as well as the direct binding to CFTR. However, in contrast to the compounds that block CFTR, a detailed understanding of how the above compounds increase the activity of CFTR has not yet emerged.

I. INTRODUCTION

Experimentally, there is a considerable need for specific high-affinity ligands that could be used to probe the structure and function of the cystic fibrosis transmembrane conductance regulator (CFTR). Insights gained from such experimentation would allow us to better understand CFTR’s role in cell biology, physiology, and ultimately the underlying mechanisms leading to clinical pathology. There is little doubt that CFTR is the Cl⁻ channel that mediates cAMP-dependent Cl⁻ secretion in various epithelia. The availability of potent and specific CFTR modulators would greatly aid our understanding of how the different domains of CFTR interact to form a protein kinase A- and ATP-regulated Cl⁻ channel; the mechanisms of anion conduction; how CFTR interacts with other ion channels; the role CFTR plays in intracellular compartments to regulate pH, glycoprotein sulfation, and vesicle fusion; as well as how mutations in CFTR cause cystic fibrosis (CF). Equally important will be the therapeutic impact of CFTR modulators in the treatment of respiratory disorders including CF, chronic obstructive pulmonary disease, asthma, bronchitis, emphysema, pneumonia as well as secretory diarrhea, polycystic kidney disease and reproductive dysfunctions (congenital bilateral absence of the vas deferens, Ref. 270; testicular and sperm...
abnormalities, Ref. 88). Compared with cation channels, nature has not been generous in providing Cl⁻ channel modulators. Here we attempt to review the status of those compounds thought to interact with or modulate the Cl⁻ channel activity of CFTR. The presentation is divided into two main sections discussing those compounds that block CFTR and those that open or increase the activity of CFTR.

The channel activity of CFTR can be altered at multiple levels (Fig. 1). Because CFTR is activated by the cAMP signal transduction pathway, alterations in the binding of an agonist to its receptor, the G protein-mediated activation of adenylyl cyclase, the hydrolysis of cAMP by phosphodiesterase (PDE), and the activity of protein kinases or of protein phosphatases can influence the activity of CFTR. The opening and closing of CFTR is also dependent on ATP and ADP (sects. III and IV) and affected by the cellular redox potential (373). Thus changes in cellular metabolism can influence the activity of CFTR. Therefore, a compound may act at one or more of these sites of action as well as bind directly to CFTR to alter channel gating. Studies using compounds to inhibit or increase the activity of CFTR must consider each of these potential sites of action when attempting to interpret results from cellular-based assays of CFTR channel activity such as 125I efflux, 6-methoxy-N-(3-sulfopropyl)quinoline (SPQ) fluorescence, or whole cell membrane patch recordings. The transepithelial secretion of Cl⁻ also depends on three basolateral membrane proteins: the Na⁺-K⁺-ATPase to maintain a Na⁺ gradient, the Na⁺-K⁺-2Cl⁻ cotransporter for Cl⁻ entry into the cell, and K⁺ channels to recycle K⁺ and maintain the necessary membrane potential to drive Cl⁻ exit across apical membrane Cl⁻ channels (24, 154). At least three biophysically and pharmacologically distinct types of K⁺ channels are thought to contribute to the basolateral membrane K⁺ conductance: a cAMP-activated K⁺ channel, a Ca²⁺-activated K⁺ channel, and a maxi K⁺ channel. In addition to CFTR, two other Cl⁻ channels, an outwardly rectifying Cl⁻ channel (ORCC) and a Ca²⁺-activated Cl⁻ channel, are reported to contribute to the apical membrane Cl⁻ conductance. Other Cl⁻ conductances such as members of the Cl⁻ channel gene family (CIC) and voltage- and osmolyte-sensitive anion conductance have not historically received as much attention, and thus their relative contribution to transepithelial ion movement remains to be defined (193, 309). The activities of each of these basolateral and apical membrane transporters and channels are tightly coordinated in the regulated secretion of Cl⁻. Thus, in addition to effects on the signal transduction pathways, compounds may have inhibitory or stimulatory effects on one or more of these transporters and channels and thereby alter the secretion of Cl⁻. In general, many of the compounds that have been used to alter the channel activity of CFTR are not specific for CFTR. We attempt, in this review, to present the pharmacology of these compounds, specifically, the documented actions of these compounds at sites other than CFTR. Our intent is to provide a basis upon which one may formulate an informed interpretation of the sometimes confusing array of published observations using these compounds as well as to assist in the design of future studies with these compounds.

The pharmacology of CFTR is driven in large measure by the desire to develop agents that will be useful in the treatment of CF. Since the discovery of the gene coding for CFTR (201, 302, 306), over 700 mutations that cause

![Diagram of a Cl⁻ secretory epithelial cell showing selected transporters and channels that participate in transepithelial Cl⁻ secretion.](http://physrev.physiology.org/)

**FIG. 1.** Schematic diagram of a Cl⁻ secretory epithelial cell showing selected transporters and channels that participate in transepithelial Cl⁻ secretion. Included are selected second messenger pathways (cAMP cascade and Ca²⁺ cascades) reported to affect ion transport mechanisms. Note that each step in these pathways and transport proteins themselves are susceptible to pharmacological modulation as discussed in text. Four types of Cl⁻ channels are indicated in apical membrane: ClORCC, an outwardly rectifying Cl⁻ channel; ClVOL, a volume-regulated Cl⁻ channel that may also be present in basolateral membrane; ClCa, a Ca²⁺-activated Cl⁻ channel, and ClCFTR, a cAMP/protein kinase A (PKA)-activated Cl⁻ channel. Molecular identities of ClORCC, ClVOL, and ClCa are not known, nor is relative contribution of various Cl⁻ channels in response to different secretory agonists. In addition to Na⁺-K⁺-2Cl⁻ co-transport and Na⁺-K⁺-ATPase, 3 different K⁺ channels are shown in basolateral membrane: Kbas, a large-conductance K⁺ channel; KAMP, a cAMP-activated K⁺ channel; and Kca, a Ca²⁺-activated K⁺ channel. Evidence suggests Kbas corresponds to recently cloned HIK-1 K⁺ channel, whereas molecular identities of KAMP and Kca remain unknown. In addition to binding to basolateral membrane receptors, agonists may also bind to apical membrane receptors (not shown) to stimulate Cl⁻ secretion. Agonists that elevate cAMP can do so by activating one of several isoforms of adenylyl cyclase, some of which are regulated by intracellular Ca²⁺, and in turn intracellular Ca²⁺ can be regulated by cAMP levels. Thus, in addition to various isoforms of PKA, protein kinase C, protein phosphatases (PPhase), and phosphodiesterases (PDE), the two signal transduction cascades can interact at the second messenger level as well as the transport protein, ion channel level to regulate secretion of Cl⁻.
CF have been reported to the CF Genetic Analysis Consortium (13; accessible electronically at http://www.genet.sickkids.on.ca). Mutations that cause one or more problems in the transcription, translation, protein processing, or channel activity of CFTR have been described (384, 395, 423, 424, 442). The end result of the mutations in CFTR is an alteration in fluid and electrolyte transport in the epithelia of the sweat glands, pancreas, intestines, reproductive organs, and airways. One approach to the pharmacological treatment of CF is to restore normal function to the mutant CFTR protein. Although the debate continues on how many functions CFTR serves, most investigators do agree the restoration of CFTR Cl⁻ channel activity in the apical membrane of the affected epithelia is a desired goal. Unfortunately, the vast majority of CF-causing mutations result in the absence or reduced expression of apical membrane CFTR protein. Thus, for most mutations, one must develop a pharmacological means of facilitating the transcription, translation, or protein processing of CFTR to deliver a mature protein to the apical membrane. If, in addition, the mutation causes the channel to be dysfunctional, then the channel activity must also be pharmacologically modified to achieve normal function. The deletion of phenylalanine at position 508 (ΔF508) in the first nucleotide binding fold (NBF) is the single, most frequent CF-causing mutation, being present on ~67% of mutant alleles (193), although the relative proportion of specific mutations varies depending on the population of inference (307). The ΔF508 mutation causes both protein processing and channel activity to be dysfunctional (85, 89, 103, 159, 178). Therefore, the treatment of ΔF508 CF patients will require the correction of both the protein processing and channel activity defects of the ΔF508 CFTR protein.

Although two entirely different classes of compounds may be required to restore normal function to ΔF508 CFTR, specific high-affinity compounds that interact with CFTR to modulate channel activity may serve both purposes. This notion is illustrated by recent exciting studies on the multidrug resistance (MDR) protein P-glycoprotein (230). P-glycoprotein, like CFTR, is a member of the ATP binding cassette (ABC) superfamily of transport proteins. Several classes of compounds are known to be transported by or act as inhibitors of P-glycoprotein. Loo and Clarke (230) have recently shown that these same compounds can assist in the protein processing of mutant P-glycoprotein that would have otherwise not reached the plasma membrane. These results lend great promise to the hoped for discovery of CFTR-specific compounds that will improve the delivery of ΔF508 CFTR to the plasma membrane. So far, the only compounds known to bind to CFTR are those that alter channel activity. Thus the discovery and the development of compounds that can restore normal protein processing to some mutant forms of CFTR may result from the further development of specific and potent CFTR channel modulators.

The certainty that specific high-affinity CFTR channel modulators can be developed is supported by the pharmacology of another member of the ABC transporter superfamily, the sulfonylurea receptor (SUR). The sulfonylurea receptor is found in pancreatic β-cells (2) and in muscle cells (185), where it associates with Kir6.2 to form an ATP-sensitive K⁺ channel complex, KₐTP (184). In this complex, SUR provides for both nucleotide and high-affinity sulfonylurea sensitivity (146). The release of insulin from β-cells can be modulated by sulfonylureas, several of which bind to SUR with high affinity [e.g., dissociation constant (KᵯD) for glibenclamide <1 nM]. The development of specific high-affinity compounds like glibenclamide for SUR required 27 years after the discovery of hypoglycemic drugs and the synthesis of at least 12,000 derivatives by numerous pharmaceutical companies (14, 41, 252). A similar investment in effort may be required to obtain CFTR channel modulators of high potency and specificity. Certain to be of importance will be a detailed understanding of the kinetics and mechanism of action of the candidate compounds affecting CFTR channel activity. The intent of this review is to present our current understanding of the mechanisms of action of those compounds now known to affect CFTR channel activity. Although there are only a few classes of compounds and fewer still to which a mechanism can be ascribed, the list is growing, as is our understanding of their mechanisms of action. Thus, although still in its infancy, the pharmacology of CFTR Cl⁻ channels is progressing and is certain to yield significant future benefit.

II. CHANNEL BLOCKERS

A. Sulfonylureas and Diarylsulfonylureas

Sulfonyamide compounds were first developed and clinically employed for their bacteriostatic activities. When patients were being treated for typhoid with 2254 RP in 1942, it was noted that they became hypoglycemic (231, 422). In 1956, the sulfonylureas, carbamidate and tobutamide (Fig. 2), were identified as nonbacteriostatic, clinically useful hypoglycemic agents. Subsequently, in excess of 12,000 sulfonylurea compounds were synthesized and tested for their ability to treat diabetes mellitus (14). This quest for a higher affinity (e.g., <1 nM) hypoglycemic agent resulted in the discovery of glibenclamide (Fig. 2) some 13 years later (252) and diazoxide, a sulfonyamide with opposite effects, i.e., a hyperglycemic agent which proved useful for the treatment of insulinomas (14, 41). It was determined that the effects of sulfonylureas on pancreatic insulin secretion are mediated by the inhibition of a K⁺ channel that is physiologically stimulated by ADP and inhibited by ATP (KₐTP; Refs. 320, 321, 370). In 1995, 43 years after identifying hypoglycemic drugs and
26 years after identifying glibenclamide as a high-affinity modulator of insulin secretion, SUR was cloned and discovered to be a member of the ABC transporter superfamily of proteins (2, 287). More recently, it was shown that the SUR regulates the activity of a 38-kDa protein (Kir6.2) that copurifies with it and together function as the K\textsubscript{ATP} channel (184, 383). Extensive structure-activity relationships (SAR) for oncolytic activity remains undefined (150, 203, 285, 286, 311). Antidiabetic sulfonylurea derivatives are largely available from commercial sources. Oncolytic diarylsulfonylureas are not commercially available, although synthetic protocols have been published (171, 260).

Numerous sulfonylurea-based structures and associated synthetic protocols have been published during their development as antibacterial and antidiabetic drugs (69, 123). Extensive structure-activity relationships (SAR) for effects on insulin secretion and modulation of ATP-dependent K\textsuperscript{+} channels from a variety of laboratories indicate that the most potent sulfonylurea-based modulators include a cyclohexyl group linked to the urea portion of the sulfonylurea backbone and a para-substituted phenyl group linked to the sulfonyl portion (e.g., glibenclamide, Fig. 2; Ref. 219). Perhaps surprisingly, meglitinide, the benzoic acid derivative of glibenclamide, retains antidiabetic effects, indicating that the sulfonylurea moiety is not required, per se, for regulation of SUR/Kir6.2 (219). Furthermore, the para-aromatic acid or aliphatic amine present in numerous high-affinity modulators of SUR is not absolutely required on the sulfonyl phenyl, since tolbutamide (Fig. 2), which includes only a para-methyl substituent, is also an effective ligand, albeit at much higher concentrations. Rather, any combination of these moieties, when appropriately linked to a sulfonylurea backbone, provides for the highest potencies (219, 310). More recently, researchers at Lilly Laboratories have developed a subclass of sulfonylureas, the diarylsulfonylureas (Fig. 2), as oncolytic agents that do not universally have antidiabetic effects (170, 171, 260, 385). Although the SAR for oncolytic activity is seemingly extensive (171, 260), the oncolytic mechanism of action remains undefined (150, 203, 285, 286, 311). Antidiabetic sulfonylurea derivatives are largely available from commercial sources. Oncolytic diarylsulfonylureas are not commercially available, although synthetic protocols have been published (171, 260).

The realization that SUR is, like CFTR, a member of the ABC transporter family is one of many parallels that have been drawn between the SUR/Kir6.2 complex and CFTR. For example, both SUR/Kir6.2 and CFTR are reciprocally regulated by ATP and ADP. Intracellular ATP decreases and ADP increases the open probability of SUR/Kir6.2 (106, 120), whereas the reverse is true for CFTR (12, 333, 410, 432). In combination with these nucleotides, Mg\textsuperscript{2+} also modulates the gating of both CFTR and SUR/Kir6.2 (14, 152, 323). Sheppard and Welsh (347) first reported that antidiabetic sulfonylureas, compounds used to pharmacologically characterize SUR/Kir6.2, reduced
whole cell Cl\textsuperscript{−} currents in NIH 3T3 cells expressing CFTR. Blocker concentrations in excess of 1,000-fold greater than those reported to inhibit SUR/Kir6.2 were required to similarly affect CFTR, although the rank order of potency remained unchanged (glibenclamide > tolbutamide). Unfortunately, compounds that open SUR/Kir6.2 (diazoxide, minoxidil; Refs. 14, 131) caused a reduction in CFTR-dependent Cl\textsuperscript{−} conductance (347). Thus CFTR does not have an activator site comparable to the diazoxide-responsive site on SUR/Kir6.2, although the rank order of potency for glibenclamide and tolbutamide suggests that the sulfonylurea-sensitive site is weakly conserved.

Both glibenclamide and tolbutamide have been shown to reduce the open probability of CFTR in a structure-dependent and concentration-dependent manner by direct interaction with the channel as recorded in cell-free membrane patches from CFTR-expressing C127 cells, Madin-Darby canine kidney (MDCK) cells, HEK 293 cells, and I cells (324, 331, 346, 409). Inhibition of CFTR channel currents in excised membrane patches of HT-29 cells, T84 cells (293), and Xenopus oocytes (250) has also been reported. In each case, the effective concentration of glibenclamide was similar with the IC\textsubscript{50} or K\textsubscript{D} in the range of 2–30 \mu M. One discrepancy, however, is that reversal of effect with washout was rapid in mammalian expression systems (293, 324, 409) but failed to occur after an extended period in Xenopus oocytes (250). The lack of reversibility in Xenopus oocytes might be explained by channel rundown in the presence of glibenclamide or by other known effects of glibenclamide (see below), since Devor et al. (90) reported that inhibition by 300 \mu M glibenclamide was immediately reversible in shark rectal gland cells.

Kinetic analysis, modeling, and simulation of results employing glibenclamide and tolbutamide showed that the compounds reversibly interact exclusively with the open state of CFTR channels to interrupt Cl\textsuperscript{−} conduction (324, 346, 409). The blocking efficiency of glibenclamide is altered by changes in pH, which changes the proportion of glibenclamide in the anionic form, voltage, and extracellular Cl\textsuperscript{−} concentration (346). The simplest kinetic models to account for nucleotide- and kinase-dependent regulation of CFTR employ a four-state model of channel gating (64, 158, 333, 410, 432).

\[
\begin{align*}
\text{Closed} & \leftrightarrow \text{Closed} \leftrightarrow \\
\text{Inactivated} & \leftrightarrow \text{Activated} \leftrightarrow \\
\text{Closed} & \leftrightarrow \text{Open} \leftrightarrow \\
\text{Nucleotide bound} & \leftrightarrow \text{Nucleotide bound}
\end{align*}
\]

Thus the interaction of a sulfonylurea with the open state sequesters CFTR in an activated, albeit nonconducting, state and, in this linear scheme, reduces the likelihood of channel inactivation. The greater potency of glibenclamide compared with tolbutamide is chiefly attributable to a reduced off rate constant (k\textsubscript{off}) (<100 vs. 1,210 s\textsuperscript{−}1; Refs. 324, 346, 409), indicating that the glibenclamide-CFTR interaction is substantially more stable than the tolbutamide-CFTR interaction. From a theoretical and clinical perspective, these conclusions have significant ramifications. First, in the resting state, there is no expected effect of sulfonylureas on CFTR. Second, if activation is slowed or inactivation is accelerated by particular mutations as has been suggested (178, 322, 333, 428), then the effect of the sulfonylureas would be to prolong the duration which CFTR remains in an activated state before inactivation. All open-channel modulators would be expected to have similar effects on the state distribution, with the most potent compounds exhibiting the longest duration of interaction (i.e., the lowest k\textsubscript{off}). Most effective in the symptomatic treatment of CF would be compounds that similarly decrease the inactivation rate by interacting with the open state but maintain some ionic conductance. Additionally, any such compound must, unlike glibenclamide or tolbutamide, be shown to have a high degree of selectivity for CFTR (see below).

On the basis of the knowledge that sulfonylureas block CFTR by a direct interaction, it has become widely accepted to employ glibenclamide-dependent inhibition of anion transport to indicate that CFTR participates in a particular physiological system of interest (e.g., mouse intestinal crypt secretion, Ref. 400; guinea pig ventricular myocyte Cl\textsuperscript{−} currents, Refs. 388, 389; rat nephron terminal collecting duct, Ref. 175; human kidney cyst epithelial cells, Ref. 155; mMCD-K2 cells, Ref. 403; shark rectal gland cells, Ref. 90; NS004-, NS1619-, 1-EBIO-, and pso-ralen-stimulated secretion in T84 cells, Refs. 93, 94; protein kinase A (PKA)-stimulated Cl\textsuperscript{−} flux across rat nephron cortical brush-border membranes, Ref. 34; rat fetal lung epithelium, Ref. 377; and cAMP-stimulated \textsuperscript{125I} efflux from MDCK cells, Ref. 261). Alternatively, the lack of effect of glibenclamide has been used as an indicator that CFTR does not mediate the response in some systems (e.g., rat choroid plexus, Ref. 202; mouse mandibular salivary gland, Ref. 212; bovine pancreatic duct cells, Ref. 5; ATP-stimulated ion transport in rabbit tracheal epithelium, Ref. 189; and ATP-stimulated ion transport in epithelial cells from Mongolian gerbil middle ear, Ref. 127).

It must be emphasized that glibenclamide-dependent inhibition should not be used as a sole indicator of CFTR participation in a response. Because glibenclamide has a K\textsubscript{D} for the SUR of <10 nM and at micromolar concentrations has been shown to have multiple effects which include the inhibition of a variety of K\textsuperscript{+} channels (32, 110), the inhibition of numerous enzymes (62) including PKA (275), and the inhibition of other Cl\textsuperscript{−} channels (255, 293, 324, 438), caution must be exercised in interpreting effects in intact tissues. Both nonunith Hill coefficients for concentration-dependent inhibition and the inability to “wash
out” the inhibitory effect of glibenclamide might reflect concerted effects on intracellular enzymes including PKA rather than an ongoing binmolecular interaction with CFTR (90, 250, 347, 389). Likewise, a lack of effect or modest effect of glibenclamide can be misinterpreted to indicate that CFTR is not present. Ionic, pH, or voltage conditions can be set up such that either CFTR or glibenclamide is not in an optimal or permissive physicochemical confirmation for inhibition to occur (346). Additionally, one must be especially careful in interpreting experiments employing <30 µM glibenclamide, since, at these concentrations, a significant block of CFTR would not be expected in any situation. Although glibenclamide can effectively block CFTR and appears to be securely entrenched in CFTR literature, it is obvious that the field would benefit from a more potent and selective inhibitor of CFTR.

Evidence from our laboratory indicates that diarylsulfonylureas provide greater selectivity and slightly higher potency than glibenclamide for the inhibition of CFTR (328, 329, 331). The mechanism of action is identical to that presented above for glibenclamide and tolbutamide, interaction only with the open state of CFTR channels to interrupt Cl\(^-\) conduction. In contrast to glibenclamide, the diarylsulfonylureas have (by definition) substituted phenyl moieties linked to both the sulfonyl and urea of the sulfonylurea backbone (Fig. 2). The binding site of CFTR is like the SUR in that the pharmacophore does not require an aromatic acid or aliphatic amine linked to the sulfonyl phenyl group for functional interaction, e.g., tolbutamide. A p-methyl substituent on the sulfonyl phenyl appears to be adequate for interaction with the binding site (329, 409), although compounds including either benzofuran (LY-295501; Fig. 2) or indanyl (LY-186641; Fig. 2) groups linked to the sulfonyl have been shown to be effective (328). Potency of CFTR inhibition varies over more than three orders of magnitude depending on the urea-phenyl substituents. Electron-withdrawing substituents (e.g., in Fig. 2, \(R' = \text{Cl, NO}_2, \text{or CF}_3\)) at the para- and/or meta-positions of the urea phenyl have been shown to provide for higher affinity interactions than electron-donating or electroneutral substituents (e.g., \(R' = \text{OH or H}\)) (329). Both LY-186641 (p-chloro) and LY-295501 (m,p-dichloro) have been shown to block CFTR, but not to affect alternative Cl\(^-\) channels when expressed in \textit{Xenopus} oocytes [GABA\(_C\) (84, 348) and rabbit gastric CIC (CIC2G; Schultz, unpublished observation and Ref. 236)],

B. Disulfonic Stilbenes

Maddy (234) synthesized SITS (Fig. 2) as a general fluorescent, impermeant covalent modifier of membrane amino groups. Subsequently, SITS and other stilbene disulfonates (25) were recognized as novel inhibitors of band 3-mediated Cl\(^-\)/HCO\(_3\) anion exchange in human red blood cells (206). Much of the work done in understanding the reversible nature of inhibition by SITS revealed that the blockade of anion transport was not the result of intrinsic chemical modification, but rather of an “explicit”
interaction between the disulfonic stilbene probe and an apparent binding site (58). Since then, several purely non-covalent reversibly acting derivatives (e.g., 4,4’-dinitrostilbene-2,2’-disulfonic acid, DNDS; Fig. 2) and covalently acting derivatives (e.g., DIDS; Fig. 2) disulfonic stilbene derivatives have been designed and synthesized (58–60). These agents have played a critical role in the definitive identification of the anion exchanger protein band 3 (58–60, 222, 441).

The disulfonic stilbenes are among the most potent inhibitors of band 3- or AE1 (anion exchanger)-mediated (6) anion exchange. The inhibitory constants (IC_{50}) of various disulfonic stilbene derivatives for AE1 span a concentration range of over four orders of magnitude (0.8–500 μM, Ref. 57). Disulfonic stilbenes also inhibit the recently cloned AE2 and AE3 anion exchangers found in a variety of tissues (7, 87, 213, 216, 226). The backbone of this class of inhibitor consists of a trans-2,2’-disulfonic stilbene structure, with varying 4,4’-substituents (26, 58). A quantitative SAR of disulfonic stilbene derivatives revealed that there is a positive correlation with the Hammett constant (σ, a measure of the capacity to exchange electrons), and the Hansch constant (π, a measure of hydrophobicity) of the 4,4’-substituents. The reversible type of inhibition has been studied dynamically using various techniques such as spectrophotometry (111, 126), fluorimetry (58, 283, 412), and NMR (117, 130), or by utilizing radioactively labeled forms of disulfonic stilbenes (58–60, 222, 351). On the other hand, the irreversible type of binding with membrane components has been studied by radioactively labeled forms of disulfonic stilbenes or by Western blotting (57). Despite the advantages of using disulfonic stilbene derivatives with noncovalently reactive 4,4’-substitutions (e.g., DNDS), most studies have used the covalently reactive derivatives DIDS or SITS. The quality of commercially available DIDS and SITS is not uniform, because some companies provide them as free acids and others as sodium salts with varying degrees of cis/trans-isomerization and different ratios of NC5 and NH2 groups. Because of the hygroscopic properties and tendency or ease of intermolecular reactions of the NC5 group with NH2 groups, especially with sodium salts of the disulfonic stilbenes, polymerization can occur yielding DIDS-DIDS, DIDS-4,4’-diaminostilbene-2,2’-disulfonic acid (DADS) (Fig. 2), and DIDS-DADS-DIDS polymers. As suggested by Racker (294) “DIDS which often ‘dids’ more than advertised” should be used with caution.

The disulfonic stilbenes, most often DIDS, have been shown to block a wide variety of Cl- channels expressed in numerous cell types (1, 17–19, 38, 42, 43, 47, 71, 72, 96, 101, 136, 138, 160, 190, 191, 200, 204, 205, 224, 225, 255, 258, 259, 267, 279, 289, 304, 308, 315–317, 341, 343, 344, 400, 401, 411, 418, 434). Remarkably, DIDS does not block transepithelial Cl- secretion across rat colonic mucosa, trachea, or T84 monolayers (49, 340). Extracellular DIDS also fails to block CFTR-mediated whole cell Cl- currents (83, 99, 139, 140, 265, 300, 309, 336, 371, 372, 384, 403, 431). Indeed, CFTR is one of the very few Cl- channels that is not blocked by extracellular DIDS. The disulfonic stilbenes do block CFTR from the intracellular side (see below) but, because the sulfonate groups of the disulfonic stilbenes are present as fully ionized divalent anions at physiological pH (57), they do not penetrate the plasma membrane. The disulfonic stilbenes, preferably DNDS, could be used diagnostically to implicate CFTR-mediated Cl- secretion. However, because one anticipates a negative result from this type of experiment, additional positive controls are needed before one can conclude Cl- secretion is mediated by CFTR.

Although the disulfonic stilbenes do not appear to directly block CFTR from the extracellular side, they may by indirect means influence CFTR-mediated Cl- secretion. DIDS has been reported to increase short-circuit current and serosal-to-mucosal Cl- fluxes across stripped rabbit colonic mucosa (364). Basolateral addition of DIDS or SITS at a concentration between 10 and 200 μM has been reported to produce a transient increase in short-circuit current, which was followed by a gradual inhibition across T84 monolayers (48). This transient stimulation with DIDS was also observed for whole cell Cl- currents in isolated T84 cells grown on permeable supports as well as the human airway cell line Calu-3 (360) and was attributed to the elevation of cytosolic Ca2+ levels. Although the exact mechanism by which DIDS increases Ca2+ levels is not known, the fact remains that the disulfonic stilbenes are capable of elevating cytosolic free Ca2+ in both confluent as well as nonconfluent T84 epithelial cells (48). The mechanistic basis for the gradual decrease in short-circuit current in T84 cells by the disulfonic stilbenes is also unknown but might result from alteration in intracellular pH due to the inhibition of anion exchange. Disulfonic stilbenes have also been shown to effect various epithelial K+ channels such as I_{kr} (55, 342, 407), K_{ATP} (128), and the Ca2+-activated K+ channel in smooth muscle cells (167).

A disulfonic stilbene-sensitive epithelial Cl- channel that has received considerable attention by CF investigators is the ORCC. DNDS reversibly blocked the ORCC incorporated into planar lipid bilayers with a K_{D} of 2–3 μM when applied to the extracellular side, and DIDS caused an irreversible inhibition (49, 353). Structure-activity studies with the disulfonic stilbenes and a structurally similar class of compounds, the sulfonated calixarenes, have led to the development of a highly potent blocker (K_{D} = 0.6 nM) of ORCC, TS-TM-calix[4]arene (5,11,17-tetrasulfonato-25,26,27,28-tetramethoxy-calix[4]arene; Fig. 2; Ref. 358). Before the discovery of CFTR, ORCC was implicated as the Cl- channel whose PKA regulation was defective in CF (see Ref. 333a). Although it is now recognized that CFTR is the defective Cl- channel in CF, it has been suggested that CFTR regulates ORCC and that
both channels may contribute to transepithelial Cl\(^-\) secretion (179, 335, 336). Schwiebert et al. (335) made use of the differential sensitivity of CFTR and ORCC to DIDS blockade in short-circuit current studies on primary cultures of rat tracheal epithelial monolayers stimulated with cAMP-dependent agonists or ATP. DIDS (1 mM) and TS-TM-calix[4]arene (1 \(\mu\)M) inhibited 31 and 21%, respectively, of the short-circuit current stimulated by 8-bromo-cAMP (8-BrcAMP). Addition of hexokinase to remove any extracellular ATP caused a similar inhibition in the 8-BrcAMP-stimulated current. In contrast, DIDS and TS-TM-calix[4]arene inhibited nearly all, 95 and 70%, respectively, of the short-circuit current stimulated by ATP. Consistent with these results, Schweibert and co-workers (179, 335) proposed a model whereby extracellular ATP, released in response to cAMP stimulation, binds to a purinergic receptor that in turn activates ORCC. The inhibition in short-circuit current by DIDS and TS-TM calix[4]arene was interpreted as the blockade of ORCC and the remaining unblocked portion of the short-circuit current attributed to CFTR. Thus both ORCC and CFTR were suggested to contribute to transepithelial Cl\(^-\) secretion. There are a few caveats that warrant consideration with this interpretation. Most importantly, DIDS is a known antagonist of purinergic receptors (53, 54, 56, 104, 105, 116, 249, 257, 366, 430, 443). The inhibition constant (\(K_i\)) for DIDS blockade of various purinergic receptors ranges between 1.6 and 300 \(\mu\)M. Therefore, it is not clear if the inhibitory effects of 1 mM DIDS in the studies reported by Schweibert and co-workers (335, 336) are due to the blockade of ORCC or an antagonism at the ATP receptor. In the studies by Hwang et al. (179), mucosal DIDS blocked the stimulation in short-circuit current by mucosal ATP but did not block stimulation by serosal ATP. These results support the notion that DIDS may be acting at a purinergic receptor, since mucosal DIDS will not have access to a basolateral membrane purinergic receptor but will have access to an apical membrane purinergic receptor. On the basis of the very close structural similarity between TS-TM-calix[4]arene and the disulfonic stilbenes, the inhibitory effects of the calixarene may also be due to ATP receptor antagonism. Second, we (357) and others have failed to see an inhibitory effect of the disulfonic stilbenes or TS-TM-calix[4]arene on transepithelial Cl\(^-\) secretion in a variety of secretory epithelia including primary cultures of rat tracheal epithelial cells stimulated with a number of different agonists. Thus, contrary to the conclusions reached by Schweibert et al. (336) and Hwang et al. (179), we suggest that the inhibitory effects of DIDS in their studies may have an alternative explanation and that ORCC does not contribute to transepithelial Cl\(^-\) secretion. Glibenclamide and diphenylamine-2-carboxylate (DPC) were also used in these studies with the intention of discriminating between CFTR and ORCC. Unfortunately, glibenclamide and DPC also block ORCC (293, 324, 353) and may interfere with the cAMP signal transduction cascade (166, 214, 426), thus complicating the interpretation of the results obtained with these compounds. These concerns extend to any studies using disulfonic stilbenes (e.g., DIDS, DNDS), sulfonylureas (e.g., glibenclamide), or arylaminobenzoates [e.g., DPC, 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB)] on cellular-based macroscopic measurements of epithelial Cl\(^-\) currents.

Linsdell and Hanrahan (227) have shown that DNDS and DIDS cause a voltage-dependent block of CFTR-mediated Cl\(^-\) currents when applied to the cytoplasmic side of excised inside-out membrane patches from baby hamster kidney cells expressing wild-type or R347D CFTR. Extracellular DNDS or DIDS did not block the CFTR-mediated Cl\(^-\) currents. Inhibition from the intracellular side by DNDS displayed a voltage-dependent affinity for block of the channel pore by a negatively charged molecule acting from the intracellular side. Fitting these data to the Woodhull equation (433) gave a \(K_i\) of 236 \(\mu\)M at 0 mV. Substitution of the positively charged arginine at position 347 to a negatively charged aspartate significantly reduced the affinity of block of DNDS by eightfold and DIDS by threefold. Tabcharani et al. (382) had previously shown that the R347D mutation reduces the single-channel conductance, eliminates channel blockade by SCN\(^-\), and abolishes anomalous mole fraction behavior seen in Cl\(^-\)-SCN\(^-\) mixtures. Tabcharani et al. (382) have suggested that R347 contributes to an important anion-binding site close to the cytoplasmic end of the channel pore. Linsdell and Hanrahan (228) suggest that CFTR channel pore may contain a relatively large inner vestibule accessible from the intracellular side to large blocking anions such as DNDS, gluconate, and glutamate and that arginine-347 may be involved in anion binding within this region of the pore. Further structure-activity studies with additional disulfonic stilbenes and perhaps calixarene derivatives together with additional mutational analysis will be useful in defining the CFTR structure at this site as well as the development of higher affinity blockers of CFTR.

### C. Arylaminobenzoates

The arylaminobenzoate DPC (Fig. 2) was developed by Di Stefano et al. (100) as a blocker of the basolateral membrane Cl\(^-\) conductance in the thick ascending limb of the loop of Henle (TAL) and in the apical membrane Cl\(^-\) conductance of shark rectal gland tubules (RGT). The DPC had an IC\(_{50}\) of 26 \(\mu\)M when added to the basolateral side of the rabbit cortical and mouse medullary portion of the TAL (cTAL and mTAL, respectively), both of which are NaCl-reabsorptive epithelia (163, 164, 417). When added to the apical side in the RGT, a NaCl-secreting
epithelia, DPC showed similar inhibition of the apical membrane Cl− conductance (143, 144, 157). The inhibition by DPC on both the renal and rectal gland epithelia was shown to be rapid and reversible. Di Stefano et al. (100) have shown the presence of a DPC (100 μM)-sensitive Cl− channel in excised inside-out patches from the apical membrane of RGT and the basolateral membrane of rabbit cTAL; however, the concentration dependence of DPC block was not evaluated. An SAR of 219 arylaminobenzoates led to the discovery of more potent blockers such as NPPB (Fig. 2), which inhibited the basolateral membrane Cl− conductance of cTAL with an IC50 of 80 nM (416). To our knowledge, the single-channel identity of a Cl− channel with an IC50 of 26 μM for DPC or 80 nM for NPPB has not been shown in the cTAL or any other epithelium.

Since the discovery of the arylaminobenzoates, high concentrations of DPC and NPPB have been widely used in several macroscopic assays as inhibitors of Cl− transport in numerous epithelia (57). These include rabbit, ca¬nine, and sheep tracheal epithelia (189, 374), luminal membrane of the rectal gland of dogfish (145), cultured human fetal alveolar epithelial cells (244), primary cultures of human and rabbit distal colonic crypt cells (312, 313), human and rat epididymal epithelia (71, 73), mouse inner medullary collecting duct (mIMCD-K2) (403), cultured A6 renal epithelial cells (50, 67, 214, 266, 268, 269, 350), equine sweat gland epithelium (209), human jejenum and colon (52), frog skin epithelium (51), epithelial cells (intestine 407) (215), retinal pigment epithelial cells (174, 385), cultured human airway epithelial cells (242, 336), human pancreatic duct cells (411), rabbit colonic epithelia (142), mouse muscle cells (142, 414), and cultured human biliary cells (305). In all of these studies, the concentrations used were considerably higher than required to inhibit the Cl− conductive pathway across cTAL and RGT, although the inhibition of Cl− transport was considered to result from the blockade of Cl− channels.

With the use of several microscopic assays such as planar lipid bilayer and excised membrane patches, DPC and NPPB have been shown to inhibit endogenous and heterologously expressed Cl− channels in several epithelial cells. These include the ORCC from the human carcinoma cell line HT-29 (102, 161), rat colonic enterocytes (353), and cultured human respiratory cells (217), a Ca2+-dependent Cl− channel from sheep airway epithelium (8, 9), volume-sensitive outwardly rectifying Cl− channels from various epithelia (1, 17, 71, 72, 138, 215, 242), and CFTR in various epithelia (see below). Tilmann et al. (387) have shown that NPPB inhibits an ORCC from HT-29 cell line with a Kc of 0.9 μM when added to the cytosolic side and a Kc of 0.1 μM when added to the outer membrane side of the channel. The reason for this difference in the Kc values was attributed to the fact that the NPPB interaction site is likely accessible only from the extracytosolic side of the channel, so cytosolic addition precludes NPPB reaching its interaction site. However, apart from being anions, with pH values of 3–5, the arylaminobenzoates are lipophilic. At a pH of 7.4, ~50–90% are distributed into the lipid phase, e.g., DPC has a partition coefficient of 0.58 (water/CH2Cl2) (100), so by the addition of these compounds on one side of the cell membrane, one cannot rule out their penetration across the cell membrane. Indeed, we have demonstrated that both DPC and NPPB showed similar inhibition from the extracellular and intracellular sides of the ORCC incorporated into planar lipid bilayer with Ki values of 600 and 25 μM, respectively (353).

The concentrations at which the arylaminobenzoates DPC and NPPB have been used in different studies do raise a question about their specificity to inhibit one type of Cl− channel as compared with other channels or intracellular processes. The arylaminobenzoates share some structural similarity with loop diuretics. In the first paper describing the SAR of this class of compounds, it was shown that some of the arylaminobenzoates also had an affinity for the Na+-K+-2Cl− cotransporter (416). For example, DPC and NPPB had IC50 values of 100 and 30 μM, respectively, for inhibiting the cotransporter. Interestingly, the loop diuretics furosemide and bumetanide have recently been shown to block CFTR (296, 408). Furosemide and bumetanide have Kc values of 40 and 5 μM, respectively, for the inhibition of CFTR (408). Arylaminobenzoates have also been shown to block nonselective cation channels (75, 132, 133, 290, 301), L-type Ca2+ channels (415), volume-sensitive basolateral K+ channels in HT-29/B6 cells (180), an inwardly rectifying Ca2+-dependent K+ channel in turtle colon (301), and a Ca2+- and cAMP-activated low-conductance K+ channel in the basolateral membrane of human colon crypts cells (229). It is especially important to consider the inhibition of basolateral membrane K+ channels and the basolateral membrane cotransporter by these compounds when studying Cl− secretion. In a typical Cl− secretory epithelia, the apical membrane Cl− conductance and the basolateral membrane K+ conductance are tightly coupled to maintain a sustained level of Cl− secretion. Inhibition of either apical membrane Cl− channels, basolateral membrane K+ channels, or the basolateral membrane cotransporter will inhibit Cl− secretion. Hence, where investigators have intended to use DPC as an inhibitor of apical membrane CFTR to differentiate between cAMP-activated Cl− secretion via CFTR and other Cl− channels, there could well have been an inhibition of the basolateral membrane K+ conductances or cotransporter that is the actual cause of the inhibition of Cl− secretion.

Diphenylamine-2-carboxylate, NPPB, and some other arylaminobenzoates have been shown to have considerable inhibitory effects on intracellular adenyl cyclase in the Cl− secretory human colonic cell lines HT-29/B6 and T84 (166, 214, 426). Kreusel et al. (214) have shown that
1 mM DPC inhibited >85% of forskolin (10 μM)-activated cAMP production in HT-29/B6 cells. Diphenylamine-2-carboxylate was unable to inhibit Cl− secretion activated by dibutyryl cAMP. In a similar study with the T84 cell line, 500 μM DPC or NPPB inhibited forskolin-stimulated cAMP production by 28 and 56%, respectively (166). Thus it is possible that the mechanism of action of DPC in this and other cellular-based studies results from the inhibition of cAMP production rather than a direct inhibition on Cl− channels. Diphenylamine-2-carboxylate has also been reported to inhibit prostaglandin D2 synthesis from arachidonic acid in primary cultures of canine tracheal epithelium (375). The likely site of action of DPC in this tissue is the inhibition of cyclooxygenase, the enzyme responsible for prostaglandin synthesis. Prostaglandins are important modulators of electrolyte transport in a number of epithelia (33, 233, 237, 262, 292, 295, 365, 391, 413). Thus the arylaminobenzoates appear to inhibit both the cAMP and eicosanoids signal transduction pathways. The arylaminobenzoates have also been shown to have profound effects on intracellular pH in LLC-PK1 cells (50) and could thereby modulate a number of intracellular processes and channel activities. The interpretation of arylaminobenzoate inhibition of macroscopic Cl− secretion is, at best, difficult because of their nonselectivity for Cl− channels coupled with their inhibition of basolateral membrane K+ channels and the Na+-K+2Cl− cotransporter. As with the disulfonic stilbenes, the use (abuse) of the arylaminobenzoates as Cl− channel blockers has become entrenched in the literature. Clearly, more specific reagents are needed to study the contribution of CFTR in anion secretion by intact epithelia.

Caution aside, DPC and NPPB have been shown to inhibit CFTR (11, 61, 83, 86, 139, 140, 243–245, 291, 300, 309, 336, 376, 381, 384, 403), and in a series of elegant studies by McCarty et al. (243) and McDonough et al. (245), DPC was successfully used to probe the conduction pathway of CFTR. In their initial studies, McCarty et al. (243) demonstrated that DPC and flufenamic acid (FDA; 3′-trifluoromethyldiphenylamine-2-carboxylic acid; Fig. 2) blocked CFTR-mediated whole cell and single-channel Cl− currents in Xenopus oocytes. Blockade was voltage dependent; currents at positive potentials were not affected, but currents at negative potentials were blocked. Both DPC and FFA blocked single-channel openings in an excised patch when applied directly to the cytoplasmic side of the channel. As expected from the whole cell data, DPC and FFA blockade of CFTR in excised patches was also voltage dependent. The onset of blockade by extracellularly applied DPC or FFA was biphasic, with an early rapid phase and a later phase that developed over several minutes. The slow development of the blockade was attributed to the permeation of the blocker into the cell to an intracellular binding site on CFTR. Blockade by DPC and FFA was fully reversible. With the use of the voltage dependence of the blockade and the Woodhull equation (433), the Ks for DPC at 0 mV was 912 μM and at 100 mV was 237 μM. Similarly, the Ks for FFA at 0 mV was 1.22 mM and at 100 mV was 289 μM. The apparent electrical distance sensed by both the blockers was 41% as measured from the inside of the membrane. The studies of McDonough et al. (245) extended these findings to demonstrate that the interaction of DPC with CFTR was consistent with an open-channel mechanism of blockade. Furthermore, site-directed mutagenesis of residues in the putative transmembrane segments (TM) 6 and 12 significantly altered DPC blockade of the channel. Most notably, mutation of serine-341 to an alanine caused a fivefold increase in the Ks at −100 mV (wild type, 276 μM vs. S341A, 1,251 μM). This result is of special interest, since the predicted position of residue 341 lies 40% through the proposed TM6. Although S1141 in TM12 is predicted to have a similar position as S341 in TM6, mutation of serine-1141 to an alanine did not show an appreciable effect on DPC binding. Furthermore, when the methionine and threonine residues immediately adjacent to S1141 were changed to isoleucine and phenylalanine to match the residues immediately adjacent to S341, DPC bound with an affinity close to that of the wild-type channel (S341A-M1140FI-T1142F). This showed that the DPC-binding site on TM6 could be transferred to TM12. Mutation of threonine residue 1134 to a phenylalanine caused a threefold improvement in the affinity for DPC (T1134F, 74 μM). Like residue 341, residue 1134 lies 40% through the proposed TM12. These results strongly support the notion that both TM6 and TM12 contribute to forming the pore. They speculated that the carboxy group of DPC interacted with S341 on TM6 and the phenyl ring with T1134 on TM12. The studies of McCarty et al. (243), and McDonough et al. (245) with DPC, Lindsell and Hanrahan (227) with DNDs, and Schultz and co-workers (324, 329), Sheppard and Robinson (346), and Venglarik et al. (409) with sulfonyleureas provide excellent illustrations of how one can judiciously use these small ligands to probe the structure and kinetics of CFTR channel activity.

III. CHANNEL OPENERS

A. Xanthines

Paleolithic man is credited with the discovery of the central nervous system (CNS) stimulatory effects of drinks made from alkylxanthine (caffeine, theophylline, theobromine; Fig. 3) containing plants (coffee, tea, cocoa) (303). Alkylxanthines are now known, in addition to their CNS effects, to relax smooth muscle, most notably bronchial smooth muscle, stimulate cardiac muscle, and act on the kidney to cause diuresis. The treatment of asthmatic patients with strong coffee was initiated more than 100
FIG. 3. Structures of compounds that have been used to stimulate Cl⁻ secretion by directly or indirectly modulating activity of cystic fibrosis transmembrane conductance regulator.

Caffeine; R = R' = R'' = -CH₃
IBMX; R = CH₃-, R' = CH₂CH₂(CH₂)₆, R'' = -H
Theophylline; R = R' = -CH₃, R'' = -H
CYP; R = R' = CH₂CH₂CH₂CH₃, R'' = -H
Theobromine; R = H, R' = R'' = -CH₃

Apigenin; 5 = 7 = 4' = -OH
Quercetin; 3 = 5 = 7 = 3' = 4' = -OH
Kaempferol; 3 = 5 = 7 = 4' = -OH
Genistein; 5 = 7 = 4' = -OH
Daidzein; 7 = 4' = -OH
Genistin; 5 = 4' = -OH, 7 = -OGluc

Milorinone; R = CH₃, R' = -CN
Aminronine; R = H, R'' = -NH₂

Benzimidazolones

NS004; R = CF₃, R' = -Cl
NS1619; R = CF₃, R' = -H
1-EBIO; R = H, R' = -CH₂CH₃

Benzoxazoles

Chloroxazole; R = Cl, R' = ===O
Zoxazolamine; R = Cl, R' = -NH₂

8-Methoxypsoralen

Levamisole; R = H
Bromotetramisole; R = -Br

The cAMP-mediated activation of Cl⁻ secretion has been recognized for more than 30 years (24, 154) and the failure of CF epithelia to respond to cAMP for nearly 20 years. Studies before the discovery of CFTR demonstrated the impaired responsiveness of CF epithelia lies distal to the formation of cAMP and the activation of PKA. Soon after its discovery, CFTR was shown to be a PKA-activated ATP-dependent Cl⁻ channel as reviewed in detail in References 129a and 333a. Salient to CFTR activation by alkylxanthines, McPherson et al. (251) were the first to demonstrate that IBMX partially restored amylase and mucin secretion in submandibular acinar cells from CF patients. Subsequently, Drumm et al. (103) were the first to show that CFTR constructs with naturally occurring mutations in the first NBF could be activated by high concentrations of IBMX. In their studies, Xenopus oocytes were injected with wild-type or mutant CFTR (e.g., ΔF508, G551D) mRNA, and Cl⁻ current was measured in response to a stimulation cocktail including 10 μM forskolin, 200 μM 8-(4-chlorophenylthio)-cAMP, and various concentrations of IBMX. Mutant CFTR were less sensitive than wild-type CFTR to IBMX. For example, oocytes expressing wild-type CFTR were nearly completely stimulated with 1 mM IBMX, but those expressing G551D or ΔF508 CFTR required 5 mM IBMX to achieve complete stimulation. The reduction in sensitivity of the mutant CFTR was also found to be correlated with the severity of the CF in patients carrying the corresponding mutations.
The differential sensitivity of CFTR bearing mutations in NBF1 or NBF2 to IBMX stimulation was further evaluated by Smit et al. (362). Mutation of a conserved glycine (G551 and G1349) in the putative linker domains of either NBF produced the sensitivity to IBMX, and mutations of this site in both NBF produced an additive effect. In contrast, substitutions in the Walker A and B motifs produced strikingly different effects in NBF1 and NBF2. Substitutions for the conserved lysine (K464, Walker A) or aspartate (D572, Walker B) in NBF1 resulted in a marked decrease in sensitivity to IBMX, whereas the same changes in NBF2 (K1250, Walker A; D1370, Walker B) produced an increase in sensitivity. Smit et al. (361) went on to show that the missense mutation G480C associated with CFTR protein mislocalization was equally sensitive to IBMX stimulation when compared with wild-type CFTR. Wilkinson et al. (428) undertook a quantitative analysis of the rates of activation and inactivation of these same mutants in response to stimulation and removal of 10 μM forskolin and 5 mM IBMX. Consistent with the steady-state current measurements of their previous studies, substitutions at G551 in NBF1 or G1349 in NBF2 reduced the rate of activation and increased the rate of inactivation. Substitutions at K464 or K1250 also reduced the rate of activation but had opposite effects on the rate of inactivation; K464 substitution increased the rate of inactivation while K1250 substitution decreased the rate of inactivation. Substitutions of D572 decrease the rate of activation and increased the rate of inactivation. In contrast, D1370 substitutions did not alter the activation rate but markedly slowed the inactivation rate. Thus D1370 mutants remained active long after the removal of the stimulation cocktail. These elegant macroscopic measurements demonstrated that mutations in either NBF1 or NBF2 can influence the activation and inactivation of CFTR and suggest the nature or the exact consequences of nucleotide binding differ for the two domains (428), observations that have since been demonstrated in numerous patch-clamp studies.

An important outcome of the above studies in oocytes was the demonstration that mutant forms of CFTR could indeed be activated and function as Cl− channels. These results thus lent great support to the suggestion by McPherson et al. (251) that alkylxanthines could be useful in the treatment of CF. This notion received further support from the studies of Yang et al. (440), who extended the observations in oocytes to murine fibroblast cells (L cells) expressing mutant forms of CFTR. With the use of the halide-sensitive fluorophore SPQ assay, 4 mM IBMX was found to activate ΔF508 and G551D CFTR-mediated anion efflux. Activation by IBMX of the ΔF508 CFTR expressing cells was observed in cells maintained at 37°C, indicating some ΔF508 CFTR protein had reached the plasma membrane. Similar stimulatory effects of IBMX were also reported by Haws et al. (159) using mouse mammary epithelial cells (C127 cells) expressing ΔF508 CFTR. Haws et al. (159) also demonstrated, with patch-clamp studies, that ΔF508 CFTR was present in the plasma membrane albeit at a lower channel density than wild-type CFTR-expressing cells and with a lower open probability (P₀) of 0.11 compared with wild-type CFTR (P₀ = 0.33).

Beavo (28) has provided a recent review of the cyclic nucleotide PDE. An estimated 25 PDE isoforms are thought to exist. The pharmaceutical industry has attempted to capitalize on the tissue-specific expression of the various PDE isoforms in the development of antiasthmatic, antithrombotic, antihypertensive, cardiotonic, and antidepressant agents. There are, as a result of these drug development efforts, a number of isoform-specific PDE inhibitors that are in clinical use. Kelly et al. (198) set out to determine which of the specific classes of PDE were involved in the activation of CFTR in epithelial cells. Milrinone and amrinone (Fig. 3), class III PDE inhibitors, were found to stimulate 125I efflux in Calu-3 and 16HBE human airway epithelial cells, whereas the class IV PDE inhibitor rolipram and the class V PDE inhibitor dipryridamole were much less effective. Stimulation of 125I efflux by milrinone and amrinone did not require the inclusion of an adenylyl cyclase activator (e.g., forskolin), nor did stimulation correlate with cAMP levels. However, stimulation by milrinone and amrinone was inhibited by the cell-permeant PKA inhibitor N-(2-[methylamino]ethyl)-5-isouquinolinesulfonamide (H-8) and the cAMP antagonist Rp-cAMP.

These results are consistent with a cAMP/PKA-mediated activation of CFTR that results from a compartmentalized pool of cAMP. These studies were extended to show that 36Cl efflux could be stimulated by milrinone plus isoproterenol in the transformed nasal polyp cells (CF-T43) homozygous for ΔF508-CFTR (197). The CFTR antisense oligonucleotides prevented the increase in 36Cl efflux in response to milrinone and isoproterenol. These results again demonstrate that some ΔF508 CFTR is functionally expressed in the plasma membrane and that it can be activated by milrinone and isoproterenol. Kelly et al. (199) have since shown the efficacy of forskolin and milrinone to hyperpolarize the nasal epithelium indicative of a Cl− secretory response in ΔF508 CFTR-expressing mice. Whereas the combination of forskolin and milrinone was ineffective in altering the nasal potential difference in the CFTR (−/−) mice, the ΔF508 CFTR-expressing mice displayed a change in potential difference of ~50% of that observed in mice expressing at least one wild-type CFTR allele. As in the in vitro studies with ΔF508 CFTR-expressing cells, both an adenyl cyclase agonist and milrinone were required to cause a response in vivo.

Collectively, these studies on human salivary acinar cells, Xenopus oocytes, human airway cells, murine fibroblasts, and murine nasal epithelia suggest the superactivation of the cAMP-PKA regulatory pathway may activate certain mutant forms of CFTR and thus be of thera-
High concentrations of IBMX (5 mM) did not augment ylline had (28). Milrinone concentration-response studies as well as only poorly inhibit phosphatases, were ineffective in slow-
tive in initiating Cl

inhibition of PDE, one must also consider the inhibition nels. Subsequently, Becq et al. (30) showed in CFTR
secretagogues that the alkaline phosphatase inhibitors
forms at 1 mM) slowed the rundown of CFTR channel rundown. Caffeine (Fig. 3) and dipyridamole, two PDE inhibitors that opened CFTR channels from CF patients with the
ion (rundown) that is often observed upon patch excision

The studies of Becq et al. (29) suggest an alternative explanation for the effects of high concentrations of IBMX. These investigators observed that channel inactiva-
tion (rundown) that is often observed upon patch excision could be slowed by theophylline, IBMX, and levamisole (Fig. 3) in wild-type CFTR-expressing CF pancreati-
cells (CFPAC-PLJ-CFTR-6). These same substances reduced the apical membrane-associated alkaline phosphatase activity by 70–75%. A polyclonal antialkaline phosphatase antibody that detected and reduced apical membrane al-
kaline phosphatase activated quiescent CFTR Cl

channels. Subsequently, Becq et al. (30) showed in CFTR
expressing Chinese hamster ovary (CHO) and human airway epithelial cells that the alkaline phosphatase inhibitors bromotetramisole, IBMX, theophylline, and vanadate (Fig. 3; each at 1 mM) slowed the rundown of CFTR channel activity in excised membrane patches. These same substances also reduced the dephosphorylation of CFTR protein in isolated membranes. 3-Isobutyl-1-methylxanthine was the most effective at slow channel rundown. Caffeine (Fig. 3) and dipyridamole, two PDE inhibitors that

with the biochemical demonstration of the presence of class III PDE in the apical membrane of airway epithelial cells are needed before one can conclude the stimulatory effects of milrinone are mediated by inhibition of class III PDE. In addition, the assumed activation of the cAMP-
PKA regulatory pathway by IBMX or milrinone is ex-
ected to lead to the phosphorylation of the mutant CFTR proteins. Protein phosphorylation studies are needed to demonstrate that IBMX and milrinone do alter the phos-
phorylation status of CFTR under the experimental condi-
tions used in these studies. Positive results from such biochemical studies would lend great support to the con-
cclusions reached by these investigators. These studies must also be reconciled with the observations of Grubb et al. (148), who evaluated the combined use of adenyllyl cyclase activators (forskolin or isoproterenol) and IBMX on normal and CF airway epithelia in vitro and in vivo. High concentrations of IBMX (5 mM) did not augment forskolin-stimulated Cl

secretion in primary cultures from normal patients. Neither forskolin nor forskolin plus IBMX had any effect in cells from CF patients with the ∆F508 mutation even in the presence of a favorable Cl

gradient. Nasal potential difference measurements failed to detect an additive ef-
fect of IBMX with isoproterenol in either normal or CF subjects. Grubb et al. (148) concluded that the combina-
tion of adenyllyl cyclase activators and IBMX is not effect-
ive in initiating Cl

secretion in CF epithelia. These authors also note that agents that raise cell cAMP in CF airways may further increase the abnormally high basal rate of sodium absorption. Thus Cl

secretagogues that elevate cAMP levels may be counterproductive in the treatment of CF airways (148).

The direct binding of the alkylxanthines to CFTR also warrants some consideration when attempting to under-
stand their mechanism of action on CFTR channel gating. We and others (322, 428) have observed that IBMX causes a decrease in the single-channel amplitude of CFTR and thus appears to act as an open-channel blocker. Thus IBMX will by mass action hold CFTR in the open (albeit partially blocked) state. If only closed CFTR channels can be dephosphorylated, IBMX, by stabilizing the open state, will slow dephosphorylation and channel inactivation (rundown). In support of this hypothesis, we have observed that lowering the bath ATP concentration in excised inside-out patches, a manipulation that lowers the P

also accelerates wild-
type CFTR rundown. The ∆F508 CFTR has a lower affinity for ATP (K

300 μM vs. wild-type CFTR 25 μM), a lower maximal P

(0.25 vs. wild-type CFTR 0.4), and tends to
rundown more rapidly than wild-type CFTR. 3-Isobutyl-1-methylxanthine tends to slow the rundown of ΔF508 CFTR. The effects of IBMX on wild-type and mutant CFTR may therefore in part be mediated by a direct interaction with the CFTR protein. Clearly, further studies are needed to determine the contributions of PDE inhibition, phosphatase inhibition, and direct binding to CFTR by the alkylxanthines on CFTR channel activity.

Another series of alkylxanthines that has received attention by CF investigators is the potent adenosine receptor antagonists. In a series of papers by Pollard and co-workers (112, 151, 192), compounds such as 8-cyclopentyl-1,3-dipropylxanthine (CPX; Fig. 3) and the xanthine amino congener (XAC) were reported to increase 36Cl efflux from the ΔF508 CFTR-expressing CFPAC pancreatic cells. The response to CPX and XAC was biphasic, with low concentrations (10–30 nM) causing stimulation and higher concentration (100 nM to 10 μM) causing inhibition of 36Cl efflux. CFPAC cells transfected with wild-type CFTR were not responsive to CPX or XAC. The stimulatory effects of CPX and XAC were blocked by exogenous adenosine agonists such as 2-chloroadenosine and were not observed in the presence of adenosine deaminase. These studies were subsequently repeated with similar results using NIH 3T3 cells expressing ΔF508 CFTR or wild-type CFTR as well as with the human airway cell line IB3–1 derived from a ΔF508/W1282X CF patient (151).

In a companion paper, structure-activity studies were performed using the CFPAC cells. The results demonstrated there was no correlation between the potency on 36Cl efflux and adenosine receptor antagonism (192). Human A1 adenosine receptor mRNA was not detected in CFPAC cells, excluding this receptor as a mediator of CPX-elicited 36Cl efflux. The authors suggest the action of CPX on ΔF508-CFPAC cells represents a novel site of action apparently unrelated to known adenosine receptors. However, Haws et al. (159) did not observe a stimulatory effect of CPX at low or high concentrations when using stably transfected mouse mammary epithelial cells (C127 cells) expressing ΔF508 or wild-type CFTR. At high concentrations (EC50 58 μM), CPX did potentiate the response to forskolin in ΔF508 CFTR-expressing cells. Thus further studies will be required to establish the generality of CPX efficacy on CF cells as well as its mechanism of action.

B. Phosphatase Inhibitors

Cystic fibrosis transmembrane conductance regulator Cl− channel activity is thought to be reciprocally regulated by kinase-dependent phosphorylation and phosphatase-dependent dephosphorylation. Functional studies indicate that Cl− channels are primarily activated by PKA in physiological settings, although phosphorylation by other kinases (e.g., protein kinase C, protein kinase G) may play significant modulatory roles. In the following paragraphs, we discuss the pharmaceutical compounds that have been used to infer phosphatase-dependent modulation of CFTR Cl− channel activity and the associated supporting observations. Although it is parsimonious to conclude that channel activation associated with exposure to kinases results from channel phosphorylation, and that channel inactivation associated with exposure to phosphatases is indicative of dephosphorylation, a healthy skepticism that awaits verification in the form of unique phosphopeptide maps associated with distinct gating modes is appropriate.

The classification of protein phosphatases can be quite confusing. In the most widely accepted nomenclature scheme, phosphatases are classified based on substrate specificity and inhibition by particular proteins, inhibitor 1 and inhibitor 2 (186, 187). Type 1 phosphatases (PP1) preferentially dephosphorylate the β-subunit of phosphorylase kinase and are sensitive to both inhibitory phosphorylations. Type 2 phosphatases have been subdivided based on requirements for divalent cations; PP2A requires no divalent cations, PP2B (also known as calcineurin) are regulated by Ca2+ and calmodulin, and PP2C require Mg2+. In this nomenclature system, tyrosine phosphatases (PTP) are classified separately, whereas neither acid nor alkaline phosphatases are represented as a unique class. Alternative classification schemes have been presented but are not yet widely used (263). For more complete discussion of phosphatase nomenclature and forms, the reader is directed to various recent reviews (20, 119, 263, 345, 425).

The discovery of okadaic acid and similarly bioactive compounds has greatly enhanced our knowledge of the role of phosphatases in physiological systems because of their selectivity for PP1 and PP2A and their unique rank order of potency for each class. In this regard, the pharmacology of serine/threonine phosphatases PP1 and PP2A is the best developed. Alternatively, PP2B is selectively inhibited by FK506 and cyclosporin after their interactions with binding proteins FKBP12 and cyclophilin, respectively. Distinct, unique pharmacological profiles for PP2C and PTP based on the actions of high-affinity blockers are not yet available, although the molecular forms and catalytic mechanisms have been reviewed (20, 119).

It was recognized early on that specific kinases were capable of activating CFTR; however, the phosphatases capable of, or responsible for, inactivation remained unknown. Tabcharani et al. (380) first showed that exogenously applied alkaline phosphatase could reduce the Po of channels in membrane patches excised from CFTR-expressing CHO cells. Furthermore, the authors (380) showed that both F− and metavanadate, two nonspecific phosphatase inhibitors, increased channel Po in this preparation and inhibited the effects of exogenously applied alkaline phosphatase. Thus it was concluded that mem-
brane-associated alkaline phosphatase might have been responsible for the inactivation of channels (rundown) that consistently accompanied patch excision. In contrast, Berger et al. (36) reported that alkaline phosphatase failed to inactivate CFTR channels in membrane patches excised from NIH 3T3 cells. Rather, exposure to alkaline phosphatase caused a reduction in $P_o$ that reversed upon washout and without the addition of kinases. These results were interpreted to indicate that dephosphorylation, which is expected to require kinases for reversal, had not occurred but that the phosphatase had reduced the ATP concentration and thus caused a transient reduction in $P_o$. Alternatively, it was shown that exogenously added PP2A, but not PP1 or PP2B, was capable of reducing $P_o$ in excised membrane patches (36). Although results from these studies demonstrate that CFTR channels expressed in these cell lines (CHO and NIH 3T3) can be inactivated by exposure to specific phosphatases (alkaline phosphatase or PP2A, respectively), the results do not indicate that these phosphatases regulate CFTR activity in vivo and especially in cells or tissues that normally express CFTR. In this vein, Travis et al. (390) reported that PP2C was present in colonic and airway epithelial cells, was capable of dephosphorylating CFTR in vitro, reduced transepithelial Cl$^-$ currents when over expressed, and inactivated CFTR in excised membrane patches. These data would argue that PP2C might have a physiological role in CFTR regulation.

Numerous pharmacological studies designed to determine the factors that physiologically mediate inactivation of CFTR have been completed. Compounds such as okadaic acid, calyculin A, and microcystin, which are known to inhibit PP1 and PP2A, have been widely used to rule out or impute a potential role for these protein phosphatases in the regulation of CFTR. Historically, the "rundown" of channel activity that accompanied patch excision and that could be reversed by addition of PKA was interpreted as dephosphorylation/inactivation (77, 380). Tabcharani et al. (380) reported that okadaic acid (10 μM) did not preclude rundown of channel activity upon patch excision from CFTR-expressing CHO cells. This observation led to the conclusion that protein phosphatases other than PP1 or PP2A inactivate CFTR upon patch excision, a conclusion supported by Becq and co-workers (29, 30) from recordings of both cell-attached and excised membrane patches. Similarly, neither the kinase-dependent activation kinetics of CFTR channels in membrane patches excised from NIH 3T3 cells expressing CFTR nor the steady-state $P_o$ of the channels was affected by the presence of either calyculin A (100 nM) or okadaic acid (1 μM; Refs. 10, 125), again indicating that PP1 and PP2A were not active in these cell-free experiments. Alternatively, Hwang and co-workers (177, 439) have reported that okadaic acid (10 μM) and microcystin (5 μM) enhanced the forskolin-stimulated current in whole cell records from guinea pig ventricular myocytes and that calyculin A (20 nM) enhanced forskolin-stimulated CFTR channel activity in cell-attached patches on Hi-5 insect cells. Likewise, okadaic acid (10 nM) prevented the inactivation of forskolin-stimulated current across permeabilized human sweat duct (297), and calyculin A ($EC_{50} \sim 0.1$ μM) stimulated $^{125}$I efflux from CFTR-transfected NIH 3T3 cells (298). Taken together, these observations suggest that, in intact cells, the inhibition of either PP1, PP2A, or both results in the stabilization of an activated form of CFTR. Such phosphatase inhibition could account for the apparent stimulation of CFTR activity if, in basal conditions, some ongoing kinase and phosphatase activities are present. Upon excision, elements responsive to these pharmaceutical compounds diffuse away from the channel and regulation attributable to PP1 or PP2A is no longer evident. Whether the phosphorylation state of CFTR is, in fact, directly affected by the protein targets of these compounds cannot be determined based on these results. An intriguing and unexplained observation in these studies was that okadaic acid increased the phosphorylation of CFTR as assessed by $^{32}$P incorporation but had no apparent effect on CFTR channel activity as assessed by $^{125}$I efflux (298) or channel rundown (30). The residues that were differentially phosphorylated in the presence of okadaic acid did not apparently impinge on channel activity.

Protein phosphatase 2B and PP2C were first defined by dependence on Ca$^{2+}$ and Mg$^{2+}$, respectively. Additionally, orthovanadate selectively inhibits PP2C, whereas F$^-$/phenothiazines selectively inhibit PP2B (345). More recently, FK506 and cyclosporin have been shown to be high-affinity inhibitors of PP2B. The authors are unaware of any studies designed to determine if either FK506 or cyclosporin directly affected CFTR channel gating, although Travis et al. (390) reported that FK506 did not inhibit cAMP-stimulated Cl$^-$ currents in airway or T84 colonic epithelial cell monolayers. The removal of divalent cations in general, and Mg$^{2+}$ in particular, has been reported to alter CFTR gating in excised membrane patches. Little effect of Ca$^{2+}$ removal was noted, indicating that PP2B did not modulate gating in excised membrane patches (323, 380). Removal of Mg$^{2+}$ has been associated with significant reductions in the nucleotide-dependent opening rate of CFTR and in its closing rate (10, 63, 153, 323). Depending on the relative effects on the opening and closing rates, $P_o$ of CFTR channels was reported to either remain relatively unchanged (153, 323) or to be dramatically decreased (10, 63). However, results from these studies along with those from studies employing orthovanadate as a panspecific phosphatase inhibitor (see below) cannot be interpreted in isolation from possible effects caused by changes in the rates of nucleotide binding and unbinding and perhaps hydrolytic events that are thought to occur at one or both of the NBF (27, 129, 152, 323).
Orthovanadate has been employed in some studies to assess the role of protein phosphatases in the regulation of CFTR (10, 30, 182, 297, 380). Other studies have employed the same compound as a hydrolytic transition state analog to investigate energy requirements for channel gating (27, 152, 323). At concentrations <1 mM (concentrations appropriate for the inhibition of P-type ATPases and PTP and known to inhibit PPI), vanadate had little or no observable effect on CFTR channel activity in excised membrane patches (10, 323, 380) or in permeabilized human sweat duct (297).

When used at 1–10 mM, vanadate significantly increases the $P_o$ of CFTR in excised membrane patches (27, 30, 152, 323). Because of the concentrations required to elicit changes in CFTR channel activity, it is difficult to draw any conclusions regarding the site or mechanism of action. Clearly, the concentration dependence is not consistent with the inhibition of previously described protein phosphatases.

Perhaps the most relevant and interpretable use of vanadate in the modulation of CFTR is that it inhibits genistein-stimulated $^{131}$I efflux and channel activity in excised and whole cell membrane patches (182, 352). In each case, significant inhibition was reported with concentrations <100 μM. These observations are consistent with the hypothesis that a tonically active tyrosine kinase maintains CFTR in an inactive state. Inhibition of this kinase by genistein and the concomitant activity of PTP results in the apparent activation of CFTR. Inhibition of PTP by vanadate maintains the inactivated phosphotyrosine form of CFTR and thus precludes an effect of genistein. This interpretation of the data is attractive but awaits supporting observations that indicate the distinct phosphotyrosine forms of CFTR that are present in the various conditions as well as a delineation of the site and mechanism of action of genistein.

Fluoride affects a variety of hydrolytic enzymes including G proteins, kinases, and phosphatases. The concentration dependence for specific enzymes ranges from low microequivalent to the milliequivalent range. Tabcharani et al. (380) showed that 10 meq F$^-$ blocked the effect of exogenously added alkaline phosphatase on excised membrane patches and reported that channel rundown was inhibited by this treatment. In our hands, F$^-$ activates CFTR activity in the cell-attached configuration (410), although stably active CFTR channels in excised membrane patches are unaffected by the presence of 10 meq F$^-$ (323, 331, 333). Alternatively, Berger et al. (36) reported that 20 meq F$^-$ increased CFTR $P_o$ by twofold. Five milliequivalents F$^-$ slightly reduced, but did not inhibit, the reduction in Cl$^-$ conductance that accompanies the washout of cAMP from a permeabilized human sweat duct (297). Taken together, these results are ambiguous in that the results were inconsistent even with the extreme concentrations that were employed. Although F$^-$-dependent effects were reported, the results cannot be attributed to phosphatase inhibition with any reasonable degree of certainty.

Becq and co-workers (29–31) have reported that the antihelminthic phenylimidazothiazole drugs (−)-p-bromotetramisole and levamisole stimulated CFTR channel activity in CHO cells. The stereoisomer (−)-p-bromotetramisole was without effect. Likewise, Illek et al. (183) have shown that (−)-p-bromotetramisole stimulated short-circuit current across Calu-3 cell monolayers. The authors interpreted these observations, along with those of Tabcharani et al. (380), to indicate that alkaline phosphatase inactivates CFTR and that the inhibition of a tonically active alkaline phosphatase accounted for stimulation. The observed stereospecific inhibition is consistent with this interpretation, although the concentration dependence is not (200 μM to 2 mM; levamisole EC$_{50}$ = 450 μM). Metaye et al. (254) first reported the stereospecific inhibition of alkaline phosphatase by phenylimidazothiazoles, but reported a $K_i$ of 1.2 μM for (−)-p-bromotetramisole and 11 μM for levamisol, two to three orders of magnitude lower than the concentrations used in the studies on CFTR. Furthermore, the authors (254) reported that, at concentrations approaching 1 mM, these compounds inhibited adénylyl cyclase, although not stereospecifically (254). The effects of phenylimidazothiazoles on CFTR-mediated ion transport are not consistent with the pharmacology at recognized sites of action, e.g., inhibition of alkaline phosphatase. Therefore, further studies are required to define the mechanistic basis for these effects. Regardless, Becq et al. (30) pointed out that enhanced secretion may explain the ability of levamisole to reduce the frequency, severity, and duration of upper respiratory tract infections in children (98, 404, 405).

Additional compounds including xanthines (29, 30) and genistein (183) have been reported to stimulate CFTR by modulating phosphatase activity. These observations are addressed elsewhere in this report.

C. Isoflavones and Flavones (Genistein)

Genistein is a plant-derived isoflavone for which a biological activity was first described in the 1940s and early 1950s (35, 46, 65, 66). Initial reports regarding genistein dealt with estrogenic effects of this class of compounds because of the economic impact on livestock production and potential clinical applications. The cellular or subcellular target mediating this effect was uncertain, but thought to be the estrogen receptor (318). In the 1980s, a related flavone, quercetin, was found to inhibit PKC (149), casein kinase (78), phosphor ylase kinase (367), and the tyrosine kinase activity as-
associated with the Rous sarcoma virus gene product pp60<sup>src</sup> (141). In each case, significant inhibition was seen at concentrations below 100 mM, making this compound one of the most “potent” kinase inhibitors available. While developing an SAR based on quercetin, Ogawara et al. (272) reported that genistein inhibited tyrosine kinase activity of the epidermal growth factor (EGF) receptor and src kinase with IC<sub>50</sub> values of 2.6 and 30 µM, respectively, while having no effect on PKA at 375 µM. Subsequent research showed that, of 28 isoflavonoids and 5 flavonoids evaluated, genistein was the most potent inhibitor of EGF receptor tyrosine kinase activity (2.6 µM) and most cytotoxic to Rous sarcoma virus-transformed cells (26 µM) (273). The reported rank order of potency for inhibition of EGF receptor tyrosine kinase activity for widely available flavones (f) and flavonones (i) is as follows: genistin (i) > kaempferol (f) = prunetin (i) = quercetin (f) > apigenin (f) = biochanin A (i) = acacetin (f) = flavone (f) > genistin (i) = daidzein (i) (273). The assay was completed in a cell-free system that would likely preclude interactions with other binding sites that could be linked to alternative and perhaps antagonistic affects. However, because cell membranes and/or vesicles were involved, the rank order of potency might have been influenced by lipid shielding of the binding site. Consistent with the rank order of potency is the observation that genistein is more hydrophobic than daidzein (22) or genistin. It is also intriguing to note that there seems to be little selectivity between flavones and isoflavones at the active site. This could, in part, be because of the freedom of rotation for the phenyl group attached to the chroman ring system at C<sub>2</sub> or C<sub>3</sub> of isoflavones and flavones, respectively (Fig. 3; Ref. 22). Certain hydroxyl substituents on the chroman ring appear to be quite important for activity. An hydroxyl at C<sub>3</sub> of flavones provides for greater kinase inhibition (kaempferol or quercetin vs. apigenin), and a substituent at C<sub>5</sub> appears to be required for activity (daidzein or flavone vs. genistein). Kinetic analysis indicated that the inhibition of EGF receptor tyrosine kinase activity by genistein is competitive with ATP (K<sub>i</sub> ~13.7 µM) but is noncompetitive or mixed inhibition with the phosphate acceptor (3, 4). A more limited pharmacological profile showed that, of six flavones and isoflavones evaluated, genistein was singular in its potency as an inducer of DNA strand breakage due to its interaction with topoisomerase II (effective concentration 5 µM) (238, 278). Genistin was 10-fold less effective, whereas prunetin, quercetin, apigenin, and biochanin A were without effect at 100 µM. Although assays were not performed to definitively determine the site of action, the ATPase activity of topoisomerase was reduced (278). On the basis of these observations, it was predicted that genistein interacted with a consensus sequence of GxGxxG, which is present in various ATP-dependent enzymes including topoisomerase II and erb-B2 (238). Akiyama and co-workers (3, 4) reported that genistein is selective by at least an order of magnitude for tyrosine kinases (EGF, gag-fes, and src) over serine/threonine kinases (PKA, PKC, phosphorylase kinase), 5′-nucleotidase, and phosphodiesterase. Thus it appears that the effective concentration of genistein along with the rank order of potency for these compounds will differentiate effects mediated by interactions with tyrosine kinases from those mediated by an interaction with topoisomerase II or the serine/threonine kinases.

There is currently tremendous interest in genistein as an oncolytic or oncostatic agent (21, 121, 284, 368; for review, see Ref. 253). Most authors have attributed oncolytic activity directly or indirectly to topoisomerase inhibition (143, 144, 156, 220, 288, 397, 419, 427). Alternatively, the oncolytic effect of genistein might be mediated by the interaction with topoisomerase II and accompanying increase in DNA strand breakage to induce apoptosis (238, 278). Additionally, genistein has been shown to bind to P<sub>1</sub> purinergic (adenosine) receptors (K<sub>i</sub> = 5 µM; Ref. 274) and to inhibit murine multidrug resistance-associated protein (MRP) transport (K<sub>i</sub> = 23 µM; Ref. 282). It should be emphasized that, when used at concentrations in excess of 100 µM, genistein has also been shown to inhibit PKC and PKA (4) and to alter cellular metabolism by inhibiting β-galactosidase (162).

In summary, genistein is the most potent of many flavones and isoflavones that have been reported to inhibit protein tyrosine kinases, topoisomerase, and certain other ATP-requiring enzymes. In the case of tyrosine kinase, inhibition is competitive with ATP. Inhibition of these enzymatic activities or interaction with yet undetermined binding sites causes estrogenic, antiestrogenic, oncolytic, angiostatic, and apoptotic events along with the plethora of other cellular processes that are reportedly affected by genistein. If genistein is to be of therapeutic value in the treatment of CF, then these activities of genistein will have to be considered in concert with the targeted cell processes.

That genistein and some related flavones and isoflavones affect epithelial ion transport is now widely accepted. Such effects of flavones on CFTR-mediated ion transport were first reported by Nguyen et al. (264), who showed that quercetin and kaempferol stimulated Cl<sup>−</sup> secretion across T84 cell monolayers. The authors speculated that because quercetin-induced stimulation was potentiated by carbachol, but not VIP, and because quercetin produced a phosphoprotein map like that observed in the presence of <sup>cAMP</sup> (increased phosphorylation of proteins

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1 A “consensus” genistein binding sequence, GxGxxG, was identified in CFTR beginning at G1125, which is located at the extracellular face of the 12th putative membrane-spanning segment (Wisconsin Package, Genetics Computer Group; CGC, version 9.0, Madison, WI).
designated p18, p23, and p37), the effects were likely mediated by a PKA-dependent mechanism. An effect of quercetin on adenyl cyclase activity was ruled out by the observation that cAMP production was unchanged. In more recent and extensive reports, the mechanisms by which stimulation of CFTR occurs have been contested.

As in the following paragraphs, valid arguments have been made that genistein affects CFTR-mediated ion transport by direct interaction with the channel, by inhibition of protein phosphatases, and by inhibition of tyrosine kinases. Perhaps what has added the greatest fuel to the debate is the variety of settings in which genistein is being applied to CFTR and the lack of extensive pharmacological profiles in any one of these settings. Since the start of 1995, no less than 15 reports have appeared which address the effects of genistein on CFTR. Numerous wild-type and mutant forms of CFTR have been evaluated in Xenopus oocytes, insect cells, fibroblasts, epithelial cell lines, cardiac cells, and shark rectal gland cells. Measurements have been made to determine the effects of genistein on Cl− secretion, short-circuit current, 125I efflux, intracellular cAMP concentrations, protein phosphorylation levels, and the activity of CFTR in whole cell, cell-attached, and excised membrane patches. On top of this diversity in constructs, assays, and expression systems is superimposed the conditions for evaluation which include differences in temperature, ionic environment, pH, and membrane potential. It is no wonder that not all systems have given consistent and thus easily interpretable answers. However, some generalizations are possible at this time.

The first question that must be addressed is, Is the effect of genistein on CFTR mediated by a tyrosine kinase? At best, the answer is that there may be a tyrosine kinase component in some systems. Undoubtedly, results first reported by Illek et al. (182) from experiments employing CFTR-expressing NIH 3T3 cells are consistent with this hypothesis. In both 125I efflux and cell-attached membrane patches, the concentration of genistein employed (40–50 μM) is appropriate for the selective inhibition of tyrosine kinases; daidzein, an analog known not to affect EGF receptor tyrosine kinase, was without effect, and a known tyrosine phosphatase inhibitor, vanadate, inhibited the response. The authors reported that tyrphostin B42, a structurally unrelated tyrosine kinase inhibitor, had similar effects to those of genistein. Like the earlier report by Nguyen et al. (264) and as an additional control, it was reported that alternative second messenger concentrations (cAMP, Ca2+) were not affected by genistein. These results were largely supported by Sears et al. (338), who showed that both genistein and tyrphostin 47 stimulated short-circuit current across T84 cell monolayers with an EC50 of 12.5 μM. Again, genistein did not affect intracellular cAMP or Ca2+ concentrations and genistein, yet another analog of genistein that does not affect EGF tyrosine kinase activity was without effect. More recently, Diener and Hug (97) reported similar observations in rat colon. None of the authors (97, 182, 338) provided evidence for a direct effect of genistein on CFTR. However, Sears et al. (338) showed that genistein potentiated, by fourfold, the effect of forskolin (10 μM) on intracellular cAMP production in T84 cell monolayers, suggesting that the target for genistein might be a part of this second messenger pathway subsequent to the activation of adenyl cyclase. Lehrich and Forrest (221) reported that genistein, but not genistin, stimulated bumetanide-inhibitable Cl− secretion in shark rectal glands with an EC50 <50 μM when applied to the apical membrane. The tissue was less sensitive to basolateral genistein application. Genistein consistently reduced the labeling of four proteins (240, 210, 55, and 53 kDa) by a monoclonal antibody (4G10) raised against phosphotyrosine residues, reportedly indicating the inhibition of a tyrosine kinase. However, the inhibition of protein phosphorylation was seen only at concentrations far in excess of those required to stimulate ion transport (≥250 μM vs. <50 μM) and, in fact, labeling of each of these proteins was increased in the presence of 100 μM genistein. Alternatively, Bischof et al. (40) reported that, in HT-29 cells, 50 μM genistein prevented carbachol-induced phosphorylation of three proteins (110, 75, and 70 kDa) and inhibited carbachol-induced Ca2+ entry. Similar effects were seen with other known tyrosine kinase inhibitors (methyl-2,5-dihydroxycinnamate, lavendustin A), but not daidzein. Finally, observations by Shuba et al. (352) regarding genistein-stimulated Cl− currents in whole cell records of guinea pig cardiac myocytes suggest the participation of a tyrosine kinase in this system; the stimulation was vanadate sensitive, and daidzein was virtually without effect. It should be noted that these authors report an EC50 of 100 μM, significantly greater than the K for tyrosine kinase inhibition reported in other systems. The observations thus far cited are consistent with tyrosine kinase involvement; however, many questions remain unanswered. Perhaps most important, in all systems tested, CFTR was not isolated either physiologically or biochemically to determine if there was a genistein-dependent change in CFTR phosphorylation or if there was reason to impute a direct effect of genistein on channel biophysics. Subsequent reports have dealt with these issues.

Hwang et al. (178) reported that, in membrane patches excised from NIH 3T3 cells containing previously activated CFTR channels, genistein did not support channel gating. This observation rules out a simple bimolecular interaction of genistein with PKA-phosphorylated CFTR resulting in an open state of the channel. Alternatively, numerous investigators have postulated that genistein inhibits dephosphorylation of CFTR resulting in increased and prolonged channel activity (76, 181, 298, 439). Thus, as discussed by Reenstra et al. (298), in cells with tonic PKA activity and proportionately greater protein phospho-
tase activities, genistein could, by inhibiting a phosphatase, increase the functional activity of CFTR. Alternatively, in cells that are strongly stimulated (i.e., PKA activity significantly greater than phosphatase activity), varying degrees of additional activation by genistein are observed. At the level of the tissue or in intact cells, this is supported by observations that genistein will increase epithelial ion transport during submaximal cAMP-dependent stimulation, but not during maximal stimulation, and that secretion is prolonged after cAMP removal (97, 181). Further support of this concept comes from the observation that CFTR is similarly phosphorylated in the presence of either forskolin or genistein (298). This model for the predominant effect of genistein has significant clinical ramifications if mutant forms of CFTR including ΔF508 CFTR are less likely to be activated by phosphorylation (178) or if inactivation is more rapid (322). Indeed, we have now reported that genistein potentiates the effect of maximally effective concentrations of IBMX (5 mM) and forskolin (10 μM) on G551D CFTR-expressing oocytes, whereas there is no effect on unstimulated or maximally stimulated oocytes expressing wild-type CFTR (327). Although the inhibition of a protein phosphatase by genistein can explain these observations, there have been no studies demonstrating that genistein does inhibit any of the known phosphatases, and the specific phosphatases that regulate CFTR inactivation remain to be elucidated.

The observation that genistein does not support channel gating in the absence of phosphorylation and/or nucleotides does not rule out the possibility for direct interaction with some unique phosphorylated or kinetic state of the channel. Hwang and co-workers (178, 439) have reported direct effects of genistein on CFTR channel gating both in the cell-attached and excised membrane patch configurations. Both variance analysis and fluctuation analysis indicated that genistein altered the gating behavior of forskolin-stimulated CFTR in cell-attached membrane patches. Results from cell-attached membrane patches were interpreted to indicate that genistein prolonged the burst duration of CFTR and thus increased $P_o$. However, the 2- or 3-fold increase in $P_o$ was not sufficient to account for the genistein-induced 45-fold increase in macroscopic current reported by the authors. Similar genistein-induced changes in $P_o$ of CFTR channels were observed in excised membrane patches for Xenopus oocytes and NIH 3T3 cells (124, 421). In each case, the authors concluded that genistein directly interacts with CFTR to increase $P_o$ and discount any role for genistein in the modulation of protein kinases or phosphatases. Speculation is provided regarding the domain that might be affected, a nucleotide binding domain, although no data are provided to support the claims (124, 421).

Hwang et al. (178) have alluded to a more unifying model for the effect of genistein on ΔF508 CFTR in which a direct interaction of genistein with the channel alters the ratio of phosphorylated to dephosphorylated channels. As other authors have noted, the $P_o$ of ΔF508 CFTR was reported to be less than that of wild-type CFTR in similar conditions (85, 159, 322). The reduced $P_o$ could reflect changes in the cell’s ability to phosphorylate the protein, changes in the stability of the phosphorylated state (i.e., the rate of dephosphorylation), or changes in the rates of nucleotide-dependent channel opening and closing. Hwang et al. (178) report that the activation rate of ΔF508 CFTR is significantly reduced compared with wild-type CFTR, while we have proposed that the dephosphorylation/inactivation rate of ΔF508 CFTR is greater than that of wild-type CFTR (322, 333). Regardless, once activated, genistein can directly interact with ΔF508 CFTR channels to prolong the phosphorylated, actively gating state of the channel. This concept is consistent with most observations reported to date.

It is tenuous to assess kinetic parameters for activation and inactivation processes involving enzymatically catalyzed reactions at room temperature if extrapolations to physiologically relevant conditions are intended. This is important to note since the only kinetic data currently available are derived from experiments completed at ambient temperature (178, 439). Because each enzyme in the activation and inactivation cascade and the kinetic behavior of CFTR itself likely exhibits a unique $Q_{10}$, the distribution of kinetic states available for modulation by genistein will be different at each temperature. Furthermore, different cell types (Hi-5 insect cells, NIH 3T3, L cells, Xenopus oocytes) would be expected to provide forms of regulatory proteins that are designed to function at the appropriate temperature for that cell type. For example, Bijman et al. (39) reported that the nucleotide dependence of CFTR channel gating changes with temperature, and we have shown that cooling excised membrane patches of L cells in the presence of PKA and ATP results in reduced CFTR channel amplitude and prolonged openings of the channels, a kinetic state that was not observed at 37°C (333). Additionally, it is important to recall that genistein may interact with multiple binding sites that might affect ion transport in any given cell type (see Fig. 1). In fact, genistein has been reported to reduce transepithelial Cl⁻ movement by inhibiting the function of a basolateral K⁺ channel (97, 181) and might have effects via interactions with purinergic receptors (194, 274).

Although it is often quoted that genistein is a specific tyrosine kinase inhibitor (3, 272), it is almost certain that the effects of genistein on CFTR are not modulated exclusively or even predominantly by an interaction with tyrosine kinase. It is, however, intriguing that some tyrosins and chemically unrelated tyrosine kinase inhibitors have been reported to affect CFTR-mediated ion transport (124, 182, 338). With the consideration of the diversity of systems evaluated, it is not remarkable to observe complementary effects of some tyrosine kinase inhibitors on
some occasions. Alternatively, it has been reported that erbastin analog, tyrphostins A23, A51, and AG126, canntharin, and herbimycin, all inhibitors of tyrosine kinase, did not mimic the effects of genistein on ion transport (76, 124, 181). This broader pharmacological perspective is not consistent with previously defined tyrosine kinases (68). Recent work in our laboratory has shown that both flavones and isoflavones modulate the activity of CFTR expressed in Xenopus oocytes and in T84 cell monolayers. Most importantly, we have noted a rank order of potency (genistein > apigenin > daidzein > flavone) that is preserved between these diverse systems (unpublished observations) and separates the effects of flavones and isoflavones on CFTR-mediated ion transport from previously reported pharmacological profiles on PTP and topoisomerase. Thus, although questions remain regarding the location of the genistein binding site(s), the physicochemical constraints of the binding site(s), and the mechanism(s) by which genistein modiﬁes mutant CFTR channel activity, it is undeniable that genistein holds great promise as a prototype therapeutic agent and research tool. The utility of this compound is underscored by recent reports in which the stimulatory effect of genistein on ion transport is interpreted as evidence that CFTR is present (183, 386).

D. Benzimidazolones

The benzimidazoles were first characterized as novel openers of large-conductance Ca2+-dependent K+ (BK, maxi K) channels in a European patent application (EPA 0477819A2) filed by NeuroSearch A/S. Olesen et al. (276) demonstrated that the most potent of these compounds, NS1619 [1-(2′-hydroxy-5′-trifluoromethylphenyl)-5-trifluoromethyl-2(3H)benzimidazolone; Fig. 3] activated BK channels from bovine aortic smooth muscle cells in a concentration-dependent (3–30 μM) manner. In contrast, NS1619 had no reported effect on a wide range of additional ion channels or seven membrane-spanning domain receptors (276). Thus this class of compounds represented the first known pharmacological openers of this important class of K+ channels. Both NS1619 and its structurally related analogs, NS004 [5-trifluoromethyl-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2H-benzimidazole-2-one; Fig. 3] and NS1608 [N-(3-(trifluoromethyl)phenyl)-N′-(2-hydroxy-5-chlorophenyl)urea] have now been shown to activate BK channels in a wide range of tissues including smooth muscle from guinea pig and human urinary bladder (172, 392), rat cerebral artery (168), porcine pulmonary artery (402), porcine (173) and canine (436) coronary artery, neurons from the ventromedial hypothalamus (339), GH3 cells (246), and Xenopus oocytes expressing the BK channel (108, 147).

Olesen et al. (276) demonstrated that NS1619 acted by inducing a parallel shift in the voltage dependence of BK activation in whole cell recordings. These studies were subsequently extended by demonstrating that the structurally related analog NS004 induced a parallel shift in the P0 versus membrane voltage relationship for single BK channels studied in lipid bilayers (246). In both of these studies, single-channel recording techniques demonstrated that the benzimidazolones directly increased the P0 of BK channels and that this increase in P0 was due to both an increase in channel open time as well as a decrease in channel closed time (246, 276).

To define the mechanism by which these benzimidazolones activate BK channels, the Ca2+ dependence of activation was investigated. Both Olesen et al. (276) and Holland et al. (168) demonstrated that NS1619 failed to activate BK channels in the absence of added bath Ca2+ in excised patch-clamp recordings. These results suggested that the observed activation was due to a shift in the Ca2+ affinity of the channel rather than NS1619 simply substituting for Ca2+ in activating the channel. However, both Hu and Kim (172) and McKay et al. (246) found that NS004 continued to activate BK channels in the nominal absence of bath Ca2+ in excised patches. Similarly, Vandier and Bonnet (402) and Edwards et al. (109) found that NS004 and NS1619, respectively, activated BK channels in the absence of Ca2+. However, the experiments of Vandier and Bonnet (402) and Edwards et al. (109) were done in the whole cell recording mode where Ca2+ at the plasma membrane may have been somewhat elevated. The disparate results in the excised patch configuration may be due to structural differences between the benzimidazolones and NS004 or NS1619 may represent differences between tissues. An additional possibility is that the regulatory status of the channel may determine the effect of the benzimidazolone. For example, the phosphorylation state of the channel may be an important determinant of benzimidazolone effects. Additional experimentation will be required to fully appreciate the mechanism by which the benzimidazolones activate BK channels.

It is now known that both voltage-activated (Kv) and maxi K channels consist of a pore-forming α-subunit coupled with a regulatory β-subunit (207, 235, 299, 337, 393). Although the Kvβ-subunit is a cytoplasmic protein (235, 299, 337), the β-subunit associated with the maxi K channel consists of two membrane-spanning domains and a large extracellular loop (207). The Kvβ-subunit has been shown to interact with the NH2-terminal end of the Kα-subunit (435), thereby inducing inactivation of the K+ current (165, 299, 435). Similarly, the expression of both α- and β-subunits of the maxi K channel has been shown to shift both the voltage dependence and Ca2+ dependence of activation (107, 247, 393). This effect of the β-subunit is similar to the effect described for NS004 (246, 276). A more detailed understanding of the subunit with which pharmacological modulators interact has emerged from

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studies employing coexpression of the α- and β-subunits of the maxi K channel. Activation of the maxi K channel by dehydrosoyasaponin I was absolutely dependent on expression of the β-subunit (247), whereas activation by NS1619 was independent of β-subunit expression (107). These results suggest that the benzimidazolones interact with the α-subunit of the channel.

Although the benzimidazolones were initially thought to be specific activators of BK channels, more recent evidence has demonstrated that they also inhibit K+ channels in smooth muscle (109, 168), as well as voltage-dependent Ca2+ channels in vascular smooth muscle (109, 168), cerebellar granule cells (277), and isolated rat heart (319). Additionally, NS004 has been shown to inhibit both a delayed rectifier K+ current and a KATP channel in smooth muscle (436). Thus it is now apparent that the benzimidazolones modulate the activity of numerous ion channels and, as such, a macroscopic effect must be interpreted with caution.

A critical breakthrough in the pharmacology of CFTR came in 1994 when Lazdunski and colleagues (147) demonstrated that one of the benzimidazolones, NS004, activated both wild-type and ΔF508 CFTR in both the Xenopus oocyte expression system as well as in excised outside-out patches from Vero cells transiently expressing the CFTR protein. More recently, these authors demonstrated, using I− efflux and whole cell patch-clamp measurements, that NS004 similarly activated an additional CFTR mutant, P574H, while failing to activate the CF-causing R560T or ΔI507 CFTR mutants (70). Thus NS004 was the first described pharmacological opener of CFTR. In Xenopus oocytes, NS004 activated wild-type CFTR current to ~30–60% of that seen with elevated cAMP. In contrast, in excised outside-out patches, no effect of NS004 was seen unless CFTR was first activated by cAMP (147). These results suggest that NS004 cannot activate CFTR unless it is in a permissive, phosphorylated state. Also, after maximal activation with cAMP, wild-type CFTR cannot be further activated by NS004 in Xenopus oocytes. In contrast to these results, ΔF508 CFTR could not be activated in the oocyte expression system unless it was first activated by cAMP (147).

We recently evaluated the effect of NS004 on both wild-type and G551D CFTR in the Xenopus oocyte expression system (327). In contrast to the results of Lazdunski and colleagues (147), we were unable to detect any activation of wild-type CFTR in the absence of first partially activating CFTR with a low concentration of forskolin plus IBMX. These differences may be explained by differing levels of basal cAMP present in our experiments and those of Lazdunski and colleagues (147). Similar to the results on ΔF508 CFTR (147), we found that after activation of G551D CFTR with a maximal concentration of forskolin (10 μM) plus IBMX (5 mM), the subsequent addition of NS004 (30 μM) induced an additional twofold increase in CFTR-dependent current (327). Unfortunately, we have been unable to routinely activate either wild-type or G551D CFTR with NS004 in excised inside-out patches from mouse L cells recombinantly expressing CFTR (unpublished observations). Similarly, excised inside-out patches obtained from oocytes expressing wild-type CFTR could only be sparcly activated by NS004 (V. Gribkoff, personal communication). Lazdunski and colleagues (147) did not report the effect of NS004 in excised inside-out patches.

The observations that NS004 and 8-methoxypsoralen (8-MOP, Fig. 3; see below) only sporadically activate CFTR in excised inside-out patches while consistently activating the channel in both oocytes and intact epithelial monolayers suggest that either these pharmacological “openers” are not working directly on the CFTR protein or that CFTR must be in a conformational state that is permissive for activation. Our own observations suggest that CFTR must be at least partially phosphorylated before NS004 is capable of activating the channel in Xenopus oocytes. Also, Lazdunski and co-workers (147) found that CFTR must first be activated by forskolin before NS004 is capable of activating CFTR during outside-out patch-clamp recording. One possibility to explain these divergent results is that a cytoplasmic component necessary for the activation of CFTR by NS004 is entrapped by the formation of the outside-out patch. A second possibility is that the phosphorylation state of the channel obtained during activation by forskolin is distinct from that after activation of the channel by exogenous PKA/ATP in an excised patch, and it is this level and/or sites of phosphorylation that define the state of the channel that can be further modulated by NS004. A final possibility that must be borne in mind is that these pharmacological modulators of CFTR activity may be operating via an unspecified second messenger to increase channel activity. Clearly, a great deal more study is required to elucidate the mechanism of action of the CFTR openers thus far defined.

Although the above results demonstrate that NS004 is capable of opening both wild-type and mutant CFTR, these studies do not address whether NS004 will stimulate a transepithelial Cl− secretory response. Thus we determined whether NS004 would stimulate transepithelial Cl− secretion across monolayers of the colonic cell line T84. We demonstrated that NS004 activated an apical membrane Cl− conductance in nystatin-permeabilized T84 monolayers but failed to stimulate a transepithelial Cl− secretory response in intact monolayers (93). Similar results were obtained with NS1619 (93). Both NS004 and NS1619 also failed to stimulate a Cl− secretory response after stimulation of Cl− secretion by forskolin (93). In contrast to this response, the benzimidazolone 1-ethyl-2-benzimidazolinone (1-EBIO; Fig. 3) stimulated a sustained charybdotoxin-sensitive Cl− secretory response (93, 95). Unfortunately, 1-EBIO has a low affinity for this effect.
eral K
we demonstrated that NS004 and NS1619 both further results suggest that the human airway possesses a basolat-
stimulate Cl
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and potentiated the response to the Ca2
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secretion via the direct activation of a basolateral secretion requires the coordinate regulation of both apical
conductance (K Ca), thereby cultures of murine airway suggest that NS004 is ineffec-
secretory re- these two culture systems. Indeed, 200
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M) stimulate transepithelial Cl
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secretion when added subsequent to 1-EBIO (100 
M) activated K Ca, whereas the subsequent addition of 1-EBIO (100 
M) induced a 10- to 15-fold increase in K Ca. Similar to NS004, 1-EBIO activated CFTR in nystatin-permeabilized monolayers (93). These results suggest that NS004 fails to stimulate transepithelial Cl
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secretion because of its inability to modulate the activity of a basolateral K+ conductance. In contrast, the related benzimidazolone 1-EBIO activates both apical Cl
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conductance (CFTR) and basolateral K+ conductance (K Ca), thereby providing the coordinate regulation necessary to stimulate a transepithelial Cl
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secretory response. These results have led us to speculate that modulating the driving force on Cl
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secretion via the direct activation of a basolateral membrane K+ conductance would be beneficial in modu-
lation Cl
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secretion across the human airway.

We have also evaluated the effects of the benzimidazolones on primary cultures of murine tracheal epithelia (MTE). Our results on the MTE were qualitatively similar to those on the T84 cells, i.e., after inhibition of basal Na+ transport with amiloride, NS004 (10 
M) failed to stimulate Cl
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secretion. However, 1-EBIO stimulated Cl
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secretion, and the subsequent addition of NS004 further augmented the Cl
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secretory response (93). In contrast to these results, Alton (personal communication) found that NS004 (20 
M), alone, induced an ~9 
A/cm
2 Cl
-secretory response in freshly excised murine trachea, after inhibition of Na+ transport with amiloride. In paired tracheas, forskolin increased Cl
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secretion by ~40 
A/cm
2, and this was not further stimulated by NS004 (E. W. Alton, personal communication). In contrast to these results, NS004 had no effect on in vivo nasal potential difference measurements in the G551D mouse in the absence or presence of forskolin (Alton, personal commu-
nication).

In contrast to our results from T84 cells and primary cultures of MTE, we recently demonstrated that both NS004 (10 
M) and 8-MOP (10 
M) stimulate transepithelial Cl
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secretion across primary cultures of human bronchial epithelium (HBE) subsequent to inhibition of basal Na+ absorption with amiloride (unpublished observations). This NS004- and 8-MOP-induced Cl
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secretory response was not blocked by charybdotoxin, suggesting that the stimulation in HBE was not due to the activation of a basolateral membrane BK channel by NS004. However, pretreatment of the tissue with a nonspecific K+ channel blocker, Ba2+, diminished the subsequent response to either NS004 or 8-MOP (unpublished observations). These results suggest that the human airway possesses a basolat-
eral K+ conductance that is constitutively active and thereby provides the driving force required to sustain a Cl
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secretory response in the presence of a Cl
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channel opener (e.g., NS004, 8-MOP). This is consistent with the observation that, after inhibition of basal Na+ absorption with amiloride, Cl
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is above electrochemical equilibrium across the apical membrane in human airway epithelia (429), such that increasing apical Cl
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secretion will result in Cl
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secretion. Indeed, in the human airway, cAMP-dependent agonists have little effect on basolateral K+ conductance will depend on the exact relationship between these two conductive pathways.

E. Benzoxazoles (Chlorzoxazone)

Based on our results with the K Ca opener 1-EBIO, we speculated that modulators of basolateral membrane K+ conductances would be beneficial in stimulating Cl
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secretion or augmenting the stimulatory response of Cl
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channel openers in the human airway. Thus one of the aims of our group is to identify novel, potent openers of basolateral membrane K+ channels in the hopes of utilizing these as therapeutic tools in CF. However, we appreciate the difficulty associated with moving an investiga-
tional new drug into a clinical setting. Therefore, we have attempted to identify drugs that are currently approved (E. W. Alton, personal communication). In contrast to these results, 1-EBIO represents the first known opener of this class of K Ca. Similar to NS004, 1-EBIO activated CFTR in nystatin-permeabilized monolayers (93). These results suggest that NS004 nor NS1619 (10±100 
M) activated K Ca whereas the subsequent addition of 1-EBIO (100 
M) induced a 10- to 15-fold increase in K Ca. Similar to NS004, 1-EBIO activated CFTR in nystatin-permeabilized monolayers (93). These results suggest that NS004 fails to stimulate transepithelial Cl
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secretion because of its inability to modulate the activity of a basolateral K+ conductance. In contrast, the related benzimidazolone 1-EBIO activates both apical Cl
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conductance (CFTR) and basolateral K+ conductance (K Ca), thereby providing the coordinate regulation necessary to stimulate a transepithelial Cl
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secretory response. These results have led us to speculate that modulating the driving force on Cl
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secretion via the direct activation of a basolateral membrane K+ conductance would be beneficial in modu-
lation Cl
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secretion across the human airway.

We have also evaluated the effects of the benzimidazolones on primary cultures of murine tracheal epithelia (MTE). Our results on the MTE were qualitatively similar to those on the T84 cells, i.e., after inhibition of basal Na+ transport with amiloride, NS004 (10 
M) failed to stimulate Cl
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secretion. However, 1-EBIO stimulated Cl
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secretion, and the subsequent addition of NS004 further augmented the Cl
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secretory response (93). In contrast to these results, Alton (personal communication) found that NS004 (20 
M), alone, induced an ~9 
A/cm
2 Cl
-secretory response in freshly excised murine trachea, after inhibition of Na+ transport with amiloride. In paired tracheas, forskolin increased Cl
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secretion by ~40 
A/cm
2, and this was not further stimulated by NS004 (E. W. Alton, personal communication). In contrast to these results, NS004 had no effect on in vivo nasal potential difference measurements in the G551D mouse in the absence or presence of forskolin (Alton, personal commu-
nication).

In contrast to our results from T84 cells and primary cultures of MTE, we recently demonstrated that both NS004 (10 
M) and 8-MOP (10 
M) stimulate transepithelial Cl
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secretion across primary cultures of human bronchial epithelium (HBE) subsequent to inhibition of basal Na+ absorption with amiloride (unpublished observations). This NS004- and 8-MOP-induced Cl
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secretory response was not blocked by charybdotoxin, suggesting that the stimulation in HBE was not due to the activation of a basolateral membrane BK channel by NS004. However, pretreatment of the tissue with a nonspecific K+ channel blocker, Ba2+, diminished the subsequent response to either NS004 or 8-MOP (unpublished observations). These results suggest that the human airway possesses a basolat-
eral K+ conductance that is constitutively active and thereby provides the driving force required to sustain a Cl
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secretory response in the presence of a Cl
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channel opener (e.g., NS004, 8-MOP). This is consistent with the observation that, after inhibition of basal Na+ absorption with amiloride, Cl
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is above electrochemical equilibrium across the apical membrane in human airway epithelia (429), such that increasing apical Cl
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secretion will result in Cl
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secretion. Indeed, in the human airway, cAMP-dependent agonists have little effect on basolateral K+ conductance will depend on the exact relationship between these two conductive pathways.
peak plasma concentrations found in vivo (see below). In addition, both chlorzoxazone and zoxazolamine activated \( K_{ca} \) in excised inside-out patches (356). We have now demonstrated, using the paradigm of Knowles et al. (208), that chlorzoxazone induces a hyperpolarization of the transepithelial nasal potential difference in normal volunteers after inhibition of the basal Na\(^+\) absorption with amiloride and establishment of a blood-to-lumen Cl\(^-\) concentration gradient (M. Gondor, personal communication). This hyperpolarization is indicative of an induced Cl\(^-\) secretory response. Thus these results demonstrate the utility of augmenting the basolateral membrane K\(^+\) conductance in sustaining a Cl\(^-\) secretory response in the human airway.

Chlorzoxazone is approved by the Food and Drug Administration as an adjunct therapy for the relief of acute musculoskeletal pain. At high doses (600–750 mg), a peak plasma concentration of 100–200 \( \mu \)M is obtained (113, 369), with a half-life of \( \sim 1 \) h. The concentration in kidney and muscle is approximately one-half that found in plasma. Although the benzodiazepines have largely displaced the use of these muscle relaxants, at one point they were included on the list of the 200 most prescribed drug products. In spite of the fact that these drugs were introduced over 40 years ago, their mechanism of action remains largely unknown, and with their limited current use in the clinical realm research into their mechanism of action has virtually ceased. These drugs preferentially suppress polysynaptic reflexes and have been shown to decrease striatal dopamine turnover and decrease neuronal firing rate (240, 248). The mechanism for this effect is unknown but does not appear to involve strychnine-insensitive glycine or GABA\(_B\) receptors (248). Although our recent results do not address the mechanism by which these agents affect neuronal firing, we have demonstrated a direct effect on \( K_{ca} \) (356), suggesting that a direct effect on an ion-conductive pathway may be important in determining their clinical efficacy.

Recently, Adelman and colleagues reported the cloning of two different classes of \( K_{ca} \): the small-conductance, apamin-sensitive K\(^+\) channels (SK; Ref. 210) and the intermediate-conductance, charybotoxin-sensitive K\(^+\) channels (IK; Ref. 188). The cloning of this IK class of K\(^+\) channels was simultaneously reported by Joiner et al. (195). Although the SK channels have a principally neuronal expression pattern (210), IK is expressed peripherally in epithelial tissues including lung and colon (188). Both rSK2 and hIK1 have been generously given to us by Dr. J. P. Adelman (Vollum Institute, Oregon Health Sciences University). We have demonstrated expression of hIK1 in both the human colonic cell line T84 as well as the human airway serous cell line calu-3, employing both Northern analysis as well as indirect immunofluorescence using an antibody directed against the COOH-terminal tail of hIK1 (45; unpublished observations). These results, coupled with the biophysical and pharmacological findings of hIK1, suggest this is the basolateral membrane \( K_{ca} \) of colonic and airway epithelia. Based on this, we determined the effect of the K\(_{ca}\) openers 1-EBIO, chlorzoxazone, and zoxazolamine on hIK1 as well as rSK2 heterologously expressed in Xenopus oocytes. Chlorzoxazone, 1-EBIO, and zoxazolamine all activate both hIK1 and rSK2 during both two-electrode voltage-clamp and excised inside-out patch-clamp recordings (378). Similar to what we previously reported on the endogenous \( K_{ca} \) in T84 cells, activation by these pharmacological agents is dependent on the presence of physiological levels of cytoplasmic Ca\(^{2+}\). Also, the primary metabolite of chlorzoxazone, 6-hydroxychlorzoxazone, fails to activate either hIK1 or rSK2 (378), similar to our results on the T84 \( K_{ca} \) (356). These results further suggest that the centrally acting muscle relaxant effects of these compounds are related to their ability to activate neuronal K\(^+\) channels (SK), which would be predicted to hyperpolarize the neuron thus decreasing firing rate as observed. In future studies, it will be important to define the mechanism by which these pharmacological K\(^+\) channel openers modulate the Ca\(^{2+}\)-dependent gating of both hIK1 and rSK2.

The major metabolic product of chlorzoxazone is 6-hydroxychlorzoxazone (369). The generation of this metabolite has been used as a means of quantifying cytochrome P-450 \( \beta \)E1 activity, since this has been shown to be the major isozyme responsible for the metabolic conversion of chlorzoxazone (15, 74, 232, 437). More recently, however, the 3A and 1B1 isozymes of cytochrome P-450 have also been shown to make a contribution to the 6-hydroxylation of chlorzoxazone (137, 349). In contrast to chlorzoxazone, we have demonstrated that 6-hydroxychlorzoxazone fails to activate \( K_{ca} \) in excised inside-out patches (356). Because the cytochrome P-450 have been shown to exist in significant quantities in lung tissue (134, 239), it will be important to determine whether the oxidative metabolites of proposed pharmacological modulators of Cl\(^-\) secretion maintain efficacy, thereby allowing the extrapolation of in vitro results from primary cultures to the intact lung where inhalation therapy may be employed.

### F. Psoralens

The psoralens (Fig. 3) are planar, tricyclic furcoumarins, being composed of a furan ring fused to a coumarin moiety. In contrast, the angelicins are angular psoralens. The psoralens are found naturally in a wide variety of plants including limes, parsnip, celery, and figs as well as in certain fungi. The photochemotherapeutic potential of these compounds was first recognized \( \sim 3.5 \) millennia ago by the herbalists of ancient India and Egypt who treated vitiligo with the extracts of plants known to contain psoralens to induce repigmentation upon exposure...
to sunlight. In 1947, El-Mofty (114) began treating patients for vitiligo with 8-MOP extracted from plants. However, the major clinical usefulness of the psoralens is in the treatment of psoriasis. This was first recognized by Pinkus in 1951 (as reviewed in Ref. 281) and eventually led to the first controlled trial by Parrish et al. (280). In these studies, it was demonstrated that psoriasis could be effectively treated with a combination of psoralens and long-wave ultraviolet light (PUVA). The psoralen most commonly used for this purpose is the naturally occurring 8-MOP, although more recently 5-methoxypsoralen (Bergapten) has been shown to be effective while having fewer side effects (135). Since this initial trial, psoralen phototherapy has been used to successfully treat more than 30 conditions including rheumatoid arthritis, cutaneous T-cell lymphoma, and numerous photodermatoses (169), although the mechanism underlying its therapeutic benefit remains unknown.

The photochemical reaction of the psoralens is complex, involving at least two separate reactions. The first, and perhaps better known, is the mechanism by which the psoralens intercalate into the DNA double helix. Upon absorption of long-wave ultraviolet light, the psoralens form a monofunctional adduct with thymine and cytosine. Absorption of additional photons allows the linear psoralen, including 8-MOP, to form an interstrand cross-link of the double helix. While the Watson-Crick hydrogen bonding is maintained throughout the double helix, the strength of the hydrogen bond is weakened at the site of cross-linking. Thus the psoralens induce significant changes in the local DNA helix, although there is no important effect on the overall helical structure (176). This DNA cross-linking interferes with replication and transcription resulting in an immediate inhibition of DNA synthesis and cell proliferation. Although it was initially believed that this inhibition of DNA synthesis was responsible for the therapeutic effect observed, more recent evidence has demonstrated that the angelicins also possess therapeutic potential while failing to form the bifunctional adducts required to inhibit DNA synthesis (80). A second photochemical reaction involves the transfer of energy to molecular oxygen resulting in reactive oxygen species and free radicals. This in turn activates the arachidonic acid metabolism cascade, resulting in eicosanoid formation. The result of this is erythema, a known clinical manifestation of PUVA therapy (135).

This ability of the psoralens to intercalate into the DNA helix, thereby inhibiting DNA replication, has recently been exploited in designing viral vectors for gene delivery. Tsung et al. (396) demonstrated that 8-MOP entered the capsid of an adenovirus, thereby cross-linking the viral DNA at a frequency of approximately one modification per 100 bp of viral DNA. Treatment with 8-MOP inactivated the adenovirus, rendering it replication incompetent. However, this treatment did not affect the ability of the adenovirus to enhance the cellular delivery of polylysine-ligand packaged DNA. More recently, Baker and Cotten (16) demonstrated that this 8-MOP-inactivated adenovirus could be used to transfer bacterial artificial chromosomes of up to 170 kb into mammalian cells. Thus the use of psoralen-inactivated virus particles may provide an alternative gene delivery system for CFTR.

In addition to its effects on DNA replication, 8-MOP has been shown to be a potent ($K_i = 10–25 \mu M$) suicide inhibitor of cytochrome P-450 (122, 218, 223). This inhibition is due to the initial activation of 8-MOP by cytochrome P-450 into a metabolite that then covalently binds microsomal proteins and inhibits cytochrome P-450 (122).

Szewczyk et al. (379) demonstrated in 1992 that 8-MOP inhibited both the ATP-sensitive K$^+$ channel of pancreatic $\beta$-cells ($K_{\text{ATP}}$) as well as a delayed rectifier K$^+$ channel similarly expressed in these cells. As detailed above, the well-known $K_{\text{ATP}}$ inhibitors glibenclamide and tolbutamide were also found to inhibit the CFTR Cl$^-$ channel initially in both whole cell (347) and single-channel studies (90, 324, 346, 409) and finally in transepithelial short-circuit current measurements (409), albeit at a much reduced affinity ($K_i$ of glibenclamide for CFTR of $\sim 30 \mu M$ and nM for $K_{\text{ATP}}$). These results suggested that other known modulators of $K_{\text{ATP}}$ might also influence the gating of CFTR. Based on these observations, we determined the effect of several psoralens on Cl$^-$ secretion in the T84 cell line as well as primary MTE cultures (94). Although 8-MOP failed to induce a Cl$^-$ secretory response on its own in the T84 cell line, it both potentiated the Cl$^-$ secretory effect of the muscarinic agonist carbachol as well as induced a sustained plateau phase to the carbachol response. These results are consistent with the activation of an apical membrane Cl$^-$ conductance by the psoralens in the absence of any change in basolateral K$^+$ conductance. This paradigm has previously been used to explain the potentiation of a Ca$^{2+}$-mediated agonist responses in T84 cells by increasing cellular cAMP (23). This hypothesis was evaluated by determining the effect of 8-MOP on Cl$^-$ secretion after activation of the basolateral membrane $K_{\text{cb}}$ by either the Ca$^{2+}$-ATPase inhibitor thapsigargin or a direct pharmacological opener of $K_{\text{cb}}$-1-EBIO (93, 95). In both cases, 8-MOP further increased Cl$^-$ secretion after activation of $K_{\text{cb}}$, indicating that K$^+$ conductance was rate limiting in these secretory responses. Similarly, 8-MOP failed to induce a Cl$^-$ secretory response in MTE when added alone. However, after activation of K$^+$ conductance by 1-EBIO, 8-MOP further increased the Cl$^-$ secretory re-
sponse (94). Finally, in nystatin-permeabilized T84 monolayers, 8-MOP increased apical Cl\(^-\) conductance while having no effect on basolateral K\(^+\) conductance. The activation of Cl\(^-\) conductance was insensitive to block by either Cd\(^{2+}\), an inhibitor of CIC-2 (334), or TS-TM-Calix[4]arene, an inhibitor of the outwardly rectifying Cl\(^-\) channel (358). In contrast, the 8-MOP-induced activation of Cl\(^-\) conductance was inhibited by glibenclamide (94). This pharmacological profile is consistent with the activation of CFTR by 8-MOP.

These results suggest that the psoralens, which are clinically useful for a variety of diseases, may be therapeutically beneficial in CF therapy by increasing apical Cl\(^-\) conductance. Indeed, we recently demonstrated that, after inhibition of basal Na\(^+\) transport with amiloride, 8-MOP stimulates a Cl\(^-\) secretory response in primary cultures of HBE (unpublished observations). Despite these observations, we have been unable to demonstrate a direct activation of CFTR by 8-MOP in excised inside-out patches in the presence of 300 \(\mu\)M ATP with or without PKA (unpublished observations). Also, in Xenopus oocytes expressing wild-type CFTR, 8-MOP fails to activate CFTR on its own. These results suggest that CFTR must be in an appropriate conformational state for the psoralens to activate the channel.

**IV. HUMAN AIRWAY EPITHELIAL STUDIES**

It is clear from the above discussion that several exciting lead compounds have been identified that are capable of stimulating Cl\(^-\) secretion across human airway. Although we have not evaluated all of these compounds to determine their ability to modulate Cl\(^-\) secretion across primary cultures of human CF (\(\Delta F_{508}\): \(\Delta F_{508}\) airway, we have studied several of these, including genistein, NS004, and 1-EBIO. The K\(^+\) channel opener 1-EBIO (1 mM) had no effect on Cl\(^-\) secretion in these cultures (change in short-circuit current = 0.06 ± 0.08 \(\mu\)A/cm\(^2\); \(n = 15\)). In comparison, in wild-type CFTR expressing HBE, 1-EBIO increased short-circuit current by 11.6 ± 0.9 \(\mu\)A/cm\(^2\) (\(n = 20\)). Similarly, when added alone, NS004 increased short-circuit current by only 0.4 \(\mu\)A/cm\(^2\) (\(n = 13\)) in \(\Delta F_{508}\) expressing HBE, whereas in wild-type CFTR expressing HBE, NS004 increased short-circuit current by 10.8 ± 1.7 \(\mu\)A/cm\(^2\) (\(n = 18\)). Forskolin also has little effect as expected for a CF airway; increasing short-circuit current by 1.5 \(\mu\)A/cm\(^2\) (\(n = 21\)) compared with 13.7 ± 1.2 \(\mu\)A/cm\(^2\) (\(n = 116\)) in wild-type CFTR expressing HBE. However, subsequent to forskolin, NS004 (10 \(\mu\)M) increased short-circuit current by an additional 2.1 \(\mu\)A/cm\(^2\) (\(n = 21\)) in \(\Delta F_{508}\) expressing HBE, and this was inhibited by bumetanide. This apparent dependence on prior forskolin stimulation is similar to our results from Xenopus oocytes as outlined above. Also, genistein (50 \(\mu\)M) stimulated a significant Cl\(^-\) secretory response in both \(\Delta F_{508}\) (2.5 ± 0.5 \(\mu\)A/cm\(^2\); \(n = 11\)) and wild-type CFTR (7.9 ± 0.8 \(\mu\)A/cm\(^2\); \(n = 17\)) expressing HBE. To our knowledge, these are the first results demonstrating an effect of a pharmacological opener (NS004, genistein) of CFTR on human CF airway and suggest that native human airway expresses sufficient \(\Delta F_{508}\) CFTR at the apical membrane to be modulated by pharmacological agonists. As further support for this hypothesis, we incubated \(\Delta F_{508}\) HBE cells at 26°C for 24 h and determined the effect of 1-EBIO, NS004, and genistein on Cl\(^-\) secretion. This treatment is expected to increase the delivery of \(\Delta F_{508}\) CFTR to the apical membrane (89). Under these conditions, the effects of 1-EBIO, NS004, and genistein on Cl\(^-\) secretion were all potentiated [1.5 ± 0.2 (\(n = 4\)), 2.0 ± 0.3 (\(n = 7\)), and 8.3 ± 2.4 (\(n = 6\)) \(\mu\)A/cm\(^2\), respectively].

In addition to determining the effect of these ion channel modulators on Cl\(^-\) secretion, it will also be important to evaluate their effects on Na\(^+\) absorption across human airway. For example, we recently demonstrated that Ca\(^{2+}\)-mediated agonists, including mucosal UTP, are potent inhibitors of Na\(^+\) absorption across CF airway suggesting they may have a dual therapeutic role: 1) the inhibition of Na\(^+\) hyperabsorption associated with CF and 2) the stimulation of Cl\(^-\) secretion (92). In contrast, we demonstrated that genistein stimulates Na\(^+\) absorption in human CF airway (92). Also, we found that 1-EBIO stimulates Na\(^+\) absorption as expected for a compound that opens basolateral membrane K\(^+\) channels and thus increases the driving force for Na\(^+\) entry across the apical membrane (unpublished observations). In contrast, NS004 has no effect on Na\(^+\) absorption across human CF airway. Thus it will be important to carefully evaluate the effects of these investigational drugs not in isolation on the intended target (CFTR) but rather in the context of the transporting epithelium where they may have potentially unwanted effects on alternative targets such as Na\(^+\) absorption.

Finally, it will be important in future studies to determine whether these modulators of CFTR stimulate a Cl\(^-\) secretory response in human airways expressing additional mutations that are normally trafficked to the apical membrane in the intact epithelium (e.g., G551D). As outlined above, pharmacological agonists have been shown to activate the G551D mutant in Xenopus oocytes. These studies will be important in determining the CF patients with appropriate genotypes amenable to pharmacological manipulation by ion channel openers versus those that will require alternative interventions including chemical chaperones or gene therapy.

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