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Commentary

CFTR Channel Pharmacology: Novel Pore Blockers Identified by High-throughput Screening

David N. Sheppard

Department of Physiology, University of Bristol, School of Medical Sciences, Bristol BS8 1TD, UK

Investigators of anion channels are frequently heard bemoaning the absence of potent, specific inhibitors of their favorite channel. The lack of such blockers has been particularly frustrating for researchers investigating the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel, which plays a central role in electrolyte transport across epithelial tissues (Welsh et al., 2001). Perhaps the complaints of CFTR researchers might soon be a thing of the past following the discovery of glycine hydrazides by Chatchai Muanprasat and colleagues, which is reported in this issue of the *Journal of General Physiology* (Muanprasat et al., 2004). In brief, the authors employ a high-throughput screening (HTS) assay for the identification of CFTR inhibitors to discover glycine hydrazides and then investigate the effects of these agents on CFTR function in experiments that range from single channels to animal models (Muanprasat et al., 2004). In the process, Muanprasat and colleagues find that glycine hydrazides have a novel mechanism of action: occlusion of the extracellular end of the CFTR pore. All in all, the work is a tour de force of CFTR pharmacology.

The pharmacology of the CFTR Cl^- channel has attracted significant interest in recent years. Much of this attention has been fuelled by the search for rational new therapies for diseases caused by CFTR malfunction. Mutations that, in general, abolish the function of CFTR cause the life-threatening genetic disease cystic fibrosis (CF; Welsh et al., 2001). By contrast, some forms of male infertility, disseminated bronchiectasis and chronic pancreatitis are caused by CFTR mutations that are likely to preserve partial CFTR function (Welsh et al., 2001). Other diseases, such as secretory diarrhea and polycystic kidney disease involve unphysiologic activation of the CFTR Cl^- channel (Sullivan et al., 1998; Al-Awqati, 2002). Besides treatments for disease and perhaps even male contraception, CFTR inhibitors are important in several other respects: (a) as probes to identify CFTR-dependent function and to investigate CFTR structure and function, (b) to study the pathogenesis of lung disease in CF, and (c) to develop animal models with which to evaluate new therapies for CF.

A wide spectrum of diverse small molecules, which inhibit the CFTR Cl^- channel, has been identified. Analysis of these molecules reveals several common themes: they are anions, most are lipophilic, and many are large in size. Moreover they inhibit CFTR by two general mechanisms: open-channel and allosteric block (Cai et al., 2002). A variety of large organic anions (e.g., glibenclamide) are open-channel blockers of the CFTR Cl^- channel. These agents inhibit CFTR by binding within the deep wide vestibule at the intracellular end of the CFTR pore and preventing Cl^- flow by occluding the permeation pathway (Fig. 1, A and B; Cai et al., 2002). By contrast, elevated concentrations of several agents that potentiate CFTR Cl^- currents (e.g., genistein) inhibit CFTR by an allosteric mechanism. These agents inhibit CFTR by interacting with the nucleotide-binding domains (NBDs), which control channel gating, and slowing greatly the rate of channel opening (Fig. 1, A and C; Cai et al., 2002). Because the characteristics of CFTR inhibition by open-channel and allosteric blockers differ, these agents can be distinguished by the effects of (a) voltage, (b) external Cl^- concentration, and (c) the inorganic phosphate analogue pyrophosphate (PP_i) that disrupts ATP-dependent gating. Inhibition of CFTR by open-channel blockers is voltage dependent and enhanced when the external Cl^- concentration is reduced, but unaffected by PP_i (Cai et al., 2002). In contrast, inhibition of CFTR by allosteric blockers is voltage independent and unaffected by reducing the external Cl^- concentration, but relieved by PP_i and elevated concentrations of ATP (Cai et al., 2002).

Despite the plethora of CFTR inhibitors identified using conventional assays of CFTR function, potency and specificity have remained intractable problems. Few agents have been identified that inhibit CFTR with nanomolar affinity. Worse, no specific blockers of the CFTR Cl^- channel have been identified. Open-channel

Abbreviations used in this paper: ADPKD, autosomal dominant polycystic kidney disease; CFTR, cystic fibrosis transmembrane conductance regulator; FRT, Fischer rat thyroid; HTS, high-throughput screening; NBD, nucleotide-binding domain.

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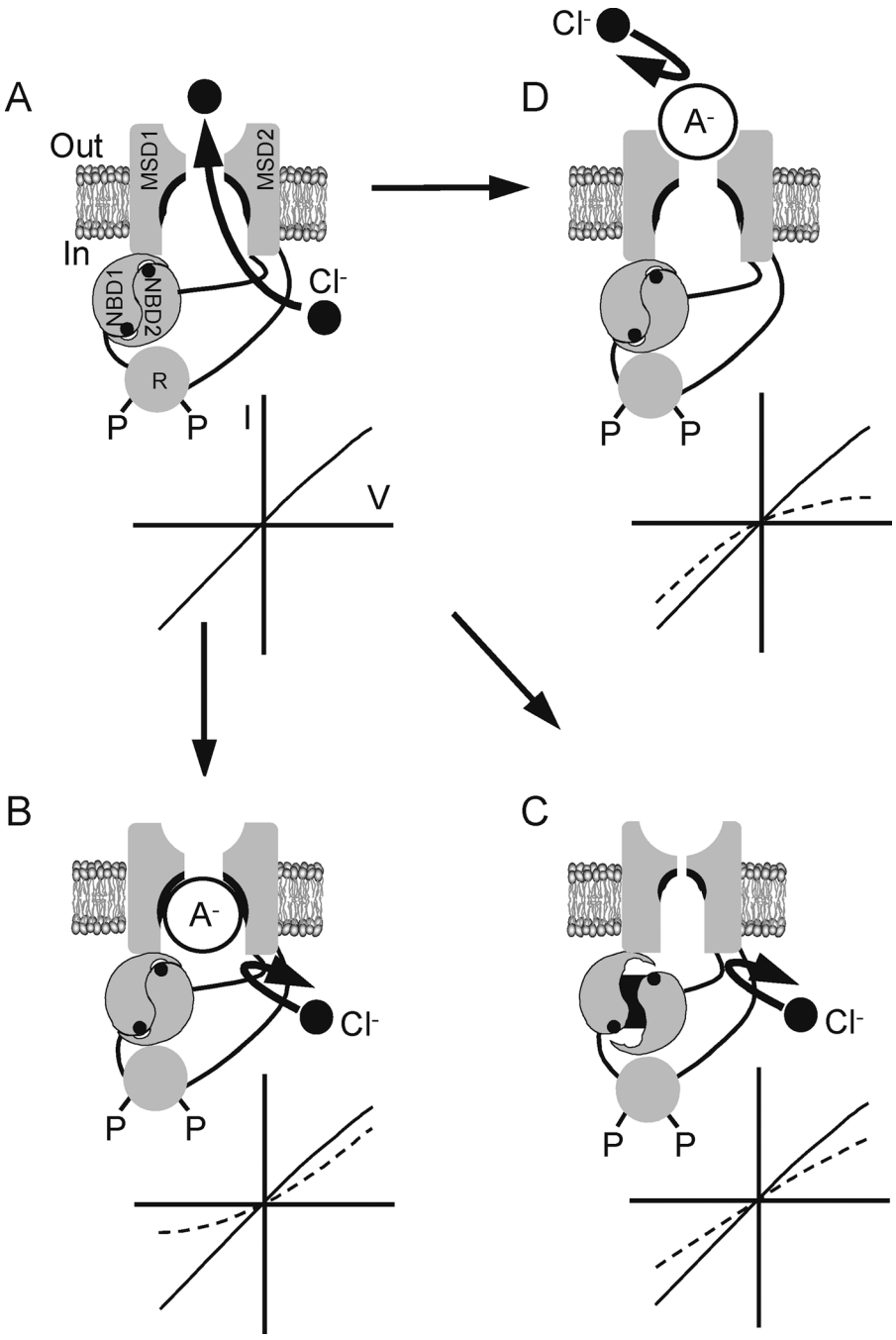

FIGURE 1. Mechanism of action of CFTR inhibitors. (A–D) Simplified models of the CFTR Cl^- channel and the effects of open-channel and allosteric blockers. Schematic representations of the I–V relationships of CFTR Cl^- currents in the absence (continuous line) and presence (dotted line) of blockers under conditions of symmetrical Cl^--rich solutions are shown beneath each model. (A) The CFTR pore contains a deep wide intracellular vestibule and a shallow extracellular vestibule separated by a selectivity filter (for review see Cai et al., 2003). (B) Large anions (A^-; e.g., glibenclamide) inhibit CFTR by occluding the intracellular vestibule (Cai et al., 2002). (C) Allosteric blockers (e.g., genistein) inhibit CFTR by interfering with channel gating (Wang et al., 1998; Cai et al., 2002). Because the NBDs likely function as a head-to-tail dimer (Vergani et al., 2003; Lewis et al., 2004), allosteric blockers might inhibit channel gating by preventing dimer formation (Ai et al., 2004). (D) The glycine hydrazide GlyH-101 (A^-) inhibits CFTR by occluding the extracellular vestibule (Muanprasat et al., 2004). MSD, membrane-spanning domain; R, regulatory domain; P, phosphorylation.

blockers of CFTR invariably block other types of Cl^- channels, whereas allosteric blockers interact with other targets within cells at concentrations similar to those that inhibit CFTR. The existence of so-called pseudo-CFTR Cl^- channels of unknown molecular identity, but properties and regulation strikingly similar to those of CFTR (Marvão et al., 1998), highlights both the importance and difficulty of discovering specific blockers of the CFTR Cl^- channel.

In recent years, there has been a revolution in drug discovery. First, the determination of the atomic structures of proteins has facilitated greatly rational drug design. Second, HTS of compound libraries has accelerated dramatically the discovery of lead compounds for drug development. In the search for drugs to treat CF, several groups have employed the latter strategy to identify novel small molecules that either restore the cell surface expression of mutant Cl^- channels (CFTR correctors) or rescue their defective channel gating (CFTR potentiators). Using Fischer rat thyroid (FRT) epithelial cells coexpressing recombinant human CFTR and a green fluorescent protein with ultra-high

halide sensitivity, Alan Verkman and his colleagues have developed an automated HTS for small molecules that potentiate the activity of wild-type and mutant CFTR either by themselves or in synergy with cAMP agonists (Yang et al., 2003). In this assay, CFTR potentiators are identified as agents that strongly enhance the time course of fluorescence decrease and, hence, the rate of iodide influx into FRT cells, following chloride replacement with iodide (Yang et al., 2003). This screening strategy has proved so successful (e.g., Yang et al., 2003) that the challenge for Verkman and his colleagues now is to carefully decide which of the many CFTR potentiators that they have identified to pursue further in their quest to develop new therapies for CF.

Concurrent with their search for CFTR potentiators, Verkman and colleagues modified their HTS assay to identify agents that interact directly with CFTR to inhibit CFTR-mediated iodide influx. Their initial screen of 50,000 compounds identified the thiazolidinone $CFTR_{inh}$-172, a CFTR inhibitor with several highly desirable properties (Ma et al., 2002). First, potency: $CFTR_{inh}$-172 reversibly inhibited CFTR-mediated Cl^- currents with a half maximal inhibitory concentration (K_i) of ~300 nM, an increase in potency of almost 500-fold compared with the widely used CFTR blocker glibenclamide (Ma et al., 2002). Second, specificity: at relevant concentrations, $CFTR_{inh}$-172 was without effect on several ion channels and transporters found in epithelial tissues including Ca^{2+}-activated and volume-regulated Cl^- channels and the ATP-binding cassette transporter P-glycoprotein (Ma et al., 2002). Third, efficacy: $CFTR_{inh}$-172 inhibited cholera toxin–induced fluid secretion in the small intestine of mice, dramatically highlighting the therapeutic potential of CFTR blockers (Ma et al., 2002). $CFTR_{inh}$-172, however, suffers two drawbacks, that might constrain the drug's usefulness: first, limited water solubility (~20 μM) and, second, reduced potency in intact cells and tissues ($K_i \sim 5$ μM; Muanprasat et al., 2004).

Aiming to do better, Verkman and his colleagues screened another 100,000 small molecules selected for their chemical diversity and drug-like properties. They identified four compounds that are potent inhibitors of CFTR-mediated iodide influx ($K_i \sim 5$ μM; Muanprasat et al., 2004). For two reasons, the glycine hydrazides were particularly attractive for further study. First, many glycine hydrazides can inhibit CFTR-mediated iodide influx (Muanprasat et al., 2004). Second, *N*-(2-naphthalenyl)-([3,5-dibromo-2,4-dihydroxyphenyl]methylene)glycine hydrazide (GlyH-101) inhibition of CFTR-mediated iodide influx was rapid in onset, readily reversible, and effective after CFTR activation by agonists that act by both direct and indirect mechanisms (Muanprasat et al., 2004). Encouraged by these results, which indicate that glycine hydrazides are amenable to chemical modification and suggest that these agents might act from the extracellular side of the membrane, the authors embarked on an exhaustive characterization of glycine hydrazides and their effects on the CFTR Cl^- channel.

To investigate the relationship between the chemical structure of glycine hydrazides and CFTR inhibition, the authors modified systematically the structure of GlyH-101 and assessed CFTR function in FRT epithelia. These experiments demonstrate that *N*-(2-naphthalenyl) and 3,5-dibromo-2,4-dihydroxyphenyl substituents in glycine hydrazides are required for maximal inhibition of the CFTR Cl^- channel. The authors also examined the physical properties of glycine hydrazides. In contrast to $CFTR_{inh}$-172, GlyH-101 is highly water-soluble (~1 mM), and between pH 6.0 and 8.0 it is a monovalent anion (Muanprasat et al., 2004).

To investigate how GlyH-101 inhibits CFTR, the authors studied whole-cell and single-channel CFTR Cl^- currents in FRT cells expressing recombinant wild-type human CFTR using the patch-clamp technique. GlyH-101 caused a voltage-dependent block of whole-cell CFTR Cl^- currents with the potency of CFTR inhibition influenced by the extracellular Cl^- concentration (Muanprasat et al., 2004). Visual inspection of single-channel records indicates that CFTR inhibition by GlyH-101 was characterized by numerous drug-induced closures interrupting channel openings, with the drug dramatically decreasing mean channel open-time without altering single-channel current amplitude (Muanprasat et al., 2004). These characteristics of GlyH-101 inhibition of CFTR are unexceptional; they suggest that GlyH-101 is an open-channel blocker of CFTR that inhibits the channel with "intermediate" speed (Hille, 2001).

The characteristic of channel block that sets GlyH-101 apart from other open-channel blockers of CFTR is the shape of the current–voltage (I–V) relationship. Typically, open-channel blockers of CFTR cause outward rectification of CFTR Cl^- currents, indicating that Cl^- flow from the intra- to the extracellular side of the membrane is more strongly attenuated than that in the opposite direction. In contrast, submaximal concentrations of GlyH-101 (<30 μM) caused inward rectification of CFTR Cl^- currents, indicating that Cl^- flow from the extra- to the intracellular side of the membrane is more strongly attenuated than that in the opposite direction (Muanprasat et al., 2004). Taken together, the simplest interpretation of the data is that positive voltages drive the negatively charged GlyH-101 into the extracellular vestibule of the CFTR pore where it binds and occludes Cl^- permeation (Fig. 1 D). Channel block is relieved both by negative voltages and reducing the extracellular Cl^- concentration; these maneuvers enable Cl^- flow from the intracellular to the

extracellular side of the membrane to flush GlyH-101 from its binding site within the extracellular vestibule.

Not content to elucidate the molecular mechanism of CFTR inhibition by GlyH-101, the authors investigated the efficacy with which GlyH-101 inhibits CFTR function in respiratory and intestinal epithelia at both the intact tissue and whole animal levels. GlyH-101 (10–50 μM) completely abolished cAMP-stimulated short-circuit current, a measure of CFTR-mediated transepithelial electrolyte transport, in respiratory and intestinal epithelia from humans and mice (Muanprasat et al., 2004). Consistent with these data, topical application of GlyH-101 (10 μM) to nasal epithelia of mice rapidly attenuated CFTR function in vivo (Muanprasat et al., 2004). Finally, like $CFTR_{inh}$-172, GlyH-101 dramatically inhibited cholera toxin–induced fluid secretion in the small intestine of mice (Muanprasat et al., 2004). However, in contrast to $CFTR_{inh}$-172, which must be administered intraperitoneally, GlyH-101 was active when added directly into the lumen of the small intestine (Muanprasat et al., 2004). Taken together, the data suggest that glycine hydrazides have great promise as blockers of the CFTR Cl^- channel.

What of the future? One pressing question and several exciting possibilities are suggested by the present work. First, the pressing question: specificity. GlyH-101 was without effect on P-glycoprotein and non-CFTR–mediated Cl^- currents in murine nasal epithelia and only inhibited Ca^{2+}-activated Cl^- channels at elevated concentrations (Muanprasat et al., 2004). While these initial data are encouraging, it will be important to test the effects of glycine hydrazides on a battery of targets, including ion channels and ATP-binding cassette transporters commonly found in epithelial tissues.

Second, the architecture of the CFTR pore. To date, studies using permeant anions, especially those that bind tightly within the CFTR pore (e.g., $Au(CN)_2^-$; Gong and Linsdell, 2003), have proved most informative. Work by Smith et al. (2001) and Gong and Linsdell (2003) indicate that arginine (R) 334, a positively charged residue located toward the extracellular end of the sixth transmembrane segment, plays a crucial role in anion permeation through the CFTR pore by concentrating anions within the extracellular vestibule. However, the identity of other residues that line the external mouth of the CFTR pore is poorly understood. The suggestion that GlyH-101 binds at the external mouth of the CFTR pore to inhibit Cl^- flow (Muanprasat et al., 2004) raises the possibility that GlyH-101 might be a valuable probe of the extracellular vestibule of the CFTR pore. In addition, GlyH-101 might be used to investigate the regulation of CFTR function by external Cl^- concentration (Wright et al., 2002).

Third, large animal models of CF. The failure of CF mice to replicate the lung disease and pancreatic destruction characteristic of CF has prompted a search for alternative animal models. Anatomical, biochemical, and functional data suggest that sheep lungs might prove an excellent model for therapy evaluation (Harris, 1997). The generation of cloned sheep argues that a CF sheep might be produced using cloning technology. However, the development of specific high-affinity inhibitors of the CFTR Cl^- channel raises the possibility that a pharmacological approach might prove successful. Consistent with this idea, $CFTR_{inh}$-172 produced viscous fluid secretions from submucosal glands of normal human and porcine airways reminiscent of those of CF airways (Thiagarajah et al., 2004). Pharmacological inhibition of CFTR function in sheep lungs might provide fresh insight into the pathogenesis of CF lung disease. Moreover, agents like GlyH-101 might be employed to investigate the physiological role of CFTR. There is increasing recognition that CFTR is expressed in a diverse variety of cells and tissues outside of epithelia. Understanding the functions of CFTR in these tissues is crucial for the development of effective therapies for CF and related diseases.

Fourth, disease therapy. Secretory diarrhea is the leading cause of death in young children worldwide (Al-Awqati, 2002). The disease results from the irreversible activation of fluid and electrolyte secretion in the intestine (Al-Awqati, 2002). Ever since CFTR was identified as the apical membrane Cl^- channel responsible for cAMP-stimulated Cl^- secretion in intestinal epithelia, there has been speculation that CFTR blockers might be of value in the treatment of secretory diarrhea. However, it was not until the discovery of $CFTR_{inh}$-172 that proof of principle for this idea was obtained (Ma et al., 2002). Based on the efficacy with which intraluminal GlyH-101 inhibited intestinal fluid secretion in a mouse model of cholera, Muanprasat et al. (2004) speculate that glycine hydrazides might be developed into a nonabsorbable drug therapy for secretory diarrhea.

Inappropriate CFTR-driven fluid accumulation also contributes to the pathogenesis of autosomal dominant polycystic kidney disease (ADPKD), the most common single gene disorder to affect the kidney (Sullivan et al., 1998). Because CFTR is located on the lumen-facing membrane of epithelial cysts, there has been speculation that it is not a suitable target for ADPKD therapy. However, CFTR blockers, including $CFTR_{inh}$-172, retard cyst formation and enlargement (Hanaoka and Guggino, 2000; Li et al., 2004), raising the possibility that therapeutically active CFTR blockers might be of value in the treatment of ADPKD.

Finally, what are the prospects for the rational design of drugs based on the atomic structure of CFTR? Excitingly, Hal Lewis and his colleagues at Structural GenomiX Inc. have recently determined the crystal structure

of NBD1 from murine CFTR (Lewis et al., 2004). However, the complexities of CFTR channel regulation (e.g., Vergani et al., 2003) argue that the structure of the complete protein will be required at atomic resolution for rational drug design. The successes of researchers investigating CLC channels (e.g., Estévez et al., 2003) should galvanize CFTR investigators to try harder.

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REFERENCES

Ai, T., S.G. Bompadre, X. Wang, S. Hu, M. Li, and T.-C. Hwang. 2004. Capsaicin potentiates wild-type and mutant cystic fibrosis transmembrane conductance regulator chloride-channel currents. *Mol. Pharmacol.* 65:1415–1426.

Al-Awqati, Q. 2002. Alternative treatment for secretory diarrhea revealed in a new class of CFTR inhibitors. *J. Clin. Invest.* 110:1599–1602.

Cai, Z., T.S. Scott-Ward, H. Li, A. Schmidt, and D.N. Sheppard. 2002. Strategies to investigate the mechanism of action of CFTR modulators. Virtual Repository of Cystic Fibrosis European Network; http://central.igc.gulbenkian.pt/cftr/vr/physiology.html

Cai, Z., T.S. Scott-Ward, and D.N. Sheppard. 2003. Voltage-dependent gating of the cystic fibrosis transmembrane conductance regulator Cl^- channel. *J. Gen. Physiol.* 122:605–620.

Estévez, R., B.C. Schroeder, A. Accardi, T.J. Jentsch, and M. Pusch. 2003. Conservation of chloride channel structure revealed by an inhibitor binding site in ClC-1. *Neuron.* 38:47–59.

Gong, X., and P. Linsdell. 2003. Molecular determinants and role of an anion binding site in the external mouth of the CFTR chloride channel pore. *J. Physiol.* 549:387–397.

Hanaoka, K., and W.B. Guggino. 2000. cAMP regulates cell proliferation and cyst formation in autosomal polycystic kidney disease cells. *J. Am. Soc. Nephrol.* 11:1179–1187.

Harris, A. 1997. Towards an ovine model of cystic fibrosis. *Hum. Mol. Genet.* 6:2191–2193.

Hille, B. 2001. Ionic Channels of Excitable Membranes. 3rd ed. Sinauer Associates Inc., Sunderland, MA. 814 pp.

Lewis, H.A., S.G. Buchanan, S.K. Burley, K. Conners, M. Dickey, M. Dorwart, R. Fowler, X. Gao, W.B. Guggino, W.A. Hendrickson, et al. 2004. Structure of nucleotide-binding domain 1 of the cystic fibrosis transmembrane conductance regulator. *EMBO J.* 23: 282–293.

Li, H., I.A. Findlay, and D.N. Sheppard. 2004. The relationship between cell proliferation, Cl^- secretion, and renal cyst growth: a study using CFTR inhibitors. *Kidney Int.* In press.

Ma, T., J.R. Thiagarajah, H. Yang, N.D. Sonawane, C. Folli, L.J.V. Galietta, and A.S. Verkman. 2002. Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J. Clin. Invest.* 110:1651–1658.

Marvão, P., M.-C. De Jesus Ferreira, C. Bailly, M. Paulais, M. Bens, R. Guinamard, R. Moreau, A. Vandewalle, and J. Teulon. 1998. Cl^- absorption across the thick ascending limb is not altered in cystic fibrosis mice: a role for a pseudo-CFTR Cl^- channel. *J. Clin. Invest.* 102:1986–1993.

Muanprasat, C., N.D. Sonawane, D. Salinas, A. Taddei, L.J.V. Galietta, and A.S. Verkman. 2004. Discovery of glycine hydrazide pore-occluding CFTR inhibitors: mechanism, structure-activity analysis, and in vivo efficacy. *J. Gen. Physiol.* 124:125–137.

Smith, S.S., X. Liu, Z.-R. Zhang, F. Sun, T.E. Kriewall, N.A. McCarty, and D.C. Dawson. 2001. CFTR: covalent and noncovalent modification suggests a role for fixed charges in anion conduction. *J. Gen. Physiol.* 118:407–431.

Sullivan, L.P., D.P. Wallace, and J.J. Grantham. 1998. Epithelial transport in polycystic kidney disease. *Physiol. Rev.* 78:1165–1191.

Thiagarajah, J.R., Y. Song, P.M. Haggie, and A.S. Verkman. 2004. A small molecule CFTR inhibitor produces cystic fibrosis-like submucosal gland fluid secretions in normal airways. *FASEB J.* 18: 875–877.

Vergani, P., A.C. Nairn, and D.C. Gadsby. 2003. On the mechanism of MgATP-dependent gating of CFTR Cl^- channels. *J. Gen. Physiol.* 121:17–36.

Wang, F., S. Zeltwanger, I.C.-H. Yang, A.C. Nairn, and T.-C. Hwang. 1998. Actions of genistein on cystic fibrosis transmembrane conductance regulator channel gating: evidence for two binding sites with opposite effects. *J. Gen. Physiol.* 111:477–490.

Welsh, M.J., B.W. Ramsey, F. Accurso, and G.R. Cutting. 2001. Cystic fibrosis. *In* The Metabolic and Molecular Basis of Inherited Disease. C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, editors. McGraw-Hill Inc., New York. 5121–5188.

Wright, A., B. Argent, and M. Gray. 2002. Regulation of Cl^- efflux through CFTR by external Cl^- concentration. *Pediatr. Pulmonol. Suppl.* 24:187.

Yang, H., A.A. Shelat, R.K. Guy, V.S. Gopinath, T. Ma, K. Du, G.L. Lukacs, A. Taddei, C. Folli, N. Pedemonte, et al. 2003. Nanomolar affinity small molecule correctors of defective ΔF508-CFTR chloride channel gating. *J. Biol. Chem.* 278:35079–35085.