CFTR Is a Conductance Regulator as well as a Chloride Channel

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IX. Update: Recent Developments
I. INTRODUCTION: CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR AS A MULTIFUNCTIONAL MOLECULE

This review discusses published data, preliminary data, and hypotheses that support the view of the cystic fibrosis transmembrane conductance regulator (CFTR) as a bona fide “conductance regulator” in addition to itself playing a role as a “conductor” of Cl⁻.

Before the identification and cloning of the cystic fibrosis (CF) gene and characterization of its protein product, CFTR, as a cAMP-regulated Cl⁻ channel (12, 39, 74, 103, 104, 125), a series of defects in cellular functions were described in CF epithelia, some of which could not be easily reconciled with “primary” mutations or abnormalities within a Cl⁻ channel protein (9, 10, 23, 25, 33, 40, 44, 61, 114, 120). These “secondary” defects included misregulation of a separate class of Cl⁻ channels, the outwardly rectifying Cl⁻ channels (ORCC), by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) in airway epithelial cells. Moreover, the ORCC Cl⁻ channel or a regulator of this Cl⁻ channel subtype was hypothesized originally to be the putative CF gene product before CFTR was identified (59, 81, 112). Another abnormality, apart from the defects in Cl⁻ channel cell biology proposed originally by Quinton (99) and identified by Boucher et al. (23), was hyperabsorption of Na⁺ across CF airway epithelia (23, 76) and hyperactivity of Na⁺ channels in isolated CF airway epithelial cells (33). This defect cannot be explained directly by mutations or abnormalities in a channel that conducts Cl⁻. Moreover, misregulation of exocytosis and endocytosis by cAMP in CF cells (25) as well as lack of sufficient acidification of CF intracellular organelles has been documented (9, 10, 61). As a consequence, defective posttranslational modification and/or trafficking of any and all membrane glycoproteins may be abnormal in CF epithelial cells, leading possibly to “tertiary” complications within the CF cell.

These studies illustrate that CF disease may be caused by a complex combination of primary defects in CFTR Cl⁻ channel function (131); secondary defects in other CFTR-regulated ionic conductances such as ORCC (40, 44, 114), epithelial Na⁺ channels (ENaC) (23, 76), and renal outer medullary K⁺ channel (ROMK) or other inwardly rectifying K⁺ channels (89); or possible tertiary perturbations of protein modification or trafficking and defective protein processing. Finally, the concept of “modifier” CF genes has emerged to complicate interpretations of CF lung disease further and may also contribute to some secondary or tertiary defects in CF cells (106). Figure 1 illustrates the concept of a multifunctional CFTR, and Figure 2 introduces the concept of primary, secondary, and tertiary defects in a CF epithelium.

Taken together, the multitude of functional defects in CF epithelial cells, illustrated in Figure 2, together with the characterization of CFTR as a Cl⁻ channel led to a key question in CF research: How does CFTR expression modify the regulation of separate populations of ion channels such as ORCC, ENaC, and ROMK and alter other cellular processes such as vesicle trafficking and Golgi acidification? Answers to this question have emerged from published and preliminary work summarized below. In actuality, the naming of CFTR as the cystic fibrosis transmembrane conductance regulator was done with much foresight (103, 104).

II. CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR-OUTWARDLY RECTIFYING CHLORIDE CHANNEL REGULATORY INTERACTION

Early studies of epithelial Cl⁻ channels and CF showed that ORCC were present in CF epithelial cells but that these channels failed to respond to PKA and/or PKC (59, 81, 112). As such, an early hypothesis was that the CF gene product would be a regulator of ORCC (59, 81, 112). After identification of CFTR as a cAMP-regulated Cl⁻ channel distinct from ORCC, the validity of research concerning the ORCC and its defective regulation by protein kinases was questioned. Complementation studies, however, showed that reintroduction of wild-type CFTR into a CF cell corrected defective protein kinase regulation of ORCC (40). Moreover, patch-clamp recordings of airway epithelia derived from CF(−/−) mice demon-
A. Importance of Cytoplasmic ATP

To determine why CFTR expression was essential for PKA stimulation of ORCC, a convenient assay was needed to record simultaneously the activity of both CFTR and ORCC Cl\(^{-}\) channel populations. Previous whole cell recordings of normal airway epithelial cells showed that only CFTR Cl\(^{-}\) channel currents were stimulated in symmetrical Cl\(^{-}\)-containing solutions (bath and pipette solutions) with 1 mM Mg\(^{2+}\)-ATP in the pipette (intracellular) solution (115). However, complete reconstitution of the physiological intracellular concentration of ATP with 5 mM Mg\(^{2+}\)-ATP yielded cAMP-stimulated whole cell recordings in which both CFTR and ORCC Cl\(^{-}\) channel currents could be measured (115). The Cl\(^{-}\) channel blocking drug DIDS could be added subsequently to inhibit ORCC while not affecting CFTR channels (115). In excised or inside-out patch-clamp recordings, 1 mM Mg\(^{2+}\)-ATP was added to the cytoplasmic face of excised membrane patches as a cofactor for PKA or PKC phosphorylation; at that time, however, it was not appreciated that intracellular ATP was important for CFTR modulation of ORCC activation by cAMP and PKA (40, 44, 59, 81, 112). Thus it was apparent that physiological concentrations of intracellular ATP were required for CFTR modulation of ORCC. The next challenge was to understand the role of intracellular Mg\(^{2+}\)-ATP in CFTR-ORCC interactions.

B. Importance of ATP Release Mechanisms and Purinergic Receptors

A combination of single-channel and whole cell recordings and Ussing chamber short-circuit current recordings revealed that ATP had to cross the membrane from the cytoplasm to the extracellular space to exert its effects on ORCC and other Ca\(^{2+}\)-dependent Cl\(^{-}\) conductances (60, 114). Addition of hexokinase or apyrase into the extracellular solution, agents which “scavenge” ATP or degrade ATP into its metabolites, prevented cAMP from stimulating ORCC without affecting cAMP stimulation of CFTR (114). In effect, ATP scavengers uncoupled CFTR from ORCC. This was a key observation, because it showed that cAMP-stimulated and CFTR-dependent release of ATP out of the cell was essential to CFTR regulatory interaction with ORCC. This finding has been confirmed by other laboratories using different experimental cell model systems (72, 114); moreover, these findings demonstrated that overexpression of CFTR not only correlated with a cAMP-regulated 9-pS Cl\(^{-}\) conductance but also a smaller 4- to 5-pS ATP\(^{-}\) conductance across the plasma membrane of a mammalian cell (114). This result served to integrate further this autocrine/paracrine ATP signaling between CFTR and ORCC. The simplest interpretation was that CFTR conducted the ATP itself; however, other possible mechanisms exist for how CFTR facilitated ATP conduction and/or release from cells (see sect. iv). Additional experiments independent of cAMP stimulation or CFTR expression revealed that extracellular UTP and ATP at nanomolar doses stimulated ORCC in both normal and CF airway epithelial cells (114). This result was consistent with the emerging model that the released
FIG. 2. Primary, secondary, and tertiary effects of CFTR dysfunction. Model of a segment of cystic fibrosis (CF) airway illustrating host of defects associated with CF. Impact of mutations with CFTR causing primary (1°) defects are subject of other reviews within this volume. Secondary (2°) and tertiary (3°) defects are also listed in this model figure and may result in a lack of CFTR function or lack of function of protein product of a modifier gene or genes that has yet to be found. Secondary and/or tertiary defect designations have been given to those defects where their relationship to CFTR function is less clear. CaCC, Ca\(^{2+}\)-dependent Cl\(^{-}\) channels; ClC, voltage-dependent Cl\(^{-}\) channels; CNG, cyclic nucleotide-gated cation channels; AQP, aquaporins; PMN, polymorphonuclear cell or neutrophil; P.a., Pseudomonas aeruginosa; asialo-GM1, carbohydrate moieties with a lack of added sialic acid residues; APM, apical membrane domain; BLM, basolateral membrane domain.

ATP was regulating ORCC through a purinergic receptor (114). Also, the demonstrations that nanomolar and low micromolar doses of ATP were sufficient to stimulate ORCC was novel (60, 114). Moreover, this study corroborated previous evidence showing that a P\(_{2x}\) or P\(_{2y}\) G protein-coupled purinergic receptor stimulates Cl\(^{-}\) channels and Cl\(^{-}\) secretion across normal and CF airway epithelia (92, 93, 121, 122). Taken together, these results helped formulate a working model for a CFTR-ORCC regulatory interaction that required physiological concentrations of 5 mM Mg\(^{2+}\)-ATP, functional CFTR, release of ATP as an agonist into the extracellular milieu, purinergic receptors that bind the ATP, and an ORCC that is regulated by ATP agonists (114). The different components of this model will be revisited below, and possible new players or stimuli that may also contribute to this model will be introduced. It is also very important to emphasize that there may be multiple mechanisms by which epithelial cells release their ATP (48), that there are multiple purinergic receptors expressed by a given epithelial cell (receptor expression may also differ between apical and basolateral domains) (1, 11, 42, 60), and that there may be multiple epithelial anion channels and, possibly, epithelial cation channels that are regulated by ATP via ATP receptors (45, 46, 60, 82, 93, 121, 122). There may also be multiple physiological roles for this CFTR-ATP autocrine signaling cascade. As we learn more information about the complexity of these and other systems, it is anticipated that novel targets for CF pharmacological therapy will emerge.

C. What Domains of CFTR Are Important for Regulation of ORCC?

To establish working models of CFTR-ORCC interaction, it is necessary to determine the molecular domains of CFTR important for its Cl\(^{-}\) channel function and its regulatory interaction with the ORCC. To identify these CFTR domains, two approaches are being employed: 1) expression of truncated forms of CFTR that contain or lack key domains of CFTR thought important for the above functions, and 2) expression of “mild,” “severe,” or “atypical” disease-causing mutations found in CFTR since the cloning of the CF gene. Mild mutations are those found in CF patients with milder forms of lung disease and pancreatic sufficiency. Of course, this is a subjective categorization. Severe mutations, such as G551D or ΔF508, are present in CF patients that are pancreatic insufficient and have
more severe lung disease. For example, atypical mutations are those that cause congenital bilateral absence of the vas deferens (CBAVD) in males without causing significant CF phenotypes or mutations that cause high sweat Cl⁻ concentrations indicative of a CF diagnosis without any other apparent disease phenotype in the lung or gastrointestinal tract. Two expression systems are being used: 1) injection of in vitro transcribed cRNA into Xenopus oocytes to study CFTR Cl⁻ channel function in the absence of endogenous CFTR or ORCC and 2) transfection of cesium chloride-tetrafluoroacetate (CsCl-TFA) purified cDNA into IB3–1 CF airway epithelial cells to study Cl⁻ channel function and regulatory interaction with the ORCC in the absence of any functional CFTR. Under the appropriate conditions, these two expression systems fail to respond to cAMP agonists with an increase in Cl⁻ current or in the activity of single Cl⁻ channels unless functional CFTR is introduced. Below is a summary of integrative results using these two approaches, both published.

Severe truncation of the NH₂ terminus of CFTR by removal of amino acids 1 to 259 but with the Kozak consensus sequence surrounding methionine-265 intact resulted in the recording of a cAMP-activated 6-pS Cl⁻ channel (compared with the 9-pS wild-type Cl⁻ channel) and a cAMP-stimulated macroscopic Cl⁻ current that was 67% that of wild-type CFTR when expressed in Xenopus oocytes (96). When expressed in IB3–1 CF airway cells, this M265 construct functioned as a cAMP-stimulated Cl⁻ channel and conferred cAMP regulation on the population of endogenously expressed ORCC. In sharp contrast, a similar truncation to amino acid 259 but with methionine-265 altered to a valine (M265V) failed to function as a cAMP-stimulated Cl⁻ channel in both expression systems. However, despite this lack of Cl⁻ channel activity, M265V conferred cAMP regulation of ORCC. The results from these initial constructs suggested that the region of CFTR critical for Cl⁻ channel function lay in the amino acids immediately distal to methionine-265. This is consistent with the mutagenesis studies showing that alterations of residues in α-helices 5 and 6 compromised single Cl⁻ channel conductance when compared with wild-type CFTR (32, 85, 117, 124). Similar to the results with M265V, insertion of “dual” arginine mutations (R334W with R347P) into CFTR eliminated Cl⁻ channel activity but conferred cAMP stimulation upon ORCC. These results also suggested that methionines alternative to methionine-1 could serve as “translation initiation” start sites for the CFTR protein (96).

Severe truncations of the COOH terminus also yielded fruitful and interesting results. Expression of a “half molecule” of CFTR (TNR-CFTR) in which amino acids 836 through 1480 were eliminated (TMD-1, NBD1, and R domains of CFTR intact) functioned in oocytes as a 9-pS single Cl⁻ channel similar to wild-type CFTR. At the macroscopic level, however, the Cl⁻ current from TNR-CFTR was only 10–20% that of wild-type current, suggesting that plasma membrane expression of TNR-CFTR was significantly less than wild-type CFTR. This result was confirmed in IB3–1 CF cells as well. Surprisingly, however, despite this low plasma membrane expression, TNR-CFTR was able to confer cAMP regulation upon ORCC in IB3–1 CF cells. Similar results were observed for a novel and naturally occurring splice variant in kidney medulla that creates a similar half molecule of CFTR (90). Thus further truncation was necessary to eliminate the portions of CFTR that were required for the regulatory interaction with ORCC. This goal was accomplished with a construct of CFTR in which only the TMD-1 domain of CFTR was left intact in the cDNA (amino acids 371–1480 removed). This construct functioned in part in oocytes and IB3–1 cells as a constitutively active Cl⁻ channel that did not require cAMP stimulation. Its activity, however, was potentiated by cAMP. This result was intriguing because this domain of CFTR lacks consensus PKA phosphorylation sites (31). This result may be explained by the fact that cAMP could cause insertion of more TMD1-CFTR from subplasma membrane vesicles (25). Because net membrane conductance can be enhanced not only by activation of channels resident in the membrane but also by insertion of new channels into the membrane, this mutant may be useful in testing this aspect of CFTR cell biology. Most important for this work, however, TMD1-CFTR failed to confer cAMP stimulation of ORCC despite its profound intrinsic Cl⁻ channel activity.

Taken together, these preliminary results utilizing truncations of the CFTR cDNA from the NH₂ and COOH termini suggest that the domains critical for Cl⁻ channel function and ORCC regulatory coupling are distinct. One function could be eliminated with the other intact, and vice versa. The part of CFTR critical for Cl⁻ channel function lies at least in part in α-helices 5 and 6 of TMD-1. In contrast, the domains of CFTR essential for interaction with ORCC are NBD1 and the R domain of CFTR. It is still not clear whether these domains are capable of transporting ATP themselves. NBD1 of CFTR does bind ATP, and there is evidence for ion channel activity intrinsic to NBD1 in bilayers (7) and mammalian cells (34) (see also below). Nevertheless, cAMP-stimulated ATP release and ORCC regulatory interaction are interconnected in CF airway epithelial cells. An alternative explanation is that NBD1 and the R domain interact with a separate ATP-specific channel or transporter or a population of ATP-filled vesicles that promotes efflux of the ATP.

With the use of insertion of mild versus severe lung disease-associated mutations, the NBD1 domain was implicated as a site important for ORCC regulation (43). A mild mutation within NBD1, A455E, caused a small reduction in Cl⁻ channel function but did not affect cAMP stimulation of ORCC. In contrast, G551D, a common and se-
vere mutation in NBD1, caused a severe reduction in Cl⁻ channel activity and eliminated cAMP stimulation of ORCC. This result agrees with the results from truncation mutants described above and implicates NBD1 as an essential domain for regulatory interaction with ORCC. Again, the important question that is addressed below is whether NBD1 with the R domain is capable of transporting ATP itself or whether NBD1 together with the R domain interact with a separate molecule that conducts or transports ATP.

### D. Integration of the CFTR-ORCC Regulatory Interaction Model: Identifying Targets for CF Pharmacotherapy

Figure 3 illustrates current hypotheses concerning the autocrine/paracrine model for CFTR-ORCC regulatory interaction. This model is an extension of the “working model” published in 1996 (114). The involvement of other regulatory molecules and, possibly, ATP-specific channels or transporters and/or ATP-filled vesicles is also shown.

First, PKA plus ATP stimulation of CFTR is required for its Cl⁻ channel activity. Second, the mechanisms of ATP release and the role of CFTR and multidrug resistance protein (mdr) in facilitating ATP release are less clear. This issue is discussed in greater detail in section IV. Third, once released under basal or stimulated conditions, ATP is free as an agonist to regulate the ORCC either directly or via purinergic receptors. Initially, the P₃y receptor was implicated. A recent explosion in cloning of purinergic receptors reveals that at least three P₃y receptors, including the P₂₆ or P₂₆y receptor, bind UTP as well or better than ATP and could underlie the responses (1, 11, 42). Future studies are necessary to document the relative expression of different receptor isoforms. Perhaps ATP receptors in addition to P₂y(2y2) could also become targets for nucleotide-based therapies. Ideally, an ATP receptor that is shared by all exocrine epithelia affected by CF could be exploited as a therapeutic target for all CF. Finally, the signaling pathways whereby the ATP receptor regulates the ORCC are unknown. They may be membrane delimited and/or involve membrane-bound G proteins, phospholipases (phospholipases C, D, and A₂), phospholipids, and protein kinases, or they may be cytoplasmic and require cytosolic second messenger pathways like cyclic nucleotide pathways or PKC pathways (1, 11, 42).

The ORCC may not be the only epithelial Cl⁻ channel regulated by external nucleotides. The emerging family of CIC channels may be regulated in this manner; emerging preliminary work suggests that newer isoforms of this family (CIC-3, CIC-5) display outwardly rectifying current-voltage relationships (73, 109). Other ubiquitously expressed CIC isoforms, such as CIC-2, CIC-6, and CIC-7, may also be candidates. External nucleotides at micromolar concentrations trigger increases in intracellular Ca²⁺ (Caᵢ²⁺) (46, 60, 108) and may, therefore, activate the recently identified Ca²⁺ family of Cl⁻ channels (37). “Maxi” Cl⁻ channels of high single-channel conductance (200–400 pS) are also expressed by a variety of exocrine epithelia and may be regulated by external nucleotides (116).

One possibility is that different purinergic receptors may be coupled to different Cl⁻ or Na⁺ channels by utilizing different second messenger pathways in different epithelia to stimulate and/or inhibit different channel subtypes. This concept has precedence in the cardiac, neuronal, and brain ion channel literature (16, 17, 26, 35, 54, 79, 105).
III. CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR REGULATORY INTERACTIONS WITH OUTWARDLY RECTIFYING CHLORIDE CHANNELS IN PLANAR LIPID BILAYERS

A. Experimental System

Planar lipid bilayers are a useful experimental technique to study regulatory interactions between membrane transport proteins. This system has been exploited to reconstitute and examine the interactions between CFTR and outwardly rectified anion channels (72) as well as the recently cloned ENaC (8, 15, 64, 65) (see sect. vi). The advantage of this system is that it provides a well-controlled model environment to study in a direct and physiologically meaningful way the functional properties of ion channels. With proper care, one can incorporate channels preferentially into the membrane with a specific and defined orientation, as well as control the number of channels present. Upon definition of the appropriate experimental conditions, the ease with which regulatory components can be added to the presumptive extracellular or intracellular side makes interpretation of these sorts of experiments unequivocal. This section describes recent studies utilizing this system to examine the regulatory relationships between CFTR and the ORCC.

B. CFTR and ORCC in Bilayers

Both CFTR and the ORCC were isolated and reconstituted simultaneously and functionally from vesicles derived from bovine tracheal epithelia (72). Remarkably, the intimate regulatory relationship between these two proteins is preserved throughout the purification procedure. Initial studies focused on the requirement of CFTR for PKA (with ATP as a cofactor) activation of the ORCC. Immunoprecipitation of CFTR from this preparation prevented PKA-induced activation of the ORCC, suggesting that the presence of CFTR is required for PKA plus ATP stimulation of the ORCC. Moreover, the hypothesis that CFTR was required to be in a “transport-capable” mode for PKA activation of the ORCC was tested. To this end, inhibitory anti-CFTR$_{95-511}$ antibodies were utilized to block CFTR transport function, or functional CFTR was replaced with a nonfunctional, mutated form, namely, G551D-CFTR (72). Only when CFTR was functional as a Cl$^-$ channel could the ORCC be activated by “cytoplasmically” added PKA plus ATP.

C. Importance of ATP and Direct Protein-Protein Coupling in CFTR-ORCC Interaction

As described above, there is also considerable debate about whether CFTR can function both as a halide and an ATP channel (2, 3, 51, 52, 60, 94, 97, 100, 102, 114). Guggino and co-workers (60, 114) proposed that the facilitation of ATP release or efflux by CFTR to the extracellular side of cells was a necessary prerequisite for PKA activation of the ORCC. The bilayer system was used subsequently to test if this same phenomenon could be observed. The addition of an ATP scavenging system [namely, hexokinase (0.5 units/ml) plus glucose (5 mM)] to the presumptive extracellular side of a bilayer containing both the ORCC channel and CFTR prevented PKA-mediated activation of the ORCC. In this experiment, no exogenous ATP was present in the “external” bathing compartment. Alternatively, in the absence of hexokinase, ATP could arrive at the extracellular surface only by passage through the bilayer membrane. Addition of ATP by itself to the extracellular side did not have any effect on the ORCC channel in the bilayer. If CFTR-facilitated ATP release was the only requirement for PKA-mediated activation of the ORCC, it should be possible to activate the ORCC by the addition of ATP to the extracellular solution and by application of PKA plus ATP to the cytoplasmic side of the system in the absence of CFTR. In these experiments, however, when ORCC was the only channel present in the bilayer membrane, the ORCC could not be activated by the addition of 100 $\mu$M ATP to the outside and PKA plus ATP to the inside or by the addition of PKA plus ATP to both sides of the membrane (72). Taken together, these results suggest that CFTR plays an additional required role in the regulation of the ORCC that may involve intramembrane or cytoplasmic interactions. This idea is supported further by the results of experiments in which the ORCC and the nonconducting G551D-CFTR were incorporated simultaneously into the planar lipid bilayer. Under these conditions, the ORCC regains its PKA sensitivity but only in the presence of extracellular ATP. The next feature examined whether the NBD1 domain of CFTR could interact with the ORCC in the presence of extracellular ATP and be sufficient as a single domain of CFTR to mediate PKA activation of the ORCC. NBD1 alone was not able to replace full-length CFTR in this function (72). These results must be viewed with some caution because NBD1 cannot conduct ions (at least in our hands) and, as such, its presence in the bilayer membrane cannot be verified. Thus, on the basis of the findings with dysfunctional G551D-CFTR in the planar lipid bilayer, it was clear that the physical presence of CFTR was essential for PKA plus ATP activation of the ORCC. This result suggests that direct intramembrane or cytoplasmic protein-protein interaction may be involved in CFTR-ORCC “cross talk.” Figure 4 is included to illustrate a working model of CFTR-ORCC regulatory interactions as deciphered by lipid bilayer studies.
IV. CAN CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR CONDUCT ADENOSINE 5'-TRIPHOSPHATE?

This question has stimulated vigorous debate in the CF community concerning the precise mechanism whereby CFTR facilitates the release of ATP out of cells. Several views exist concerning the role of CFTR and other ATP-binding cassette (ABC) transporters such as mdr in ATP release in particular and the physiological validity of ATP release from cells in general. Investigators in the laboratories of Cantiello and Ausiello (3, 97, 102) as well as those of Guidotti and Abraham (2, 3, 102) have published much data originally that support the simplest conclusion that CFTR and mdr conduct ATP. Investigators from other laboratories have also published reports (2, 3, 94, 97, 102, 114) or have generated preliminary data (see below) that show that cAMP-stimulated ATP release is dependent on CFTR, that expression of CFTR is associated with an ATP conductance, and that there are multiple interpretations of this ATP transport phenomenon. In these studies, the precise route of ATP through the membrane was not defined; however, ATP transport across the membrane occurred only in the presence of CFTR and mdr. There is an equally large number of laboratories that have published results showing that they could not demonstrate CFTR-dependent ATP conductance or release (51, 52, 80, 100, 101). Many laboratories and investigators have tackled this question and have debated over the various findings (2, 52, 101). One more recent study suggested that it was likely that these ATP transport phenomenon were observed because of mechanical artifacts during patch-clamp recordings or because of cell damage or lysis during ATP bioluminescence release assays (51).

This section of the review summarizes these findings, outlines the current views surrounding this question, and attempts to integrate these findings and propose hypotheses concerning this issue to move this research forward.

A. Lessons From Airway Epithelial Cells

Original studies by Guggino and co-workers (114) focused on normal, CF, and wild-type CFTR-complemented CF airway epithelial cells and established the CFTR-ORCC regulatory interaction model in which ATP linked CFTR to ORCC via an autocrine/paracrine mechanism involving ATP. From this work, a 4- to 5-pS ATP conductance was recorded in parallel with a 9-pS Cl⁻ conductance only from excised membrane patches of wild-type CFTR-complemented IB3–1 cells (specifically, clone S9) and stimulated with the catalytic subunit of PKA not from parental IB3–1 cells (114). This ATP conductance was discernible only in nominally Cl⁻-free solutions in which Tris-Cl or NaCl was replaced completely with Tris-ATP or Na₂ATP (114). These conditions may be critical and may explain other results below.

Because of this work and because of the variability in findings between many laboratories, assays are being developed to study ATP release into the apical and basolateral medium from normal, CF, and wild-type CFTR-complemented epithelial cells grown as monolayers in air-fluid interface culture on collagen-coated permeable supports. Alternatively, heterologous cell cultures overexpressing CFTR or mdr either stably or transiently grown on collagen-coated 35-mm culture dishes can also be studied. An important aspect of these studies is that the cells are studied in real time inside the sealed chamber of a luminometer while in a serum-free medium designed to maintain cell viability during transfections for several hours. These studies are in progress, the assay is being perfected, and the data are preliminary. Cells are grown.
to confluence or as a monolayer tight to fluid, washed twice in phosphate-buffered saline, and placed in a sealed chamber of a luminometer in an OptiMEM serum-free medium containing luciferase-luciferin reagent (113). Any ATP molecule released from the cells will catalyze the luciferase-luciferin reaction and yield a photon of light collected by the luminometer in arbitrary light units (113). Non-CF epithelia and heterologous cells expressing or overexpressing CFTR and mdr have a significantly higher basal ATP release magnitude than their CF or null-expressing counterparts, observed without any stimulation by cAMP agonists, Ca\(^{2+}\) agonists, or other stimuli (Schwiebert, unpublished data). This difference in basal ATP release is a reproducible finding and has been observed in at least six pairs or panels of CFTR- or mdr-expressing cells versus their counterpart controls. Moreover, a hypothesis concerning the role of CFTR- and mdr-facilitated ATP release in cell volume regulation is being explored. Hypotonicity-induced ATP release is potentiated markedly by the expression of the ABC transporters, CFTR and mdr (Schwiebert, unpublished data). An involvement of CFTR and mdr in regulatory volume decrease during cell volume regulation has been proposed (47, 127, 128).

Grygorczyk and Hanrahan (51, 52) have used bioluminescence to address this issue as well. No differences were observed between CFTR-expressing cells and their counterparts in terms of basal release or cAMP-stimulated release. It was demonstrated, however, that mechanical perturbation (bending of coverslips held freely in a luminometer cuvette with forceps or injection of cell samples into the injection port of a luminometer) caused ATP release from cells. This was not surprising, since cell damage or cell lysis could occur; however, it was suggested that mechanical stimulation of cells may cause ATP release and may be important for epithelial cell biology (51). These methods are similar to those used by Cantiello and co-workers (97) and Abraham and co-workers (2, 3), but they are very different from others being employed as described above by the Schwiebert and Guggino laboratories and below with respect to ATP release from single *Xenopus* oocytes by the Engelhardt laboratory in which the integrity and viability of the cells is maintained by studying the cells in a serum-free medium or in their standard Ringer solution and in which the cells are placed inside the luminometer on the support on which they are grown normally and studied for a luminescence signal without perturbation.

### B. Lessons From Other Epithelial Cell Preparations

Preparations of nonairway tissues such as sweat duct epithelia or Calu-3 submucosal gland serous cells failed to show CFTR-associated ATP single-channel or macroscopic conductance (100). No macroscopic ATP conductance was observed in isolated sweat duct preparations, whereas a robust Cl\(^{-}\) conductance was observed routinely. In single-channel or whole cell patch-clamp recordings of Calu-3 cells, no ATP single-channel or macroscopic conductance was observed, although Cl\(^{-}\) channels were observed routinely (100). One possible explanation for the differences between these studies and others may be the difference in the origin of the cells used. Airway surface epithelial cells were used exclusively for the studies by Guggino and co-workers, whereas cells derived from sweat gland/duct and airway submucosal gland were used by these investigators. These ATP release/transport mechanisms and their regulation may be very different depending on what epithelium is being studied.

### C. Lessons From Heterologous Mammalian Cells

Contradictory data concerning ATP conductance in heterologous mammalian cell systems including wild-type CFTR stably transfected Chinese hamster ovary (CHO) cells have been reported (53, 94). In a series of single-channel and whole cell experiments in which chemical gradients of Cl\(^{-}\) and ATP were manipulated on each side of the membrane, Cl\(^{-}\) conductance referable to CFTR was observed with a consistent lack of any measurable ATP conductance (53). In contrast, Pasyk and Foskett (94) were able to show that ATP and another adenine nucleotide, adenosine 3’-phosphate 5’-phosphosulfate, were conducted across the plasma membrane and the endoplasmic reticulum membrane only in CFTR-complemented CHO cells and not in parental CHO cells. As with the study of Guggino and co-workers (114), Pasyk and Foskett (94) were able to show ATP conductance at negative voltages and Cl\(^{-}\) conductance at positive voltages under bi-ionic conditions. However, these observations could not be confirmed by Wine and co-workers in Calu-3 cells (100) or Hanrahan and co-workers in these CHO cells (53).

This variability may be explained by the fact that additional regulators may need to be expressed in concert with CFTR and/or that additional cofactors may need to be present to transport the ATP and that these cofactors are regulated favorably by CFTR. These regulatory subunits or cofactors may be sensitive to factors within the serum added to the cell culture medium. Different order of solution changes, the concentration or ionic strength of anions in the solutions, the nature of the solution changes, or the osmotic strength of the solutions may also explain this variability.

### D. Lessons From the Oocyte Expression System

Engelhardt and co-workers have developed a single *Xenopus* oocyte ATP bioluminescence release assay that
studies the ability of CFTR to promote ATP release. Their preliminary results may shed light on many of the results discussed above. This cAMP-stimulated ATP release response in oocytes is variable and has many requirements (J. Engelhardt and Q. Jiang, unpublished data). First, only certain frogs yield oocytes that have cAMP-stimulated and CFTR-facilitated ATP release capability; however, if the frog yields oocytes in which the response is observed, it is observed consistently. Second, when the response is observed, it requires CFTR; uninjected or water-injected oocytes do not display the response. Wild-type and Δ259-M265V-CFTR promote cAMP-stimulated ATP release from oocytes, whereas TMD-1 CFTR fails to promote cAMP-stimulated ATP release. These truncated constructs of CFTR are described above (see sect. II). Finally, a strict protocol of solution changes must be followed to trigger this response in wild-type CFTR cRNA-injected oocytes. Oocytes must first be incubated in a 0 Cl⁻ solution for 5 min, followed by cAMP agonist stimulation in 0 Cl⁻ solution, with a subsequent change to a solution that contains >90 mM Cl⁻ with the continued presence of cAMP agonists. Upon the change to a Cl⁻-containing solution under cAMP-stimulating conditions, a robust ATP release response is observed that is immediate and sustained. Taken together with results above, specifically with regard to the variability from frog to frog and from oocyte batch to oocyte batch, these results underscore the idea that additional regulators or cofactors must be expressed simultaneously with CFTR in the oocyte to observe ATP release (see Figs. 3 and 5).

E. Lessons From the Bilayer System

With the use of immunopurified and functionally reconstituted CFTR from bovine tracheal epithelia and the nonconducting G551D-CFTR from transformed L cells, it has been shown that functional CFTR is required for the activation of the ORCC by PKA plus ATP unless ATP is supplied exogenously to the extracellular bathing solution (72). Cystic fibrosis transmembrane conductance regulator is, therefore, essential for the transport of ATP from the cytoplasm to the extracellular bathing solution where it can then interact with some external domain of the ORCC. It is unlikely, however, that CFTR itself expressed in isolation can function as an ATP channel. Results from other laboratories (K. Gunderson and R. R. Kopito; C. E. Bear) using different CFTR protein and bilayer preparations (80, 100) and preliminary data generated by Ismailov and Benos indicate that puriﬁed CFTR by itself cannot transport ATP in the planar lipid bilayer (I. I. Ismailov and D. J. Benos, unpublished data). Another possibility is that an ATP channel is present in some puriﬁed protein preparations and that CFTR facilitates the function of this ATP-conducting molecule. Indeed, preliminary work from Ismailov and Benos shows that CFTR reconstituted functionally within vesicles isolated from bovine tracheal epithelia, however, does result in appearance of an ATP-specific conductance (Ismailov and Benos, unpublished data).

The fact that immunopurified CFTR cannot conduct ATP (80, 100) but that CFTR within a vesicle that contains many other proteins including ORCC can conduct ATP suggests two possible explanations for this CFTR-dependent ATP conductance: 1) a separate channel protein conducts this ATP specifically or 2) CFTR requires additional “signals” to occur or “regulators” to be present to be
“coaxed” into conducting this ATP itself. Some of these possible signals or regulators have been proposed in Figures 3 and 5.

In short, the precise molecular mechanisms that underlie CFTR-associated ATP release are becoming clearer but have not been defined fully. However, all of this published and preliminary work discussed above is of considerable value and has provided much information upon which to attempt to explain how CFTR and other ABC transporters facilitate ATP release. It is clear, however, that if CFTR can facilitate ATP release, CFTR must itself be modulated by regulators or stimuli in a concerted manner or CFTR itself must modulate an ATP release pathway or pathways that is expressed universally in epithelial cells, heterologous cells, and Xenopus oocytes.

V. CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR-EPITHELIAL SODIUM CHANNEL REGULATORY INTERACTION IN AIRWAY EPITHELIA AND HETEROLOGOUS CELLS

Of equal or perhaps greater importance for CF airway pathophysiology, Na\(^+\) has been shown to be hyperabsorbed across CF airway epithelium when compared with its non-CF counterpart. It was postulated and subsequently proven by Stutts et al. (120) that CFTR dysfunction was responsible for this basal amiloride-sensitive hyperabsorption and for defects in cAMP regulation of amiloride-sensitive ENaC currents across CF airway epithelium. This section summarizes these results, provides new preliminary results, and hypothesizes about the possible mechanisms of CFTR-ENaC interactions. In contrast to the positive regulation of ORCC described in sections II and III, CFTR regulation of ENaC is negative or inhibitory in nature.

A. Function of CFTR and ENaC in Airway Epithelial Ion Transport

1. Cystic fibrosis transmembrane conductance regulator as a cAMP-dependent cellular Cl\(^-\) conductance available for Cl\(^-\) secretion or absorption

   Early studies to characterize the pattern of ion transport in normal airway epithelia detected consistently Na\(^+\) absorption as the dominant net ion flux in airway epithelia from normal human and adult mammalian proximal airways (20, 119). This Na\(^+\) absorption was blocked by amiloride and, therefore, mediated by amiloride-sensitive Na\(^+\) channels. The remaining current in the presence of amiloride was shown by flux analysis to be Cl\(^-\) secretion that was induced by hyperpolarization of the apical membrane by amiloride and by clamping the transepithelial voltage to zero (“short-circuit conditions”) (22). Agonists that increased cAMP stimulated Cl\(^-\) secretion in the presence of amiloride under short-circuit conditions (5, 21). In CF airway epithelium, amiloride-resistant current was greatly reduced and was not stimulated by raising intracellular cAMP (24, 76). This phenotype is now understood as “dysfunctional or missing CFTR” phenotype, and, at the time, was interpreted as defective Cl\(^-\) secretion by CF airway epithelia. It is important to note, however, that in the absence of amiloride (i.e., under open-circuit conditions in vitro and under basal conditions in vivo), the transepithelial electrochemical gradient for Cl\(^-\) movement in proximal airways is absorptive. Thus CFTR might provide a pathway in airways, as in sweat duct, for Cl\(^-\) secretion. It has been shown that CFTR dysfunctions expand the Na\(^+\) conductance available for Cl\(^-\) secretion by CF airway epithelia, increased intracellular cAMP stimulated further amiloride-sensitive short-circuit current (i.e., Na\(^+\) absorption), whereas there was no effect on the magnitude of Na\(^+\) absorption in normal airway epithelia (24). In retrospect, these abnormalities in Na\(^+\) transport in CF airways seem to indicate a negative modulatory effect of CFTR on the rate-limiting step in Na\(^+\) absorption by airway epithelia, the apical membrane amiloride-sensitive Na\(^+\) channel.

B. Heterologous Cell Models of Interaction Between CFTR and ENaC

1. Decreased cAMP stimulation of ENaC in two coexpression systems

   The precedent that CFTR regulates other ion channels was established by the positive cAMP regulation
CFTR conveyed on the outward-rectifying Cl\textsuperscript-- channel (40, 44). After ENaC was cloned (15, 19, 28, 29) and determined to mediate Na\textsuperscript+ absorption in airway epithelia at birth (58), it became possible to formally test the hypothesis that CFTR also negatively regulated epithelial Na\textsuperscript+ channels. Two heterologous expression systems were engineered in which CFTR and ENaC could be expressed independently or together. A clone of high-resistance Madin-Darby canine kidney (MDCK) cells that expressed very low endogenous amiloride-sensitive Na\textsuperscript+ absorption and cAMP-stimulated Cl\textsuperscript-- secretion were first stably transfected with cDNA of rat renal ENaC subunits and then infected transiently with an adenoviral vector containing the CFTR cDNA (Ad5-CBCFTR) (69) to generate high levels of CFTR protein. In the MDCK/renal ENac (rENaC) cells, amiloride-sensitive short-circuit current was recorded on the order of 40–60 \mu A under basal conditions, and this current was stimulated 25% upon exposure to forskolin (120). This result was similar to the stimulation of ENaC-mediated Na\textsuperscript+ absorption observed in salt-conserving epithelia (111). On the other hand, however, this result could be contrasted with abnormal stimulation of amiloride-sensitive short-circuit current in CF nasal epithelia (24). Madin-Darby canine kidney/rENaC cells transfected with CFTR had reduced basal current and, remarkably, responded to forskolin with a slight decrease in amiloride-sensitive current (120). Similar results were obtained measuring amiloride-sensitive whole cell currents of fibroblasts stably transfected with rENaC alone or rENaC-CFTR (120). These studies were performed with the whole cell voltage-clamp technique and, thus, demonstrate an effect of CFTR on regulation of ENaC permeability. These studies are also strongly supported by a recent paper by Kunzelmann et al. (78) showing that intracellular domains of CFTR (NBD1 and the R domain) interact directly with the COOH-terminal tail of \alpha\textsubscript{-}rENaC in the yeast two-hybrid analysis (78). Direct protein-protein interaction supports strongly the functional data from several laboratories showing negative modulation of ENaC-mediated current by CFTR (24, 49, 64, 82, 120). Indeed, insertion of the G551D mutation into NBD1 disrupted functional interaction (78), an observation also documented for the disruption of CFTR-ORCC regulatory interaction (43) and for CFTR-ROMK2 interaction (see sect. vi).

More recently, preliminary results from single-channel recordings that correlate with the studies above showing negative modulation of ENaC by CFTR have been obtained in fibroblasts stably transfected with ENaC alone or in combination with CFTR. The single-channel properties of ENaC expressed alone were similar to those observed for the cloned channel in other heterologous systems (28, 29) and from the native channel in renal tubules (91) and in A6 epithelia (86), including a conductance of 4–6 pS, amiloride sensitivity, and slow gating kinetics (123). The open probability (\(P_0\)) of ENaC in this system was strongly dependent on the activity of PKA. Under optimal phosphorylating conditions, high \(P_0\) and mean open time of the channel (MOT) of many seconds were observed, whereas a specific peptide inhibitor of PKA markedly reduced \(P_0\) and MOT by inducing a gating mode in which the channels were closed except for very brief openings. Coexpression of ENaC with CFTR did not have noticeable effects on the basal properties of ENaC but had a dramatic effect on the regulation of the Na\textsuperscript+ channels by PKA. Optimal phosphorylating conditions in cell-attached or excised patches induced a decrease in single-channel \(P_0\) and MOT. No effect on the incidence of ENaC was discerned (123).

C. Potential Mechanisms of Interaction

The molecular basis for negative regulation of ENaC by CFTR has not been identified. Several possibilities are depicted in Figure 6. First, although controversial, a role for CFTR in the export of intracellular ATP has been suggested (114). Adenosine 5\textsuperscript{-}triphosphate, released into the extracellular domain, could inhibit apical membrane Na\textsuperscript+ channels, either through activation of phospholipase C and PKC linked to P\textsubscript{2y2} purinergic receptors or after metabolism of ATP by ectonucleotidases into adenosine and subsequent inhibition of adenyl cyclase linked to \(A_1\) adenosine receptors (84). Theoretically, CFTR could accomplish the transport of yet unidentified substrates that affect Na\textsuperscript+ channels. Members of the related \textit{mdr} transporters move phospholipids across membranes (13, 50, 129); thus phospholipid messengers may play a role in modulation of ENaC by CFTR. A second possibility is that CFTR interacts directly with one or more ENaC subunits, as suggested by the studies in planar lipid bilayers (see sect. vi). However, this possibility may be difficult to reconcile with the observation that the functional regulation of ENaC by CFTR seen in the airways is not seen in every tissue where CFTR and ENaC appear to be expressed in a tissue where CFTR and ENaC appear to be expressed in concert, such as the sweat duct epithelium. However, the recent correlative studies by Kunzelmann et al. (78) underscore the idea that direct-direct protein interaction may be likely. The third possibility is that CFTR and ENaC interact via elements of the membrane-associated cytoskeleton and that this indirect interaction is involved somehow in the ability of CFTR to interfere with the regulation of ENaC by PKA. Although the details of this mechanism have not been established fully, both CFTR (30) and ENaC (15) have been shown to be affected by interactions with cytoskeletal components, namely, actin. Variable expression of a key regulatory protein or proteins linking CFTR or ENaC to the cytoskeleton or to each other in different tissues could explain tissue-specific regulation of ENaC by CFTR.
VI. CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR REGULATORY INTERACTIONS WITH EPITHELIAL SODIUM CHANNELS IN PLANAR LIPID BILAYERS

Although fluid and electrolyte secretion is inhibited in CF patients because of CFTR transport dysfunction, fluid absorption across the airway is enhanced because of a simultaneous increase in the rate of Na⁺ transport (24, 76). These transport events result in the accumulation of mucus, pulmonary congestion, and excessive dryness of the epithelial surface. The increased Na⁺ absorption observed in CF patients has been attributed to a two- to threefold greater activity of the amiloride-sensitive Na⁺ entry pathway when compared with epithelium derived from non-CF individuals (24, 76). Currently, amiloride inhalation is being pursued as a possible therapy for CF lung disease (14, 70, 75). As indicated earlier, the molecular pathogenesis of the aforementioned events lies in the ability of the CFTR molecule itself to inhibit tonically the activity of amiloride-sensitive Na⁺ channels. The results of Stutts et al. (120) support the hypothesis that the physical presence of functionally competent CFTR is necessary for the downregulation of ENaC channels.

A. CFTR and αβγ-ENaC in Bilayers

Several other issues are important regarding the interaction of αβγ-ENaC and CFTR. First, the αβγ-ENaC subunits of ENaC are expressed in unequal proportions in airway epithelia (27). The α-subunit is sufficient to produce functional amiloride-sensitive Na⁺ channels, although the presence of β- and γ-subunits enhances significantly amiloride-sensitive macroscopic currents in oocytes as well as changes substantively the gating pattern of α-ENaC (29, 64, 65). The importance of ENaC β- and γ-subunits for channel function and their coupling to regulatory processes is unknown, although varying the ratio of α-, β-, and γ-subunits did not produce a variety of channel intermediates in bilayers (64, 65). Second, although the message for αβγ-ENaC was found in the lung (27), this channel exhibits a single-channel conductance, ion selectivity, gating kinetics, and amiloride-sensitivity profile similar to a low-conductance, highly selective amiloride-sensitive Na⁺ channel found in native Na⁺-reabsorbing epithelia other than in the airways (91). This is intriguing because the Na⁺ channels identified thus far in airway epithelia display quite variable biophysical characteristics (33, 71, 87, 134). Single-channel conductances vary from 4 to 28 pS, their Na⁺-to-K⁺ permeability ratios vary from 1 to >10, and the affinity for amiloride varies from an inhibitory constant of 0.9 to 8 μM. Moreover, the ethylisopropyl analog of amiloride (ethylisopropylamiloride) can inhibit pneumocyte Na⁺ channels better than amiloride itself (87).

Wild-type CFTR was found to decrease the P₀ of αβγ-ENaC in planar lipid bilayers without affecting the unitary conductance of the ENaC channels (64). However, this reduction of P₀ was only moderate (~20% reduction). Other preliminary experiments revealed that CFTR had no effect on the Pᵢ of this channel formed by the α-subunit alone in contrast to inhibition of all three subunits expressed together. These preliminary results suggest that the β- and/or γ-subunit is important for CFTR to exert its regulatory influence on ENaC. However, this tentative conclusion is paradoxical in light of the recently published report of Kunzelmann et al. (78), who based on correlative yeast two-hybrid analysis and patch-clamp analysis concluded that CFTR interacts with the α-subunit of ENaC via cytoplasmic domains.
B. Influence of Actin on CFTR-ENaC Interactions

The cytoskeletal protein actin interacts with αβγ-ENaC and affects dramatically the biophysical properties of the channel. Actin decreases the single-channel conductance, confers sensitivity of the channel to PKA, and changes the gating of the channel such that the mean open and closed times become more long lived (from milliseconds to second time constants). Because of this effect of actin on ENaC single-channel properties, the hypothesis that CFTR may exert an enhanced regulatory influence on αβγ-ENaC in the presence of actin was tested. Moreover, we wanted to examine the influence of CFTR on the stimulatory effect of PKA on the activity of αβγ-ENaC. These results showed that immunopurified bovine tracheal epithelial CFTR coreconstituted into a bilayer with αβγ-ENaC lowered the single-channel P₀ of αβγ-ENaC in the presence of actin by >60% (compared with only 20% in the absence of actin). In the presence of actin, PKA plus ATP or ATP alone activated ENaC both in the presence and in the absence of CFTR in a transient manner, with peak activation occurring ~40 min after PKA plus ATP addition. The presence of CFTR, however, attenuated greatly the activation of ENaC by PKA plus ATP. We also found that actin, but not CFTR, could interact directly with the channel-forming α-subunit of αβγ-ENaC. As indicated above, CFTR did not affect any properties of the channels formed by the α-subunit of rENaC alone but did affect the channels only if either or both the β- and γ-subunits were present. Taken together, our results suggest that actin enhances the ability of CFTR to downregulate ENaC epithelial Na⁺ channels and that the interactions between αβγ-ENaC, CFTR, and actin involve different subunits of this cloned epithelial Na⁺ channel. Figure 7 illustrates a working model of CFTR-ENaC cross talk as elucidated by lipid bilayer recordings.

VII. CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR REGULATION OF RENAL OUTER MEDULLARY POTASSIUM CHANNEL SENSITIVITY TO GLIBENCLAMIDE: CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR AS A SULFONYLUREA RECEPTOR

Not only may CFTR regulate other ion channels directly or indirectly via several protein-protein or signaling interactions, but CFTR may also be capable of conferring effects of agonists or antagonists on other proteins. These functions of CFTR derive from analogies to the functions of other ABC transporters such as mdr, MRP, SUR, HisP, the TAP, and yeast STE6 (55, 56). These transporters facilitate the transmembrane movement of a large variety of compounds such as anionic and cationic hydrophobic drugs, peptides, phospholipids, and, in some cases, large proteins (55, 56). In part, the concept that CFTR could be a regulator of many other cell functions derived from its membership in the ABC transporter family. Moreover, because CFTR regulates two transmembrane-spanning motif ion channels such as ENaC and ROMK (see sect. VII), it is possible that there is a common motif that CFTR recognizes and modulates in these channel subtypes. Because CFTR also regulates ORCC, it is possible that this channel may be an anion channel with a similar topological motif.

A. Sulfonylurea Receptors: Members of the ABC Transporter Family

Sulfonylureas are a family of compounds that inhibit ATP-sensitive K⁺ channel current (I_{KATP}). The I_{KATP} chan-
nals are found in a number of tissues including the brain, heart, smooth muscle, kidney, and pancreatic β-cells (41, 98). They are a subgroup of the inwardly rectifying K⁺ channels (IRK) channels. A defining characteristic of IKATP channels is their inhibition by ATP or other nucleotides (41, 98). In native tissue, these channels are inhibited by sulfonylureas. It was believed originally that sulfonylureas were specific inhibitors of IKATP channels. However, it is clear that the sulfonylurea glibenclamide can also inhibit CFTR channel activity (118), suggesting a link between this Cl⁻ channel and the family of ATP-sensitive K⁺ channels.

The IKATP channels are believed to provide a link between the metabolic status of the cell and membrane potential. In the β-cell of the pancreas, modulation of IKATP channels controls insulin release. Clinically, sulfonylureas, such as glibenclamide and tolbutamide, are used to treat diabetes mellitus. They act by inhibiting IKATP channels in pancreatic β-cells, thereby leading to cell depolarization. The depolarization in turn activates L-type Ca²⁺ channels, and the resulting Ca²⁺ influx triggers exocytosis of insulin. It was shown that sulfonylurea sensitivity of the pancreatic IKATP (Kir6.2) (62, 95) is conferred by SUR, a high-affinity sulfonylurea binding protein and a newly identified member of the ABC superfamily (4).

The binding protein SUR is a 140-kDa molecule that has pharmacological characteristics of a high-affinity receptor. Although SUR possesses a number of structural motifs that are characteristic of ABC transporters, such as nucleotide binding folds as well as transmembrane-spanning domains (4), there are a number of unique aspects to its structure. For instance, unlike CFTR, which has both its NH₂ and COOH termini located in the cytoplasm, the predicted structure for SUR suggests that its NH₂ terminus is located extracellularly while its COOH terminus is intracellular. Moreover, its transmembrane-spanning domains are asymmetrical, that is, there are nine transmembrane-spanning helices before NBF1 (TMD1) and only four transmembrane spanning helices before NBF2 (TMD2). This asymmetry is similar to that seen in MRP, another ABC family member which has an eight/four split of its transmembrane helices, again unlike the TMD symmetry of CFTR (4, 55, 56). However, the most striking difference between SUR and CFTR is how they function. Although CFTR appears to be multifunctional acting as a Cl⁻ channel as well as a channel regulator, when SUR is expressed alone, it does not demonstrate ion channel activity (4), suggesting that it acts only as a channel regulator and not as an ion channel itself.

The binding protein SUR has been isolated in brain, heart, and pancreas. Another isoform of SUR has been identified, SUR2, which has 68% identity to SUR (SUR1). The isoform SUR2 is expressed in the heart, skeletal muscle, and ovary in abundance. It has also been identified in the brain, tongue, and pancreatic islets in moderate abundance. Finally, it is also expressed in the lung, testis, and adrenal gland in lower amounts (63). The SUR2 isoform has a lower affinity to glibenclamide than does SUR1. Most recently, another isoform of SUR2 has been identified in the mouse, SUR2B, that has similar properties to the original SUR2 (also named SUR2A). This SUR2B isoform appears to have a fairly ubiquitous pattern of expression (66).

B. Sulfonylurea Sensitivity of Inwardly Rectifying K⁺ Channels

As stated above, IKATP channels are a subgroup of the inwardly rectifying K⁺ channel superfamily (41). Recently, several inwardly rectifying K⁺ (IRK) channels have been cloned that contain two transmembrane-spanning domains characteristic of this rapidly expanding family of K⁺ (41, 98). The cloned channels retain many of the characteristics of the native channels, except that they are each insensitive to sulfonylurea compounds. Hence, it was postulated that another subunit, such as SUR, must confer this regulation. It has also been shown that SUR can confer sulfonylurea sensitivity to a variety of cloned IKATP channels, including ROMK1, an IKATP channel isolated from kidney, Kir6.1, a ubiquitously expressed KATP channel, and an endogenous K⁺ channel in HEK 293 cells (6). Taken together, these data suggest that this ABC transporter modulates IKATP channel activity. However, SUR expression does not mirror the expression of all IKATP channels, suggesting there are other sulfonylurea receptors (62, 63).

C. CFTR Interactions with K⁺ Channels

Studies have reported that the expression of CFTR alters K⁺ currents in several cell types (83, 88, 107). For instance, in CFPAC cells, an immortalized CF-affected pancreatic epithelioid cell line, CFTR expression is associated with cAMP-dependent regulation of inwardly rectifying K⁺ currents (83). Inwardly rectifying K⁺ channels have been identified on the basolateral cell membrane in airway epithelia, where they are believed to play a role in K⁺ recycling; however, there have not been any reported abnormalities in their activity in CF. Activation of these channels provide, in part, the driving force for apical Cl⁻ channel activation; therefore, they play a critical role in maintaining normal Na⁺ and Cl⁻ balance in the airway. Recently, it has been suggested that similar K⁺ channels may exist on the apical membrane of airway epithelial cells (83). Such apical channels have been identified in the lacrimal acinar cells, in the distal colon (83), and in secretory renal epithelia. One member of this family ROMK1 (Kir1.1) has been identified in the lung (57, 135).
D. CFTR and ROMK

The ROMK family is made up of $I_{\text{K}_{\text{ATP}}}$ channels derived from kidney and has a number of isoforms (ROMK 1, 2, and 3) (57, 135). In renal tissue, $I_{\text{K}_{\text{ATP}}}$ renal channels are present in the apical membrane of distal nephron segments of the mammalian kidney, where they play a major role in $K^+$ homeostasis (57, 110, 135). The $I_{\text{K}_{\text{ATP}}}$ renal channels share many of the properties and characteristics of other members of this family found in tissues such as heart and pancreas. However, $I_{\text{K}_{\text{ATP}}}$ renal channels are insensitive to tetraethylammonium (TEA), have less sensitivity to ATP (mM concentrations are required to induce channel inhibition), and have a lower affinity for sulfonylureas such as glibenclamide than required for inhibition of other $I_{\text{K}_{\text{ATP}}}$ channels. The ROMK2 isoform shares many characteristics with “native” renal $I_{\text{K}_{\text{ATP}}}$. However, unlike the native channel that is sensitive to sulfonylureas, ROMK2 exhibits only low and highly variable sensitivity to sulfonylurea compounds such as glibenclamide (57, 135). It is hypothesized that sulfonylurea sensitivity of renal $I_{\text{K}_{\text{ATP}}}$ may be either conferred to or enhanced by a separate channel subunit, that is similar to SUR. Neither SUR1 nor its isoform SUR2a has been identified in the kidney (4, 63), making it unlikely that either is responsible for the sulfonylurea sensitivity of the $I_{\text{K}_{\text{ATP}}}$ renal channel. SUR2B is thought to be expressed ubiquitously as it has been identified in every tissue studied by RT-PCR; however, no localization studies nor protein data are yet available on this isoform (66). In contrast, CFTR is expressed abundantly along the nephron (36, 38, 90, 110). In a recent study, Devuyst et al. (38) have demonstrated that CFTR is present in the apical membrane of the cortical collecting duct. Immunohistochemical studies have shown that ROMK2 is also present in the apical membrane of the cortical collecting duct (126). Given these data, it is possible that CFTR may play a regulatory role with regard to ROMK2 channel activity in vivo.

In patch-clamp studies, the coexpression of CFTR with ROMK2 in *Xenopus* oocytes resulted in the appearance of an ATP-sensitive $K^+$ channel with a 47-pS single-channel conductance that was sensitive to glibenclamide, an inhibitory sulfonylurea compound. Both CFTR and ROMK2 channel activity could be discerned in these patches before excision. Exposure to 0.1 mM glibenclamide decreased channel $P_o$ by 90%; however, concentrations as low as $10^{-7}$ M resulted in a 40% decrease in $P_o$ (88). Thus exposure to glibenclamide resulted in a dose-dependent inhibition of $K^+$ channel activity (inhibitory constant $= 1 \mu\text{M}$), a value similar to that demonstrated by Yang and co-workers (18) for the native renal channel. In these studies, neither the ATP sensitivity nor the single-channel conductance was altered by the coexpression of CFTR with ROMK2 (88). Similar findings have been reported with regard to SUR coexpression with a variety of other inwardly rectifying $K^+$ channels. In these studies, coexpression altered only glibenclamide sensitivity. Other channel properties were unchanged. Preliminary results using two-microelectrode voltage clamp of oocytes confirm these findings (130).

These results should be contrasted with a preliminary report by Welling and co-workers (107). They have reported a decrease in single-channel conductance of ROMK1 when it is expressed with CFTR (107). In addition, ATP sensitivity of ROMK1 was altered, suggesting that CFTR provides the necessary domain for the channel’s ATP sensor. A similar hypothesis has been suggested by Inagaki et al. (63) with regard to SUR and BIR (Kir6.2) interactions. These data may imply that the additional 19 amino acids that are present at the NH$_2$ terminus of ROMK1 when compared with ROMK2 alter this interaction with CFTR.

Cystic fibrosis transmembrane conductance regulator was not stimulated by PKA and ATP in any of the coexpression studies, indicating that active transport by
CFTR is not necessary for the interaction. The simplest explanation for these findings is that CFTR interacts directly with ROMK. Cystic fibrosis transmembrane conductance regulator could be coupled with either ROMK1 or ROMK2 in the cell membrane, providing a missing domain to the cloned channels, thus restoring sulfonylurea sensitivity. Alternatively, the mechanism that controls the CFTR-ROMK2 interaction could involve a regulatory protein or a cytoskeletal element. Further studies will need to be done to elucidate the mechanism of interaction. Figure 8 provides a series of hypotheses, presented in membrane model format, describing future directions in the definition of CFTR-ROMK regulatory interactions.

**E. Effects of Phosphorylation on CFTR-ROMK2 Interaction**

Patch-clamp studies of the coexpressed peptides have demonstrated that phosphorylation modifies the response to glibenclamide: phosphorylation attenuates the inhibitory response to the sulfonylurea (88). In these studies, ATP and PKA were added to the bath solution of excised inside-out patches from oocytes expressing both CFTR and ROMK2. The ROMK2 channel activity was present in all patches; however, in contrast to the previous studies of the coexpressed peptides, when these patches were then exposed to glibenclamide, channel activity was not inhibited. Additional studies showed exogenous ATP and PKA could reverse the inhibition by glibenclamide (88). In these experiments, the application of 0.1 mM glibenclamide led to a 95% decrease in ROMK2 channel activity; however, this channel activity could be fully restored by the application of PKA and MgATP, suggesting that the inhibitory effect of glibenclamide could be reversed by rephosphorylation of either ROMK2 or CFTR. Finally, when these patches were then reexposed to glibenclamide, the sulfonylurea compound failed to decrease ROMK2 channel activity. Thus, after addition of PKA and ATP, glibenclamide had no effect on ROMK2 K⁺ channel activity (88). These data suggest the interaction between CFTR and ROMK2 is altered by a phosphorylation-dependent process.

**F. CFTR Domains Important to the CFTR-ROMK Interaction**

Preliminary studies have suggested that NBF1 may be a necessary domain for CFTR-ROMK2 channel interaction (130). In two-microelectrode voltage-clamp studies, when ROMK2 was expressed with the CFTR mutant G551D, which is a severe lung disease-causing mutation and affects ATP binding to the first nucleotide binding fold (NBF1), there was only a 14% decrease in Ba²⁺-sensitive current, demonstrating minimal glibenclamide sensitivity, similar to that observed with ROMK2 alone. In contrast, when a truncated CFTR construct that contains a functioning NBF1 (K593X) was expressed with ROMK2, ~50% of K⁺ current was glibenclamide sensitive, demonstrating a significant but not complete inhibition (130). The G551D/ROMK2 and K593X/ROMK2 data suggest that the interaction between CFTR and ROMK2 may require a functional CFTR nucleotide binding fold (CFTR-NBF1) and provide evidence for subunit interactions between CFTR and ROMK2. The importance of NBF1 for CFTR-ORCC regulatory interaction and CFTR-ENaC regulatory interaction has already been documented and complements these studies nicely. Preliminary data with similar truncation mutants also suggest that NBF1 and, possibly, the R domain are important for CFTR-ORCC interactions. An unphosphorylated R domain present with NBF1 may be critical for complete CFTR-conferred inhibition of ROMK2 by glibenclamide, as phosphorylation with PKA prevents glibenclamide inhibition.

**VIII. SUMMARY AND CONCLUSIONS:**

**CORRELATION OF REGULATORY FUNCTIONS WITH SEVERITY OF CYSTIC FIBROSIS LUNG DISEASE**

It is clear that much work still needs to be done to define the cellular and molecular mechanisms that underlie CFTR conductance regulator function. It is also clear that an additional layer of cellular defects in vesicle trafficking, protein processing, and immune function also exists in CF cells that was mentioned briefly in this review and was summarized within other submissions to this volume. These defects may correlate directly with CFTR function, may correlate indirectly with CFTR function (i.e., relate to a role for CFTR as an intracellular Cl⁻ channel), or may not correlate with CFTR at all but may be caused by defects in “modifier” genes or loci.

Nevertheless, these layers of cellular defects caused or not caused directly or indirectly by CFTR dysfunction may explain fundamentally why genotype does not necessarily correlate with phenotype, especially with respect to CF lung disease. Cystic fibrosis lung disease in one individual may result unequivocally from a defect in Cl⁻ channel function, whereas CF lung disease in the next three affected patients may result from defective regulation of ENaC and/or defective protein processing and/or impaired innate immunity. Moreover, these defects may arise phenotypically at different times within a patient’s lifetime. Finally, the severity of disease may be modified by associated genetic factors such as modifier gene loci. It is tempting to speculate that these loci may encode for molecules important for conferring CFTR regulation on these other ionic conductances, on vesicle trafficking and/
or protein processing, or on immune/inflammatory mediators.

It is the hope that this review has provided new insight into the ability of CFTR to be a conductance regulator and creative thought as to how CFTR might perform all of these functions in a normal cell and cause such layers of chaos in a CF cell. It is a likelihood that elucidation of the mechanisms whereby CFTR performs as a cellular regulator and a conductance regulator will identify novel and specific targets for CF therapies in the foreseeable future.

**IX. UPDATE: RECENT DEVELOPMENTS**

During the publication of this review, several additional studies were published that provided support for some of the concepts and hypotheses introduced here. In section II, the molecular mechanisms of how domains of CFTR regulate ORCC were discussed. A paper by E. M. Schwiebert et al. was published in the *Proceedings of the National Academy of Sciences* (116a). In this paper, the data discussed in this review can be assessed as to its merit. In short, it may provide more insight into what domains of CFTR are critical for Cl− conductance and what domains are critical for regulation of other ionic conductances.

Two studies were published by Foskett and co-workers (123a) and by Linsdell and Hanrahan (81a) that have implications on the ability of CFTR to conduct ATP and/or regulate a separate ATP channel (see sect. IV). In short, ATP channels endogenous to MDCK cells were shown to be regulated positively by CFTR. They were shown to be separate, because MDCK cells that lacked CFTR occasionally had ATP channel activity. Evidence for CFTR gating of the separate yet closely associated ATP channel was shown. Linsdell and Hanrahan (81a) showed that large organic anions (not unlike the size of ATP) could permeate asymmetrically from the cytoplasmic side of the membrane to the extracellular side. When “locked open” with S′-adenylylimidodiphosphate, this large anion permeation became symmetrical. The authors suggest that ATP may be permeable under these conditions, but this experiment was not performed.

Egan and co-workers have also published a paper concerning domains of CFTR that interact with ROMK channels (89a). In this study, they show that a functional NBD1 within CFTR is required to confer glibenclamide inhibition upon ROMK channels.

Very recent studies have identified the presence of a PDZ-binding domain at the extreme COOH terminus of CFTR that may associate with anchoring proteins that link transmembrane glycoproteins to actin-binding proteins and the actin cytoskeleton. These studies solidify the hypotheses concerning accessory proteins, linker proteins, or regulatory cofactors that may confer CFTR regulation on separate yet closely associated ion channel proteins. Of the many studies in various stages of publication, the first two papers to hit the press are References 118a and 129a.

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