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ACTIVATION BY EXTRACELLULAR NUCLEOTIDES OF CHLORIDE SECRETION IN THE AIRWAY EPITHELIA OF PATIENTS WITH CYSTIC FIBROSIS

Michael R. Knowles, M.D., Lane L. Clarke, D.V.M., Ph.D., and Richard C. Boucher, M.D.

Abstract *Background.* Cystic fibrosis is characterized by abnormal electrolyte transport across the epithelia of the airways. In particular, there is excessive sodium absorption and de..cient chloride secretion. Drugs that block excessive sodium absorption may provide clinical benefit in cystic fibrosis, but there are no available therapeutic agents to improve chloride secretion. In vitro studies in cultured human-airway epithelia indicate that triphosphate nucleotides (ATP and UTP) induce chloride secretion through apical-membrane purinergic receptors.

Methods. We tested the ability of nucleotides to induce chloride secretion in vivo in 9 normal subjects and 12 patients with cystic fibrosis by measuring responses of nasal transepithelial potential difference (PD) to superfusion of nucleotides. Changes in transepithelial bioelectric properties and the permeability of the apical membrane to chloride in response to extracellular (apical) UTP were determined with ion-selective microelectrodes in cultured nasal epithelia.

Results. ATP and UTP induced chloride secretion in vivo in both groups. At their maximal effective concentrations of 10^{-4} M, ATP and UTP were more effective chloride secretagogues in the patients with cystic fibrosis (mean [±SE] change in PD, −19.8±1.4 mV and −15.0±1.7 mV, respectively) than in the normal subjects (−6.9±0.6 mV and −8.1±0.9 mV, respectively). Microelectrode studies established that extracellular UTP stimulated a larger increase in PD and chloride secretory current in epithelial cells from patients with cystic fibrosis than in cells from normal subjects, by actions localized to the apical membrane.

Conclusions. Extracellular nucleotides are effective in vivo chloride secretagogues in the nasal epithelia of patients with cystic fibrosis. The equipotency of ATP and UTP suggests that the effect is mediated by P_2 nucleotide receptors. Selected nucleotides, such as UTP or nucleotide analogues, should be investigated as therapeutic agents for lung disease in cystic fibrosis. (N Engl J Med 1991; 325:533-8.)

THE pathogenesis of lung disease induced by cystic fibrosis reflects in part the effects of mutations in the cystic fibrosis transmembrane regulator protein on electrolyte transport by airway epithelia.[1] Both the defective regulation of the secretory chloride channel[2-6] and the accelerated rate of sodium absorption[4,7] in the airway epithelia of patients with cystic fibrosis probably contribute to the dehydration of airway secretions. Recently, the sodium-channel blocker amiloride was shown to be effective in inhibiting sodium absorption in airway epithelia from patients with cystic fibrosis[7] and, when given as an aerosol, in improving mucociliary clearance[8] and slowing the rate of decline in vital capacity.[9] These results suggest that therapy designed to correct the chloride secretory defect also may be beneficial for lung disease in cystic fibrosis.

Agents that stimulate chloride secretion through pathways mediated by cyclic AMP (cAMP) in the airway epithelia of normal, healthy subjects, such as beta-adrenergic agonists, are ineffective in patients with cystic fibrosis.[2-6] Therefore, we tested compounds reported to initiate chloride secretion in epithelia through signal-transduction pathways independent of cAMP metabolism. To improve the likelihood that effective compounds would be suitable for clinical use and could ultimately be administered selectively as aerosols, we focused on endogenous biologic compounds that are active at the apical (ciliated) surface of airway epithelia. This approach led to the observation that extracellular triphosphate nucleotides are effective chloride secretagogues when applied to the apical surface of cultured human-airway epithelia.[10]

In this study, we investigated whether triphosphate nucleotides are effective chloride secretagogues in vivo and compared their relative effectiveness in patients with cystic fibrosis and in normal subjects. The nasal epithelium was studied because it is a convenient site for the measurement of the transepithelial potential difference (PD); in human-airway epithelia, the rate of sodium absorption and of chloride secretion correlates with the absolute magnitude of the transepithelial PD. Changes in PD in response to pharmacologic agents provide evidence of changes in the rate of ion transport and in the types of ions transported. The effects induced by triphosphate nucleotides in vivo were confirmed and extended with in vitro studies of primary cultures of nasal epithelia from patients with cystic fibrosis and from normal controls.

Methods

We studied the effects of nucleotide triphosphates in vivo in 9 normal, healthy subjects (6 women and 3 men; age range, 18 to 28 years) and 12 patients with cystic fibrosis (7 women and 5 men; age, 19 to 38). Among the patients with cystic fibrosis, eight were homozygous for the phenylalanine deletion (ΔF_{508}) and four were heterozygous (ΔF_{508}/unknown mutation). Specimens of nasal epithelium for cell culture were obtained from five other patients with cystic fibrosis (one woman and four men; age range, 9 to 32 years) and four normal, healthy men (age, 24 to 64) who were undergoing clinically indicated nasal reconstructive surgery. All procedures were approved by the University of North Carolina Committee on the Rights of Human Subjects, and each subject gave informed consent for the studies.

Lactated Ringer's solution (37°C) was the usual superfusion (topical perfusion) vehicle for drug delivery in vivo. The chloride concentration was reduced by replacing sodium chloride with sodium gluconate (Sigma).[11] Amiloride (gift of Merck Sharp &

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Dohme), ATP, UTP, adenosine, and uridine (all from Boehringer–Mannheim) were freshly prepared for each study.

An established technique was used to measure nasal PD.[11,12] The transepithelial PD of human respiratory epithelium is a negative value, as referenced by convention to the submucosal space.[11] Amiloride (10^{-4} M) was perfused for two to three minutes (steady state), followed by perfusion with amiloride-containing solutions of ATP, UTP, adenosine, and uridine (six minutes). The chloride-free solution containing amiloride was perfused for three minutes before perfusion of the nucleotide-containing solution was begun.

For cell culture, epithelial cells were enzymatically disaggregated from excised nasal specimens, plated on collagen membranes suitable for transport studies, maintained in hormone-supplemented medium, and studied when PD was maximal.[13]

Cultured cells were placed in a modified Ussing chamber and perfused on each surface with warmed (37°C) and gassed (95 percent oxygen and 5 percent carbon dioxide) Ringer's solution. From measurements of PD and resistance, the equivalent short-circuit current was calculated from Ohm's law.[14] Double-barreled chloride-selective microelectrodes were used to measure the electrochemical driving force for chloride flow across the apical membrane before and during the addition of nucleotides[14]; the absolute permeability of the apical membrane to chloride was calculated.[15] UTP was selected for study because its nucleoside metabolite, uridine, was ineffective; this reduced the possibility of secondary effects mediated by P_1 purinergic receptors.

The change in nasal epithelial PD in vivo was calculated by subtracting the base-line steady-state PD from the PD recorded during each maneuver. The significance of changes within a study group was estimated with paired t-tests. The significance of differences in the dose effect between normal subjects and patients with cystic fibrosis was tested by repeated-measures analysis of variance; differences in the magnitude of the responses between the two groups were assessed by unpaired t-tests corrected for multiple comparisons.[16] The UTP-induced changes in cultured epithelial cells from normal subjects and from patients with cystic fibrosis were compared by unpaired t-tests. Values are given as means ±SE. A P level of less than 0.05 was considered to indicate statistical significance.

Results

Effect of Extracellular ATP on the Bioelectric Correlate of Chloride Secretion in Vivo

The normal human nasal epithelium is converted from a sodium-absorbing to a chloride-secreting system by the application of amiloride to the apical surface.[14,17] In the normal subjects (Fig. 1), amiloride reduced the base-line nasal PD by approximately 50 percent; in the presence of amiloride, ATP increased the PD by −7 mV over a three-minute interval, a finding consistent with accelerated chloride secretion. In the patients with cystic fibrosis (Fig. 1), the base-line PD was higher, reflecting an abnormally increased rate of sodium absorption. Amiloride reduced this PD by approximately 80 percent; in the presence of amiloride, ATP increased the PD by −20 mV within 30 seconds, a response consistent with the induction of chloride secretion. The pattern of response to 10^{-4} M UTP was similar in the two groups.

Dose–Effect Studies of ATP and UTP in Vivo

To define the in vivo pharmacologic characteristics of extracellular nucleotides and characterize the type of P_2 receptor involved in the chloride secretory response, dose–effect studies relating the concentration of ATP or UTP perfused to the change in PD in amiloride-treated nasal epithelia were performed. As shown

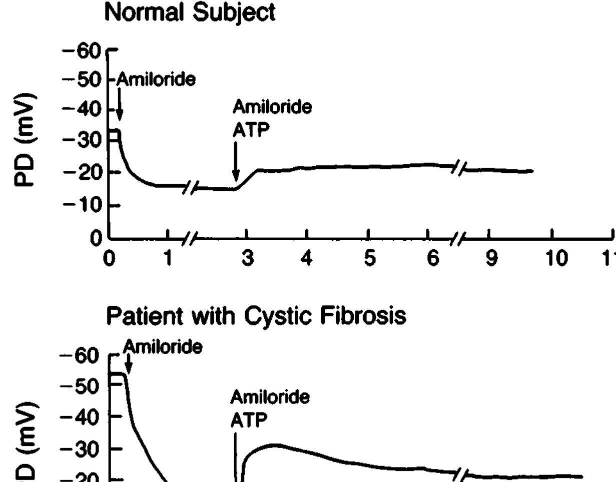


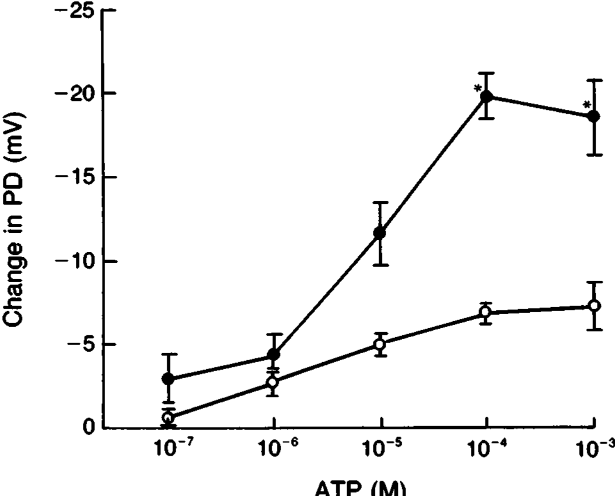
Figure 1. Response of Nasal Transepithelial PD in a Normal Subject and in a Patient with Cystic Fibrosis to Superfusion with 10^{-4} M Amiloride, Followed by 10^{-4} M ATP in Amiloride in a Ringer's Solution.

By convention, the transepithelial PD is a negative value.

in Figure 2, the dose of ATP that increased nasal PD to 50 percent of the maximal value in normal subjects and in patients with cystic fibrosis was approximately 3 to 5×10^{-6} M, and the maximal effective concentration was approximately 10^{-4} M. In both groups, UTP (Fig. 2) was equipotent with ATP (50 percent of maximal increase, approximately 2 to 4×10^{-6} M) and as effective as ATP (maximal effective concentration, approximately 10^{-4} M). The maximal effective concentrations of both ATP and UTP were more effective in inducing chloride secretion in patients with cystic fibrosis (mean change in PD, −19.8±1.4 and −15.0±1.7 mV, respectively) than in the normal subjects (change in PD, −6.9±0.6 and −8.1±0.9 mV, respectively; $P<0.01$ for the response to ATP and $P<0.02$ for that to UTP). The persistence of the PD response (percentage of the maximal response persisting at six minutes) during continuous perfusion of maximal concentrations was similar for ATP and UTP in the five patients with cystic fibrosis (59.1±3.9 and 67.8±4.0 percent, respectively) and the five normal subjects (43.5±10.9 and 52.4±6.5 percent, respectively).

Effects of Extracellular Adenosine and Uridine on Chloride Secretion in Vivo

Because the actions of ATP and UTP could be mediated by the action of their breakdown products, adenosine and uridine, respectively, on P_1 receptors, we tested the effect of these nucleosides on chloride secretion. In five normal subjects, superfusion of 10^{-4} M adenosine onto nasal epithelium pretreated with



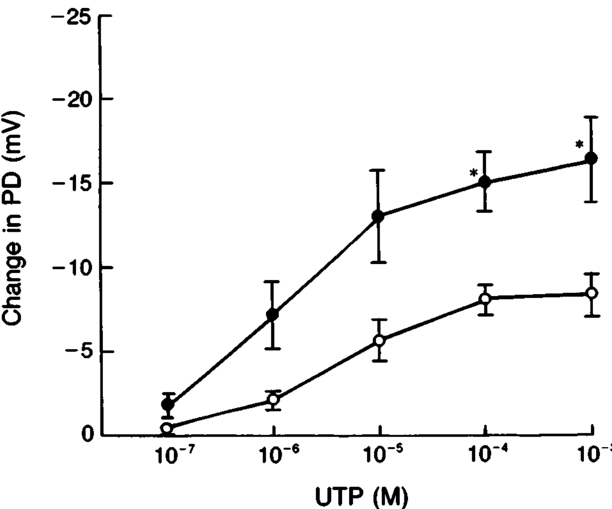


Figure 2. Dose–Effect Relation of the Mean Change in the Magnitude of the Transepithelial PD in Response to Superfusion of Various Concentrations of ATP or UTP onto 10^{-4} M Amiloride-Treated Nasal Epithelia of Five Normal Subjects (○) and Five Patients with Cystic Fibrosis (●).

The vertical bars indicate standard errors. Asterisks indicate a significant difference (P<0.02) between groups.

amiloride increased the PD (change in PD, −4.0±1.3 mV; P<0.05) and the addition of 10^{-4} M ATP further increased the PD (change in PD, −4.4±0.4 mV; P<0.05). Adenosine was ineffective in three patients with cystic fibrosis (change in PD, 0.0±1.2 mV), but the addition of 10^{-4} M ATP increased the nasal PD (change in PD, −17.0±3.1 mV; P<0.05). Equimolar concentrations of uridine (10^{-4} M) were ineffective in patients with cystic fibrosis (n = 3) and normal subjects (n = 3) (data not shown).

Action of Triphosphate Nucleotides in Cultured Epithelia

First, we sought to test by direct measurements whether the ability of nucleotides to increase the PD in vivo reflected activation of a secretory chloride current. Second, because chloride secretion can be induced by increases in the permeability of the apical membrane to chloride, activation of basolateral-membrane potassium channels,[18,19] or both, we used intracellular double-barreled chloride-selective microelectrodes to assess the effects of nucleotides on the permeability of the apical membrane to chloride and the basolateral membrane potential, an index of basolateral potassium channels. Figure 3 shows that, as it did in vivo, 10^{-4} M UTP induced a greater increase in PD in amiloride-pretreated epithelial cells from patients with cystic fibrosis than in those from normal subjects in vitro, and this response was paralleled by a greater stimulation of a chloride secretory current in the cells from the patients than in those from normal subjects. As shown in Figure 4, the greater chloride secretory response in cells from patients with cystic fibrosis reflected the lower rate of chloride secretion observed during amiloride perfusion in these patients and the similar rates observed during perfusion with UTP. Figure 4 shows that the effects of UTP on the permeability of the apical membrane to chloride in cells from patients with cystic fibrosis and from normal subjects paralleled the chloride secretory responses, indicating that the apical-membrane permeability to chloride was the rate-limiting step for UTP-activated chloride secretion. No changes in basolateral-membrane potential were detected in cells from either group, indicating that apical UTP has little effect on basolateral potassium channels. Quantitatively similar responses have been noted for ATP in human-airway epithelia.[20]

Mechanism of Nucleotide-Induced Chloride Secretion in Vivo

To test whether ATP and UTP increased chloride secretion in vivo by increasing the permeability of the apical membrane to chloride, we reduced the chloride concentration of the perfusate to zero to generate a large chloride electrochemical gradient across the api-

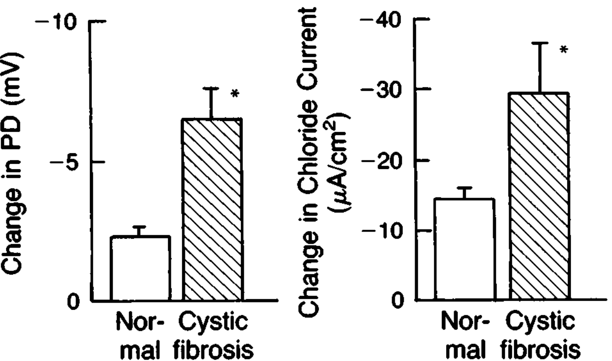


Figure 3. Effects of UTP on Epithelial Ion Transport in Vitro in Cultured Nasal Epithelial Cells from Four Normal Subjects and Five Patients with Cystic Fibrosis.

The left-hand panel shows the change in transepithelial PD and the right-hand panel shows the change in secretory chloride current induced by the addition of 10^{-4} M UTP to the apical surface of nasal epithelia pretreated with 10^{-4} M amiloride. Asterisks indicate a significant difference (P<0.05) between groups.

cal membrane. Nasal superfusion of chloride-free solution containing amiloride increased the PD in normal subjects but not in the patients with cystic fibrosis (Fig. 5), a response that is consistent with reduced permeability of the apical membrane to chloride in the basal state in cystic fibrosis. In normal subjects, the addition of ATP to the perfusate further increased the PD, which is consistent with an increase in the permeability of the apical membrane to chloride. The addition of ATP induced a greater increase in PD in the patients with cystic fibrosis than in the normal subjects. Figure 5 summarizes the PD values obtained during this protocol. The ATP-induced change in PD was significantly larger in the patients with cystic fibrosis than in the normal subjects (−33.6±2.0 vs. −20.6±1.7 mV). The greater efficacy of ATP in the patients reflects the fact that the PD was smaller in this group before nucleotide perfusion and that the magnitude of the PD during perfusion with ATP was similar in both groups. The results for UTP were similar.

Necessity of Amiloride Pretreatment for Nucleotide Activation of Chloride Secretion

In the basal sodium-absorbing state (i.e., without amiloride), superfusion of 10^{-4} M ATP onto the nasal epithelium of three normal subjects for three minutes had no effect on PD (change in PD, −0.5±0.8 mV). The absence of an effect reflects the fact that chloride is in electrochemical equilibrium across the apical membrane in the basal state, so that an increase in the permeability of the apical membrane to chloride is not associated with ion flow.[14] Without amiloride, superfusion of ATP onto nasal epithelium of four patients with cystic fibrosis induced a small decrease of the PD (from −59.8±5.1 to −51.0±4.3 mV; change in PD, 8.8±2.1 mV; P<0.05). This reduction in PD probably reflects the fact that chloride was below electrochemical equilibrium across the apical membrane,[21] leading

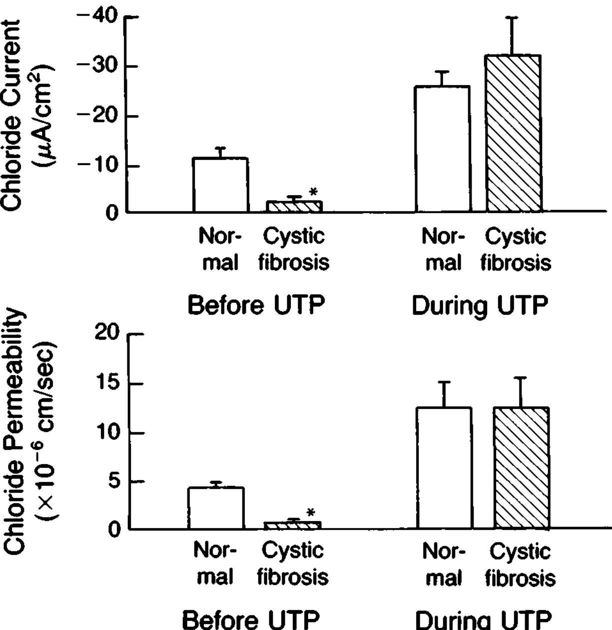


Figure 4. Secretory Chloride Current and Permeability of the Apical Membrane to Chloride in the Presence of 10^{-4} M Amiloride before and during the Perfusion of 10^{-4} M UTP onto the Apical Surface of Cultured Nasal Epithelial Cells from Four Normal Subjects and Five Patients with Cystic Fibrosis.

Asterisks indicate a significant difference between groups (P<0.05).

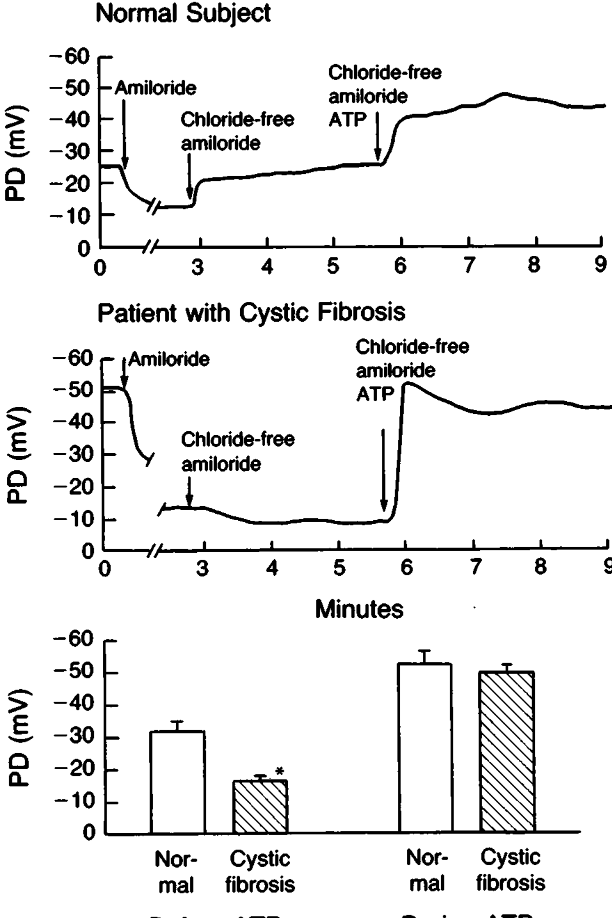


Figure 5. Response of Nasal Transepithelial PD in a Normal Subject and a Patient with Cystic Fibrosis to Sequential Superfusion with 10^{-4} M Amiloride in a Ringer's Solution, Amiloride in a Ringer's Solution Free of Chloride (Gluconate Substituted for Chloride), and Amiloride in a Ringer's Solution Free of Chloride but Containing 10^{-4} M ATP (Top and Middle Panels) and Mean Nasal PD Measured during Superfusion with Chloride-free Amiloride Solutions in Seven Normal Subjects and Eight Patients with Cystic Fibrosis before and during the Addition of ATP to the Perfusate (Bottom Panel).

The values obtained before the addition of ATP represent the steady-state PD generated by superfusion with chloride-free Ringer's solution containing amiloride. The values obtained during the addition of ATP represent the maximal PD achieved after the addition of 10^{-4} M ATP to the perfusate. The change in PD induced by ATP was larger in the patients with cystic fibrosis than in the normal subjects (−33.6±2.0 vs. −20.6±1.7 mV) (P<0.05). These data were corrected for tip potentials as appropriate.[11] The vertical bars indicate standard errors. The asterisk indicates a significant difference between groups (P<0.05).

to the influx of chloride across the apical membrane (absorption) and a decrease in the PD. The pattern of response to 10^{-4} M UTP was similar.

Discussion

Both purine (ATP) and pyrimidine (UTP) triphosphate nucleotides are highly effective chloride secretagogues when applied to the apical (ciliated) surface of human-airway epithelia in vivo as well as in vitro. This action is mediated by a class of cell-surface receptors, termed purinergic receptors, that have only recently been detected in human-airway epithelia.[10]

The identification and subclassification of purinergic receptors is a rapidly evolving area of research.[22] Mammalian cells express different types of cell-surface receptors that selectively respond to either extracellular triphosphate nucleotides or their dephosphorylated metabolites.[23] P_2 purinergic receptors are activated by triphosphate nucleotides and are linked to a variety of effector systems, including phospholipase C[24] and ion channels.[25] P_1 receptors regulate adenylate cyclase activity and are activated by the ATP metabolite adenosine.[26]

Our results indicate that extracellular nucleotides stimulate chloride secretion in airway epithelia in vivo through interaction with a subclass of P_2 receptors termed nucleotide receptors, because of their similar responsiveness to ATP and UTP.[27] As demonstrated by the magnitude of the changes in the PD of amiloride-treated nasal epithelia in vivo, ATP and UTP were equipotent chloride secretagogues, and their maximal effective concentrations (Fig. 2) and the duration of effect were similar. Adenosine affected the PD in normal nasal epithelia, indicating the presence of P_1 receptors, and the responses to maximal concentrations of adenosine and ATP in normal subjects were additive, a finding that is consistent with the expression of both P_1 and P_2 receptors. The failure of adenosine to modify the PD in patients with cystic fibrosis is congruent with the failure of cAMP-dependent mechanisms to activate chloride channels in airway epithelia in these patients[2,3,5,6] and does not imply absence of P_1 receptors. The lack of effect of adenosine in epithelia from patients with cystic fibrosis provides evidence that ATP interacted with P_2 receptors to initiate the chloride secretory response in these patients and probably in normal subjects as well. The lack of effect of uridine clearly indicates that UTP acted through P_2 nucleotide receptors to initiate chloride secretion in both groups of subjects.

Both ATP and UTP were more effective in inducing chloride secretion in vivo in patients with cystic fibrosis than in normal subjects (Fig. 2). In vitro studies of UTP confirmed that the larger change in PD in patients with cystic fibrosis was due to a larger change in the chloride secretory current (Fig. 3). The changes in chloride secretory rates reflect changes in permeability of the apical membrane to chloride, indicating that UTP initiated chloride secretion by activation of this pathway (Fig. 4). The in vivo responses during the protocols of amiloride and chloride-free solutions are consistent with this conclusion (Fig. 5). Both the in vivo and in vitro studies indicate that the increased efficacy of nucleotides in initiating chloride secretion in epithelium of patients with cystic fibrosis reflected the fact that nucleotides recovered a basal permeability of the apical membrane to chloride in patients with cystic fibrosis, and that they raised the levels of permeability to maximal levels that were similar in the patients and the normal subjects. The simplest hypothesis to account for these findings is that nucleotides activate the defective chloride transport path in cystic fibrosis, possibly the cystic fibrosis transmembrane regulator protein itself.

The action of these nucleotides on the PD in vivo was sustained during continuous drug perfusion, as previously noted in vitro.[10] The prolonged duration of action raises the question of the mechanism of activation of the permeability of the apical membrane to chloride. Two mechanisms may be operative. The first involves activation by P_2 receptors of a phospholipase C, generating inositol triphosphate and triggering an increase in cytosolic calcium concentration.[28] This activation may be short-lived[29] and therefore contribute only to the early phase of the nucleotide-induced chloride secretory response. More recently, a direct gating effect of ATP on the apical-membrane chloride channel in airway epithelia from both patients with cystic fibrosis and normal subjects has been proposed.[30] The relations among the nucleotide receptor, the type of chloride channel, and the cystic fibrosis transmembrane regulator protein[31,32] are not known. In patch–clamp studies of outside-out patches from airway epithelial cells, tachyphylaxis was not observed for this method of ATP-induced activation of the apical-membrane chloride channel under calcium-free conditions.[30] Thus, this mode of action may sustain the secretory response in vivo.

Our studies of nasal epithelium do not establish nucleotides as effective pulmonary pharmacotherapy for patients with cystic fibrosis, and the ability of nucleotides to modulate the function of inflammatory and secretory cells in the respiratory tract must be considered.[22-24] However, these studies provide data pertinent to the potential therapeutic role of aerosolized nucleotides in the treatment of lung disease in cystic fibrosis. The maximally effective concentrations of ATP and UTP were approximately 10^{-4} M. These concentrations can be easily achieved on airway surfaces with the use of current aerosol technology.[33] The duration of action of the nucleotides in vivo indicates that therapeutic effects may be maintained in the lung for long periods. Of the nucleotides tested, it is unlikely that ATP will be the nucleotide of choice for therapy because it is rapidly converted by ectonucleotidases to products (ADP, AMP, and adenosine) that induce bronchoconstriction when inhaled by subjects with asthma.[34] Nonhydrolyzable ATP compounds, such as ATPγS, are effective chloride secretagogues in vitro[10] and may provide another approach. Alternatively, it

may be preferable to use pyrimidine compounds, such as UTP, that may yield inactive products after degradation. Finally, activation of chloride channels by nucleotides in the absence of amiloride pretreatment did not induce acute chloride secretion in human-airway epithelia. Because nucleotide therapy in cystic fibrosis would be designed to initiate the secretion of chloride (and water) in the direction of the lumen, thus enhancing hydration of the airway surface, a combination of a sodium-channel blocker and a nucleotide will probably be required for therapy.

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