Polarized Signaling via Purinoceptors in Normal and Cystic Fibrosis Airway Epithelia

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ABSTRACT Airway epithelia are confronted with distinct signals emanating from the luminal and/or serosal environments. This study tested whether airway epithelia exhibit polarized intracellular free calcium (Ca$^{2+}$) and anion secretory responses to 5′ triphosphate nucleotides (ATP/UTP), which may be released across both barriers of these epithelia. In both normal and cystic fibrosis (CF) airway epithelia, mucosal exposure to ATP/UTP increased Ca$^{2+}$, and anion secretion, but both responses were greater in magnitude for CF epithelia. In CF epithelia, the mucosal nucleotide-induced response was mediated exclusively via Ca$^{2+}$, interacting with a Ca$^{2+}$-activated Cl$^{-}$ channel (CaCC). In normal airway epithelia (but not CF), nucleotides stimulated a component of anion secretion via a chelerythrine-sensitive, Ca$^{2+}$-independent PKC activation of cystic fibrosis transmembrane conductance regulator. In normal and CF airway epithelia, serosally applied ATP or UTP were equally effective in mobilizing Ca$^{2+}$. However, serosally applied nucleotides failed to induce anion transport in CF epithelia, whereas a PKC-regulated anion secretory response was detected in normal airway epithelia. We conclude that (1) in normal nasal epithelium, apical/basolateral purinergic receptor activation by ATP/UTP regulates separate Ca$^{2+}$-sensitive and Ca$^{2+}$-insensitive (PKC-mediated) anion conductances; (2) in CF airway epithelia, the mucosal ATP/UTP-dependent anion secretory response is mediated exclusively via Ca$^{2+}$; and (3) Ca$^{2+}$, regulation of the Ca$^{2+}$-sensitive anion conductance (via CaCC) is compartmentalized in both CF and normal airway epithelia, with basolaterally released Ca$^{2+}$, failing to activate CaCC in both epithelia.

KEY WORDS: cystic fibrosis transmembrane conductance regulator • purinergic receptors • triphosphate nucleotides • protein kinase C • anion secretion

INTRODUCTION

The pathogenesis of cystic fibrosis (CF)1 lung disease is complex, but likely involves abnormal regulation of the airway surface liquid. Airway surface liquid volume regulation reflects the integrated function of cystic fibrosis transmembrane conductance regulator (CFTR) as a Cl$^{-}$ channel and as a regulator of the epithelial Na$^{+}$ channel. Further, there is compelling evidence that a second, calcium-activated Cl$^{-}$ channel (CaCC) pathway exists in the apical membrane of airway epithelia (Al-Bazzaz and Jayaram, 1981; Barthelson et al., 1987; Welsh, 1987; Boucher et al., 1989; Willumsen and Boucher, 1989; Hartmann et al., 1992), which is regulated by increases in intracellular free Ca$^{2+}$ (Ca$^{2+}$i). In the airway of the CFTR$^{-/-}$ knockout mouse (Snowaert et al., 1992), the CaCC pathway is preserved, and, in some regions, upregulated (Grubb and Boucher, 1999). Similarly, in human CF airway epithelia, this Ca$^{2+}$-regulated Cl$^{-}$ conductance may also be upregulated, as initially revealed by studies with Ca$^{2+}$ ionophores (Boucher et al., 1989; Willumsen and Boucher, 1989).

Extracellular triphosphate nucleotides are released in response to local stresses in the airways and may exert autocrine/paracrine effects on ion transport (Leuba et al., 1996; Taylor et al., 1998; Homolya et al., 2000). Triphosphate nucleotides have been reported to stimulate Cl$^{-}$ secretion through non-CFTR, CaCC-dependent mechanisms (Mason et al., 1991), both in normal and CF airway epithelia (Knowles et al., 1991; Clarke and Boucher, 1992). External ATP and UTP mediate their effects in airway epithelia, at least in part, via interactions with the P2Y$_{2}$ purinergic receptor (P2Y$_{2}$-R; Parr et al., 1994). In human airway epithelia, P2Y$_{2}$-R is linked to PLC-generated inositol(1,4,5) trisphosphate (IP$_{3}$)-mediated release of Ca$^{2+}$ (Brown et al., 1991). However, the transduction pathways that link occupancy of airway P2Y$_{2}$-Rs to Cl$^{-}$ secretion are complex, and previous reports raise questions with respect to differences in the polarity of nucleotide responses in normal airway epithelia and differences in the pattern of nucleotide responses exhibited by normal and CF airway epithelia (Mason et al., 1991; Clarke and Boucher, 1992). The following three examples, juxtaposing independent ob-

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1Abbreviations used in this paper: CaCC, Ca$^{2+}$-activated Cl$^{-}$ channel; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; Ca$^{2+}$i, intracellular free Ca$^{2+}$; DAG, diacylglycerol; IP$_{3}$, inositol 1,4,5-trisphosphate; P2Y$_{2}$-R, P2Y$_{2}$ purinergic receptor.
servation from electrophysiologic (Cl− secretion) and imaging studies (ΔCa^{2+}), illustrate these questions.

First, whereas activation of apical P2Y_{2}-Rs by nucleotides evoked smaller changes in Ca^{2+} than basolateral P2Y_{2}-R activation (Paradiso et al., 1995), experiments by Clarke and Boucher (1992) showed that the relationship for Cl− secretion was inverse, i.e., the magnitude of the Cl− secretory response was greater after apical than after basolateral additions of extracellular nucleotides. These studies raised the issue of whether the membrane location (apical/basolateral) of nucleotide-induced Ca^{2+} release, and hence local Ca^{2+} concentration, are important for Cl− secretion in airway epithelia. Second, in cultured normal airway epithelial cells prestimulated with ionomycin to maximally raise Ca^{2+} activity, treatment with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin evoked no additional Cl− secretion, whereas luminal ATP activated an additional Cl− secretory response with a further rise in Ca^{2+} (Stutts et al., 1994). This finding suggested the possibility that extracellular nucleotides regulate Cl− secretion via Ca^{2+}-independent pathways (e.g., PKC) and, perhaps, via a different Cl− channel (e.g., CFTR).

Third, in normal human airways, basolateral application of ATP induced a Cl− secretory response, whereas this response could not be detected in CF (Clarke and Boucher, 1992) despite similar changes in Ca^{2+} (Mason et al., 1991), raising the possibility of compartmentalization of Ca^{2+} and Cl− signaling in columnar airway epithelial cells (Paradiso, 1997).

In the present study, we sought to test the relative roles of polarized Ca^{2+}, mobilization and PKC activation in response to mucosal versus serosal nucleotide administration in normal and CF airway epithelia. We specifically explored the hypotheses that (1) in normal airway epithelia, luminal addition of nucleotides activates both CaCC and CFTR, whereas basolateral addition activates only CFTR; (2) in CF airway epithelia, apical P2Y_{2}-R activation is effective in activating only CaCC; and (3) Ca^{2+} signals evoked by serosal nucleotides in both normal and CF are functionally confined to the basolateral domain. For these experiments, we developed the necessary measurement systems that permitted simultaneous measurements of Ca^{2+}, and anion secretion.

Materials and Methods

Tissue Samples

Nasal epithelial cells were obtained from 10 normal subjects (34 ± 7 yr old [four males and six females]) undergoing elective surgery for standard medical indications (e.g., sleep apnea secondary to nasal obstruction), and eight CF patients (16 ± 5 yr old [four males and four females]). All procedures were approved by the University of North Carolina Committee for the Rights of Human Subjects.
the atmosphere. Both half-chambers have inlet and outlet ports for solution flow. When mounted and tightly sealed, the monolayer is ~1.5 mm from the glass coverslip. For passing current, two circular Ag/AgCl electrodes are placed in the two half-chambers to secure a uniform density of current through the preparation. For measurements of the \( V_t \), polyethylene bridges contain- ing 2 M KCl in 2% agar are positioned in the two half-chambers and connected to calomel electrodes (Radiometer).

The chamber system also has a perfusion incubator with eight mucosal and eight serosal reservoirs containing Ringer solution, which are uniformly prewarmed to ~42°C with a small heater and fan. The prewarmed Ringer solutions are delivered to the cham- ber by gravity flow (rate = 3–5 ml/min) through tubing that is kept as short as possible (~20 cm) to minimize heat loss. Serosal and mucosal solutions are changed by using two eight-port valves (Hamilton). The half-time for solution exchange is <3 s.

For bioelectric measurements, \( V_t \) was monitored by a voltage clamp/pulse generator (model VCC600; Physiologic Instruments) and the signal was recorded on a two-channel recorder (model L2005; Linseis). All experiments were performed under open circuit conditions. To calculate the equivalent current from changes in \( V_t \) in response to purinergic receptor activation, a defined (1 or 2 μA) 1-s current pulse was delivered across the tissue every 5 s (see Fig. 1), and from the magnitude change in the deflection of \( V_t \), the chord resistances, and subsequent equivalent currents, were calculated using Ohm’s law. To convert the tissue from its native Na\(^+\) absorptive state and increase the transepiphe- lial Cl\(^-\) driving force, nasal monolayers were exposed to Na\(^+\)- free/low Cl\(^-\) Ringer in the mucosal bath with KBR remaining constant in the serosal perfusate.

**Fluorimeter and Measurements of Ca\(^{2+}\)**

Measurements of Ca\(^{2+}\), in monolayers were obtained using a RadioMaster fluorimeter (Photon Technology International) coupled via fiber optics to the microscope. Fura-2 fluorescence from 30–40 cells (spot diameter ~65 μm) was acquired alternately at 340 and 380 nm (emission > 450 nm). Excitation slit widths were minimized to reduce photodamage to cells and bleaching of the dye. At a given excitation wavelength (340 or 380 nm), background light levels were measured by exposing cells to digitonin (15 μM) and MnCl\(_2\) (10^-3 M) and subtracted from the corre- sponding signal measured in Fura-2-loaded cells before taking the ratio (340/380). The corrected ratio was converted to Ca\(^{2+}\), by using external Ca\(^{2+}\) standards as described previously (Para- diso et al., 1995).

**Data Analysis**

Where applicable, data are presented as the mean ± SEM for a given experimental condition. All of the data presented in summary form are expressed as the absolute change (Δ) in Ca\(^{2+}\), and anion secretion (peak basal values) before and after the addition of ATP/UTP to monolayers. Negative equivalent currents refer to the luminal side of the monolayer, negative with respect to the serosal side (Clarke and Boucher, 1992). The mean effective dose (ED\(_{50}\)) was calculated from the Boltzman model (Willard et al., 1974). Statistical significance was determined using the \( t \) test, with \( P < 0.05 \) being considered significant.

**R E S U L T S**

**Simultaneous Measurements of Cell Ca\(^{2+}\) and Anion Transport in Nasal Monolayers**

To compare the effects of apical versus basolateral trisphosphate nucleotides on changes in Ca\(^{2+}\), and an-
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have shown that ATP (Clarke and Boucher, 1992) or ionomycin (Willumsen and Boucher, 1989) elicited no significant changes in shunt resistance. Thus, the change from basal Vₑ evoked by triphosphate nucleotides likely reflects changes in the electrical diffusion potential dominated by transcellular Cl⁻ secretion as previously reported (Lazarowski et al., 1997). However, we cannot rule out other anions secreted during purinergic receptor activation in human airway epithelia. For example, HCO₃⁻ has been implicated as a relevant anion during transepithelial ion transport (Illek et al., 1997; Clarke and Harline, 1998). Moreover, CFTR has a
finite, but small, permselectivity for $\text{HCO}_3^-$ (Linsdell et al., 1999), and CaCC has been reported to conduct $\text{HCO}_3^-$ in CFTR knockout gallbladder epithelium (Clarke et al., 2000). Therefore, the more appropriate designation for the secretory pattern induced by P2Y2-R activation, in our present study, is anion secretion.

Asymmetric Responses of $\text{Ca}^{2+}$, and Anion Secretion to Serosal and Mucosal ATP

In normal airway epithelia (Fig. 1 A), serosal ATP induces an initial rapid (6–8 s) increase in $\text{Ca}^{2+}$ (spike), followed by a relaxation of $\text{Ca}^{2+}$ levels to a sustained plateau over the next 2–3 min. We have previously shown in normal nasal monolayers that the initial spike in $\text{Ca}^{2+}$, in response to serosal ATP, was entirely due to an internal $\text{Ca}^{2+}$ release that was functionally confined to the basolateral domain of cells, whereas the sustained plateau phase results from influx of $\text{Ca}^{2+}$ solely across the basolateral membrane of cells (Paradiso et al., 1995). Concomitant with these changes of $\text{Ca}^{2+}$, serosal ATP elicited changes in $V_t$, reaching peak hyperpolarizing values more slowly (i.e., $\Delta V_t$ in which the luminal side becomes more negative with respect to the serosal bath) than the $\text{Ca}^{2+}$ peak (30–60 s), then relaxing to a sustained plateau level over the next 2–4 min.

As with serosal ATP additions, mucosal ATP elicited a similar, but moderately smaller, biphasic change in $\text{Ca}^{2+}$ in normal airway epithelia (Fig. 1 A; also see Fig. 2). We have previously demonstrated (Paradiso et al., 1995) that the initial spike of $\text{Ca}^{2+}$, in response to a mucosal ATP challenge results from internal $\text{Ca}^{2+}$ release from stores in the apical domain of cells, and the sustained plateau results from $\text{Ca}^{2+}$ influx solely across the apical membrane of these cells. However, in contrast to the pattern of $V_t$ changes noted with serosal ATP, mucosally applied ATP induced a larger hyperpolarizing change in $V_t$, a more rapid rise to peak values (15–30 s), and sustained levels that were higher (see Fig. 2) than those detected for serosal application of the triphosphosphate nucleotide.

For CF nasal epithelium, ATP added to the basolateral surface of cells elicited an identical pattern of change of $\text{Ca}^{2+}$, as was noted with serosal application of ATP in the normal airway epithelium (Fig. 1 B; also see Fig. 2). However, rather than eliciting a hyperpolarization of $V_t$, the serosal addition of ATP resulted in a small depolarization of $V_t$ (i.e., $\Delta V_t$ in which the luminal side becomes more positive with respect to the serosal bath) in CF (Fig. 1 B). Mucosal administration of ATP resulted in a markedly larger (Figs. 1 and 2), biphasic change of $\text{Ca}^{2+}$ in CF compared with normal nasal cells. These changes in $\text{Ca}^{2+}$ were associated with a very rapid (8–10 s) hyperpolarization of $V_t$, reaching higher initial (relative to normal nasal) peak values within 10–15 s, before relaxing to sustained levels over the next 1–2 min.

Fig. 2 summarizes the dose–response relationships between $\text{Ca}^{2+}$ and anion transport induced by mucosal (Fig. 2 A) or serosal (Fig. 2 B) ATP in normal and CF human airway epithelia. Mucosal addition of ATP (Fig. 2 A) was more efficacious in eliciting both changes in $\text{Ca}^{2+}$ and anion secretion in CF as compared with normal airway epithelium. For both normal and CF, maximal responses of $\text{Ca}^{2+}$ and anion secretion were obtained at $10^{-5}$–$10^{-4}$ M. In both tissues, ATP was equipotent for $\Delta \text{Ca}^{2+}$, and $\Delta$ anion secretion: for the normal airway, the $\text{ED}_{50}$ for $\text{Ca}^{2+}$ mobilization and anion transport were $1.04 \times 10^{-6}$ M and $1.02 \times 10^{-6}$ M, respectively; for CF preparations, the $\text{ED}_{50}$ for $\text{Ca}^{2+}$ and anion transport were $0.69 \times 10^{-6}$ M and $0.78 \times 10^{-6}$ M, respectively.

Several key observations in normal and CF tissues were revealed by serosal administration of ATP (Figs. 1 and 2). First, in contrast to mucosal ATP, serosally applied ATP was equally effective in mobilizing $\text{Ca}^{2+}$ in normal and CF tissues, with the maximal efficacy obtained at $10^{-5}$–$10^{-4}$ M. In terms of potencies, the $\text{ED}_{50}$ for $\text{Ca}^{2+}$ in normal and CF were $1.44 \times 10^{-6}$ M and $1.58 \times 10^{-6}$ M, respectively. These potency values of nucleotide-mobilized $\text{Ca}^{2+}$ were not distinguishable from values obtained for mucosal administration of ATP determined in normal and CF airway epithelia. Second, in normal airway epithelia, the anion secretory response to mucosal ATP was greater than after serosal ATP addition, despite the larger $\Delta \text{Ca}^{2+}$, for serosal compared with mucosal ATP administration. Finally, in stark contrast to normal nasal tissue, serosally applied ATP failed to induce anion secretion in CF airway tissue.

Effects of UTP on Changes in $\text{Ca}^{2+}$, and Anion Secretion in Airway Epithelia

Because ATP can be hydrolyzed to other agonists (e.g., adenosine) that modulate anion secretion via cAMP-dependent regulation of CFTR (Lazarowski et al., 1992), we next examined changes in $\text{Ca}^{2+}$, and anion transport to UTP. Note that UTP is a potent P2Y2-R agonist (Mason et al., 1991) and its hydrolysis product, uridine, does not activate adenosine receptors present in airway epithelia (Lazarowski et al., 1992). Because of the limited availability of CF tissues, only a single dose of UTP (100 μM) was tested.

As presented in Fig. 3, mucosal/serosal administration of UTP elicited the same asymmetric pattern of responses in cell $\text{Ca}^{2+}$ and anion secretion as ATP in normal and CF airway epithelia. Mucosal additions of UTP (Fig. 3 A) were effective in mobilizing $\text{Ca}^{2+}$, and increasing anion transport in normal and CF airway preparations, but both responses were again of greater magnitude in CF. Serosal administration of UTP was again effective in raising $\text{Ca}^{2+}$ to the same extent in normal and CF airways (Fig. 3 B). Like the ATP-induced anion
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**Figure 4.** Effects of \( \text{H}_2\text{DIDS} \) and UTP applied to the mucosal (A) or serosal (B) compartments on the transepithelial potential difference \( (V_t) \) and intracellular \( \text{Ca}^{2+} \) (\( \text{Ca}^{2+}_i \)) in normal and cystic fibrosis (CF) nasal monolayers. For these protocols, monolayers were perfused serosally with Kreb's bicarbonate Ringer and mucosally with Na⁺-free/low Cl⁻ Ringer before adding \( \text{H}_2\text{DIDS} \) (1.5 mM) to the mucosal perfusate as indicated in the traces. Serosal (S) or mucosal (M) UTP (100 \( \mu \mathrm{M} \)) was applied to the apical or basolateral membrane as shown. Each trace is representative of four or more independent experiments (three different individuals).

**Figure 5.** Summary data on anion secretion and intracellular \( \text{Ca}^{2+} \) (\( \text{Ca}^{2+}_i \)) in normal and cystic fibrosis (CF) nasal epithelia pretreated with luminal \( \text{H}_2\text{DIDS} \) (1.5 mM) and exposed to a single concentration (100 \( \mu \mathrm{M} \)) of mucosal (A) or serosal (B) UTP. Data are included (from Fig. 3) for tissues not treated with \( \text{H}_2\text{DIDS} \) for comparison with tissues treated with the disulfonic stilbene. Values for \( \text{H}_2\text{DIDS} \)-treated tissues and tissues not treated with \( \text{H}_2\text{DIDS} \) are significantly different (\(*P < 0.01\)) or otherwise not different (\(P > 0.05\); symbol not shown) from each other. For \( \text{H}_2\text{DIDS} \)-treated tissue, each bar is representative of four or more independent experiments (three different individuals).
responses in normal airway epithelium, the anion secretory response upon serosal UTP administration was smaller than after mucosal addition, despite the larger change in Ca$^{2+}$i (Fig. 3). Again, in contrast to normal nasal tissues, serosal UTP failed to evoke anion secretion in CF tissues (Fig. 3 B).

As noted above, because of the limited availability of tissues, only a single dose of UTP was tested on CF airway epithelia. However, in experiments performed in normal airway, mucosal/serosal UTP ($10^{-9}$–$10^{-4}$ M; $n = 5$ per dose; three or more individuals) was equipotent with ATP for $\Delta$Ca$^{2+}$i and $\Delta$ anion secretion. For mucosal addition of UTP, the ED$_{50}$ for Ca$^{2+}$i mobilization and anion transport was $0.98 \times 10^{-6}$ M and $1.06 \times 10^{-6}$ M, respectively; for serosal application of UTP, the ED$_{50}$ for Ca$^{2+}$i and anion transport was $1.12 \times 10^{-6}$ M and $1.08 \times 10^{-6}$ M, respectively.

Protocols Using H$_2$DIDS to Block the Ca$^{2+}$-activated Anion Conductance

Because CFTR-mediated Cl$^{-}$ secretion in normal airway is resistant to inhibition by disulfonic stilbene derivatives (e.g., DIDS), whereas Ca$^{2+}$-mediated Cl$^{-}$ transport is blocked by these drugs (Stutts et al., 1994), we next examined the contribution of CFTR to UTP-induced anion secretion when CaCC was inhibited by H$_2$DIDS. Representative traces shown in Fig. 4 depict the responses of Ca$^{2+}$i and anion transport activities in normal and CF nasal epithelial monolayers treated first with mucosal H$_2$DIDS (1.5 mM) before UTP (100 nM) additions. In the normal human airway, H$_2$DIDS markedly inhibited anion secretion (~75%) in response to mucosal UTP, and H$_2$DIDS abolished anion transport activity in the CF airway tissue exposed to mucosal UTP (Figs. 4 A and 5). In contrast to mucosal UTP addition, pretreatment of normal nasal tissues with H$_2$DIDS had no inhibitory effects on anion secretory responses to serosal UTP (Figs. 4 B and 5). In CF airway epithelium, serosal UTP elicited a depolarization in $V_t$ in the presence of H$_2$DIDS, similar to that detected in H$_2$DIDS-free experiments (Fig. 4 B). Importantly, mucosal H$_2$DIDS did not block changes in Ca$^{2+}$i in response to mucosal or serosal UTP in either normal or CF nasal tissues (Fig. 4), suggesting that the inhibition of anion secretion with mucosal UTP in both normal and CF nasal tissues was not due to inhibition of P2Y$_R$-R-dependent Ca$^{2+}$i signals. The data derived from these studies are summarized and compared with the data generated in tissues that were not pretreated with H$_2$DIDS (Fig. 5).

Effects of ATP/UTP on Anion Transport in Ca$^{2+}$i-clamped Airway Epithelial Cells

The residual anion secretory response in normal airway epithelia resulting from mucosal UTP addition after 

[Figure 6. Separation between intracellular Ca$^{2+}$ (Ca$^{2+}$i) and the transepithelial potential difference ($V_t$) in response to ATP in normal (A and B) and cystic fibrosis (CF; C) nasal epithelia. For these protocols, monolayers were perfused serosally with Krebs's bicarbonate Ringer and mucosally with Na$^+$-free, low Cl$^{-}$ Ringer before adding 300 nM ionomycin (Iono) and 500 nM thapsigargin (Tg) to the mucosal perfusate as indicated in the traces. Serosal (S) or mucosal (M) ATP (100 µM) was applied to the basolateral or apical membrane as shown. Each trace is representative of four or more independent experiments (three different individuals).]
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(Fig. 6, A and B) and CF (Fig. 6 C) nasal monolayers were first exposed to mucosal ionomycin (300 nM), which, in the presence of symmetrical extracellular Ca\textsuperscript{2+} (1.3 mM), resulted in a maximal increase of Ca\textsuperscript{2+}\textsubscript{i}; subsequent addition of serosal ionomycin (300 nM; not shown) or mucosal thapsigargin (500 nM; Fig. 6) caused no additional change in V\textsubscript{i} and cell Ca\textsuperscript{2+}. We next tested the effects of unilaterally applied triphosphate nucleotides on changes of Ca\textsuperscript{2+}\textsubscript{i} and V\textsubscript{i}. In the normal airway, serosal (Fig. 6 A) and mucosal (Fig. 6 B) ATP (100 μM) induced an additional increase in V\textsubscript{i} without an apparent additional change in Ca\textsuperscript{2+}. In contrast to normal nasal tissues, neither serosal nor mucosal administration of ATP elicited changes in V\textsubscript{i} in CF airway epithelium (Fig. 6 C). The summary data derived from these protocols using ATP or UTP as the nucleotide agonist were analyzed sequentially, first, for the effects of ionomycin alone on Ca\textsuperscript{2+}\textsubscript{i} and anion secretion (see Fig. 7) and, second, for the effects of mucosal and serosal ATP/UTP on Ca\textsuperscript{2+}\textsubscript{i} and anion transport (see Fig. 8) after pretreatment with ionomycin/thapsigargin in normal and CF airway preparations.

As shown in Fig. 7, Ca\textsuperscript{2+}\textsubscript{i} increased to the same extent in normal and CF airway epithelia in response to ionomycin (Fig. 7 A). However, in contrast to ionomycin-induced changes in Ca\textsuperscript{2+}\textsubscript{i}, anion secretion in response to ionomycin was of greater magnitude in CF as compared with normal nasal tissues (Fig. 7 B). The differences in ionomycin-induced changes in anion secretion between normal and CF nasal epithelia, as reported here, are consistent with previous reports of upregulation of ionomycin-stimulated anion secretion in cultured human CF nasal epithelium (Johnson et al., 1995).

The ATP/UTP summary data are shown in Fig. 8. Under conditions in which the cell Ca\textsuperscript{2+} was clamped at high levels with ionomycin/thapsigargin pretreatment,
In CF preparations, ionomycin (Iono; 300 nM) raised Ca\textsuperscript{2+}i, but increased Vt, in normal nasal epithelium. The responsiveness of preparations in the presence of BAPTA was tested by the response to the cAMP-mediated agonist forskolin. Mucosally applied forskolin (10 μM) evoked an additional increase in Vt in normal airway tissue (Fig. 9, A and B). In marked contrast to the normal nasal preparation, neither serosal nor mucosal ATP, nor mucosal forskolin, elicited changes in Vt in CF (Fig. 9 C). However, when monolayers were exposed bilaterally to high extracellular Ca\textsuperscript{2+} (1.3 mM) plus mucosal ionomycin (300 nM), Ca\textsuperscript{2+}i slowly increased, followed by a small anion secretory response in CF, verifying the responsiveness of the preparation. It should be noted that BAPTA is a very effective buffer of Ca\textsuperscript{2+}. To detect a change in cell Ca\textsuperscript{2+} when applied to either the mucosal (Fig. 8 A) or serosal (Fig. 8 B) compartment in normal nasal monolayers. However, the anion secretory response was of a greater magnitude to mucosal addition of triphosphate nucleotides as compared with serosal additions of the agonists. Under these same conditions, neither ATP nor UTP applied to either membrane (apical/basolateral) induced an anion secretory or Ca\textsuperscript{2+}i response in CF airway cells (Fig. 8). In the second approach, normal and CF airway epithelia were exposed to BAPTA-AM to clamp Ca\textsuperscript{2+}i to low levels. To reduce Ca\textsuperscript{2+}i influx that would result from P2Y\textsubscript{2}-R activation (Paradiso et al., 1995), nasal monolayers were exposed to bilateral low Ca\textsuperscript{2+} (300 μM) solutions. As illustrated in Fig. 9, the addition of serosal (Fig. 9 A) or mucosal (Fig. 9 B) ATP (100 μM) failed to raise Ca\textsuperscript{2+}i, but increased Vt, in normal nasal epithelium. The responsiveness of preparations in the presence of BAPTA was tested by the response to the cAMP-mediated agonist forskolin. Mucosally applied forskolin (10 μM) evoked an additional increase in Vt in normal airway tissue (Fig. 9, A and B). In marked contrast to the normal nasal preparation, neither serosal nor mucosal ATP, nor mucosal forskolin, elicited changes in Vt in CF (Fig. 9 C). However, when monolayers were exposed bilaterally to high extracellular Ca\textsuperscript{2+} (1.3 mM) plus mucosal ionomycin (300 nM), Ca\textsuperscript{2+}i slowly increased, followed by a small anion secretory response in CF, verifying the responsiveness of the preparation. It should be noted that BAPTA is a very effective buffer of Ca\textsuperscript{2+}. To detect a change in cell Ca\textsuperscript{2+} when applied to either the mucosal (Fig. 8 A) or serosal (Fig. 8 B) compartment in normal nasal monolayers. However, the anion secretory response was of a greater magnitude to mucosal addition of triphosphate nucleotides as compared with serosal additions of the agonists. Under these same conditions, neither ATP nor UTP applied to either membrane (apical/basolateral) induced an anion secretory or Ca\textsuperscript{2+}i response in CF airway cells (Fig. 8). In the second approach, normal and CF airway epithelia were exposed to BAPTA-AM to clamp Ca\textsuperscript{2+}i to low levels. To reduce Ca\textsuperscript{2+}i influx that would result from P2Y\textsubscript{2}-R activation (Paradiso et al., 1995), nasal monolayers were exposed to bilateral low Ca\textsuperscript{2+} (300 μM) solutions. As illustrated in Fig. 9, the addition of serosal (Fig. 9 A) or mucosal (Fig. 9 B) ATP (100 μM) failed to

**Effects of PKC Activation and Inhibition on UTP-stimulated Anion Secretion in Ca\textsuperscript{2+}i-clamped Normal Nasal Tissue**

We have previously reported (Boucher et al., 1989) no detectable difference in total PKC activity in normal and
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CF airway, despite the fact that the PKC activator PMA had the capacity to stimulate anion transport in normal, but not CF, nasal epithelia, via PKC-dependent phosphorylation of CFTR. Based on this earlier work, and our data showing that ATP/UTP applied to either membrane (apical/basolateral) of normal airway epithelial cells induce anion secretion independently from changes in Ca\textsuperscript{2+} (Figs. 8 and 10), we tested whether a Ca\textsuperscript{2+}-independent PKC was a functional regulator of CFTR-mediated anion secretion after P2\textsubscript{Y}2-R activation. In these studies, monolayers of normal nasal epithelia were Ca\textsuperscript{2+}-clamped by BAPTA, and the effects of PKC activation or inhibition by PMA or chelerythrine, respectively, on UTP-elicited anion secretion were examined.

As shown in Fig. 11, mucosal addition of PMA (100 nM) to normal airway epithelia caused an increase in V\textsubscript{t} with no change in Ca\textsuperscript{2+} (Fig. 11 A). Importantly, subsequent exposure of nasal cells to serosal and mucosal UTP (100 μM) failed to cause an additional increase in V\textsubscript{t}, despite the fact that mucosal addition of forskolin (10 μM) markedly stimulated anion transport activity (Fig. 11 A).

At the concentration used in the study, chelerythrine chloride is a specific but broad spectrum inhibitor of PKC isozymes (Herbert et al., 1990). Therefore, we next examined whether this agent could block both the UTP- and PMA-stimulated anion transport in Ca\textsuperscript{2+}-clamped normal nasal monolayers. As shown in Fig. 11 B, pretreating nasal monolayers with chelerythrine (1 μM) completely blocked serosal and mucosal UTP-stimulated increase in anion secretion, and markedly inhibited both PMA- and forskolin-stimulated anion transport. The data generated from these studies are summarized in Fig. 11 C.

**DISCUSSION**

Purinoceptors Involved in Airway Epithelial Ion Transport Regulation

Extracellular nucleotides regulate cellular processes via interactions with cell-surface ion-gated (P2X) and G protein–coupled (P2Y) receptors. Previous studies in human airways (Paradiso et al., 1995) have identified a major role for P2Y-Rs and a little role for P2X receptors in the lumen facing (columnar) cells of the superficial epithelium of airways. Moreover, the observation that ATP and UTP were equipotent in mediating Ca\textsuperscript{2+} (Fig. 2 and associated text of Fig. 3) argues that the P2\textsubscript{Y}2-R, and not P2X-Rs, mediated the nucleotide responses on both the apical and basolateral surfaces of our airway preparations.

P2\textsubscript{Y}2 purinoceptors activate PLC\textsubscript{β} in a heterotrimeric G protein–dependent manner (Boeynaems et al., 1998) and increase Ca\textsuperscript{2+} through two distinct, but related pathways. Initially, IP\textsubscript{3} is formed from PLC\textsubscript{β} induced breakdown of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) and activates IP\textsubscript{3} channel receptors in the endoplasmic reticulum, resulting in channel opening and release of stored calcium into the cytoplasm. Subsequently, depletion of intracellular Ca\textsuperscript{2+} stores activates a pathway for Ca\textsuperscript{2+} influx across the plasma membrane,
which was originally termed “capacitative Ca\(^{2+}\) entry” or, more recently, “store-operated calcium entry” (Clapham, 1995; Putney, 1986, 1990). Both phases of Ca\(^{2+}\) mobilization can act in concert for the subsequent modulation of multiple plasma membrane transport processes, including the Ca\(^{2+}\)-activated Cl\(^{-}\) channel (i.e., CaCC) in airway epithelia.

Besides PLC-mediated changes of Ca\(^{2+}\)i, another consequence of PLC-dependent PIP2 breakdown is the formation of diacylglycerol (DAG) and its activation of PKC. PKC activation may principally regulate human airway secretion via interactions with CFTR. For example, regulation of the CFTR Cl\(^{-}\) channel by PKC has been reported in previous studies performed in a variety of epithelial cell lines expressing CFTR (Dechecchi et al., 1992, 1993; Bajnath et al., 1993; Winpenny et al., 1995; Jia et al., 1997). Moreover, studies by Picciotto and co-workers (Picciotto et al., 1992) showed that PKC phosphorylated CFTR in a Ca\(^{2+}\)-independent manner, and their findings agree with more recent studies by Liedtke and Cole (1998), who reported that Ca\(^{2+}\)-independent PKC-ε regulates cAMP-dependent stimulation of the CFTR Cl\(^{-}\) channel in Calu-3 cells, an airway epithelial cell line.

Compelling evidence has been reported that the regulation of Cl\(^{-}\) secretion in normal airway epithelia may reflect more than a simple change in Ca\(^{2+}\)i mediated by extracellular triphosphate nucleotides. For example, Hwang et al. (1996) reported that triphosphate nucleotides activated multiple types of Ca\(^{2+}\)-sensitive and Ca\(^{2+}\)-insensitive Cl\(^{-}\) conductances in rat tracheal epithelial cells. A dissociation between the regulation of Cl\(^{-}\) conductance and Ca\(^{2+}\) activity by extracellular ATP was also seen in voltage clamp studies in CFT1 cells by Stutts and co-workers (Stutts et al., 1994), suggesting multiple modes of regulation of Cl\(^{-}\) transport rates by extracellular ATP. However, these earlier studies used separate experimental systems for monitoring ion transport activities and for measurements of Ca\(^{2+}\)i (either directly or indirectly) in airway epithelial preparations.

To better quantify the role of intracellular Ca\(^{2+}\) in modulating anion transport activity in polarized tissues, we used a unique experimental technique that allowed us to measure simultaneous changes of Ca\(^{2+}\)i and anion secretion in polarized normal and CF human airway epithelia (Fig. 1). Because the results of these studies were extensive and complex, we have elected to present them with respect to nucleotide addition selectively at each barrier, starting with the simplest system (i.e., CF) in which CFTR function is absent. We then present data in the more complex normal airway epithelium.

**Apical P2Y\(_2\)-R Regulation of Anion Secretion in CF Airway Epithelia**

The large anion secretory responses of CF tissues to mucosal ATP/UTP (Figs. 2 and 3) are consistent with previous results seen in CF airway epithelia (Clarke and Boucher, 1992). The Cl\(^{-}\) secretory response in cultured CF airway epithelia in response to ionomycin or ATP was shown directly by microelectrode studies to arise principally from activation of a Cl\(^{-}\) conductance (Figs. 2 and 7) in the apical membrane (Willumsen and Boucher, 1989; Clarke and Boucher, 1992).

A comprehensive set of protocols was developed to directly test the linkage between apical (versus basolateral) nucleotide–induced Ca\(^{2+}\)i and anion secretion. Contrasting maneuvers were used to clamp Ca\(^{2+}\)i at different levels in CF nasal tissues. The first approach involved maximally elevating Ca\(^{2+}\)i by the addition of ion-
omycin, followed by thapsigargin. CF nasal tissue pre-treated with these agents exhibited no additional change in Ca\textsuperscript{2+}, in response to apically or basolaterally applied ATP/UTP, and ATP/UTP failed to increase the anion transport rate (Figs. 6 and 8). In the second approach, nasal monolayers were pretreated with BAPTA-AM to clamp Ca\textsuperscript{2+} to low levels. These experiments revealed that the addition of mucosal ATP/UTP to BAPTA-treated CF cells caused no change in Ca\textsuperscript{2+}, and again failed to increase anion secretion in CF epithelium (Figs. 9 and 10). The data showing that Ca\textsuperscript{2+}-clamped CF epithelium failed to elicit an anion secretory response to mucosal ATP/UTP, coupled with data that showed that anion transport induced by mucosal UTP in CF epithelium was completely inhibited by H\textsubscript{2}DIDS (Figs. 4 and 5), argue that anion secretion in CF in response to ATP/UTP is mediated exclusively via Ca\textsuperscript{2+}, regulation of CaCC.

A key observation of this study was the markedly larger Ca\textsuperscript{2+} response of CF as compared with normal tissues to mucosal ATP/UTP (Figs. 2 and 3), raising the possibility that regulation of Ca\textsuperscript{2+} metabolism is different in CF. Based on our functional data alone, it is not apparent how mucosal ATP/UTP activated a larger Ca\textsuperscript{2+} signal in CF compared with normal cells. However, in human proximal airway epithelia, we have recently performed preliminary studies using immunofluorescent confocal imaging of the endoplasmic reticulum (the site of IP\textsubscript{3}-releasable Ca\textsuperscript{2+} stores) markers, calreticulin, and IP\textsubscript{3} receptors, which show that endoplasmic reticulum Ca\textsuperscript{2+} stores are preferentially distributed to the apical domain, and that CF cells exhibit a greater expression of apical ER Ca\textsuperscript{2+} stores (Ribeiro et al., 1999).

### Basolateral P2Y\textsubscript{2}-R Regulation of Anion Secretion in CF Airway Epithelia

A central observation of this study was that basolateral activation of P2Y\textsubscript{2}-R with ATP or UTP raised Ca\textsuperscript{2+} to the same extent in both CF and normal airway epithelia, but failed to induce anion secretion in CF. This observation strongly indicates that Ca\textsuperscript{2+}, mobilized by activation of the basolateral P2Y\textsubscript{2}-R, is functionally confined to that barrier, i.e., the Ca\textsuperscript{2+} released from basolateral stores cannot activate apical Ca\textsuperscript{2+}-sensitive Cl\textsuperscript{-} channels. Our data in normal tissues imply that this compartmentalized release/regulation of Ca\textsuperscript{2+}, in response to basolateral ATP/UTP is also a feature of normal airway epithelia (Figs. 4 and 5). Previous microelectrode studies demonstrated that apical nucleotide administration, unlike basolateral nucleotide administration, did not activate basolateral Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (Clarke and Boucher, 1992), suggesting that this compartmentalization is symmetric. The mechanism for compartmentalization of Ca\textsuperscript{2+} signaling in airway epithelia is unknown, but it may involve the actions of mitochondria acting as a “Ca\textsuperscript{2+} fence” (Tinel et al., 1999) and/or Ca\textsuperscript{2+} binding proteins (Nomiya et al., 1998).

### Apical Membrane P2Y\textsubscript{2}-R Regulation of Anion Secretion in Normal Airway Epithelia

For apical P2Y\textsubscript{2}-R–regulated anion secretion in normal airway tissue, we propose that two distinct Cl\textsuperscript{-} channels (e.g., CaCC and CFTR) mediate anion efflux across the apical membrane, and that the linkage coupling activation of apical P2Y\textsubscript{2}-R to the induction of anion transport involves both Ca\textsuperscript{2+}-regulated CaCC and Ca\textsuperscript{2+}-independent PKC-regulated CFTR. We make these speculations based on the following observations. In normal human airway epithelial cells, the majority of anion secretion mediated by apical P2Y\textsubscript{2}-Rs reflects Ca\textsuperscript{2+}, regulated activation of CaCC. In support of this notion, anion transport activity was substantially blocked (~75%) by luminal H\textsubscript{2}DIDS in response to mucosal UTP (Figs. 4 and 5). Additional support for CaCC as the major efflux pathway during ATP/UTP-regulated anion secretion came from experiments that showed markedly reduced anion transport activity in Ca\textsuperscript{2+}-clamped normal airway monolayers in response to apically applied ATP/UTP (Figs. 8 and 10).

However, the observation that mucosally applied ATP/UTP-stimulated anion secretion was reduced, but not abolished, in normal airway epithelium by H\textsubscript{2}DIDS or Ca\textsuperscript{2+}-clamping agents indicates that apical P2Y\textsubscript{2}-R activation modulates multiple Cl\textsuperscript{-} channels via Ca\textsuperscript{2+} dependent (i.e., CaCC) and Ca\textsuperscript{2+}-independent signaling pathways. We propose that the Ca\textsuperscript{2+}-independent signaling pathway linking apical P2Y\textsubscript{2}-R activation to anion secretion reflects Ca\textsuperscript{2+}-independent PKC regulation of CFTR-mediated anion secretion for several reasons. First, as noted above, activation of the apical P2Y\textsubscript{2}-Rs by ATP/UTP increased anion secretory activity in a Ca\textsuperscript{2+}-independent manner (Figs. 8 and 10). Second, the PMA-stimulated anion secretion abolished UTP-regulated anion secretion (Fig. 11) in Ca\textsuperscript{2+}-clamped monolayers, suggesting that the PMA- and UTP-regulated signaling pathways occur via the same cellular mechanism (i.e., Ca\textsuperscript{2+}-independent PKC). Finally, PKC inhibition by chelerythrine completely abolished apically applied UTP-regulated anion secretion under Ca\textsuperscript{2+}-clamped conditions (Fig. 11), which is consistent with the notion that a Ca\textsuperscript{2+}-independent PKC is the intracellular second messenger relevant to CFTR-mediated anion secretion in normal airway epithelia.

### Basolateral Membrane P2Y\textsubscript{2}-R Activation of Anion Transport in Normal Airway Epithelia

For basolateral P2Y\textsubscript{2}-R–regulated secretion, we propose that (1) only a single Cl\textsuperscript{-} channel (i.e., CFTR) mediates anion efflux across the apical membrane, and (2) that the linkage between P2Y\textsubscript{2}-R activation and CFTR in-
volves regulation via PKC rather than Ca$^{2+}$i. Several observations support these hypotheses. In normal nasal epithelium, the addition of basolateral ATP/UTP consistently increased the anion secretory rate, but no changes in anion secretion were noted in CF. These data clearly point to the requirement for functional CFTR to mediate anion secretion after basolateral addition of ATP/UTP. The identification of CFTR and not CaCC as the apical membrane anion efflux pathway during basolateral P2Y$_2$-R activation is further supported by data that show that mucosal H$_2$DIDS failed to block anion secretion in normal nasal tissues induced by serosal UTP, but abolished anion secretion in response to mucosal UTP in CF (Figs. 4 and 5).

The notion that the apical membrane CFTR-mediated anion conductance is regulated by a Ca$^{2+}$-independent PKC is based on several interrelated observations derived from the Ca$^{2+}$i-clamped studies. First, as noted above, activation of the basolateral P2Y$_2$-Rs by ATP/UTP increased anion transport activity in a Ca$^{2+}$-independent manner (Figs. 8 and 10). Second, no additivity was observed between the PMA-stimulated anion secretion and basolateral UTP-regulated anion transport (Fig. 11) in Ca$^{2+}$i-clamped monolayers, again suggesting that the PMA- and UTP-regulated signaling pathways occur via the same cellular mechanism, i.e., Ca$^{2+}$-independent PKC. Finally, PKC inhibition by chelerythrine completely abolished serosally applied UTP-regulated anion secretion under Ca$^{2+}$i clamp conditions (Fig. 11), which is again consistent with the notion that a Ca$^{2+}$--independent PKC is the intracellular messenger relevant to CFTR-mediated anion secretion in normal human airway epithelia.

**Ion Transport Model**

In summary, the model depicted in Fig. 12 shows that, in normal epithelium, basolateral P2Y$_2$-R activation couples to apical anion secretion through two related pathways. First, the Ca$^{2+}$i signal resulting from PLC-generated IP$_3$ activates Ca$^{2+}$i-dependent K$^+$ channels on the basolateral membrane, promoting membrane hyperpolarization and generation of a loop current responsible for CFTR-mediated anion secretion. Second, PLC-generated DAG activates a Ca$^{2+}$-independent PKC which, directly or indirectly, activates CFTR-dependent anion transport. In this model, PKC (or one of its targets) translocates from the basolateral to the apical domain where it can modulate CFTR function, whereas the Ca$^{2+}$i signal generated by basolateral nucleotide application is restricted to the basolateral compartment. In contrast, apical P2Y$_2$-R activation in normal epithelium increases anion secretion as a result of Ca$^{2+}$-dependent activation of CaCC as well as CFTR regulation by a Ca$^{2+}$-independent PKC.

In CF epithelium, although basolateral P2Y$_2$-R activation stimulates PLC to the same extent as in normal ep-
imelium, Ca\(^{2+}\)-activated K\(^+\) channel–dependent membrane hyperpolarization and DAG-activated PKC do not generate anion secretion because of the lack of functional CFTR expression at the apical barrier. However, apical P2Y\(_2\)-R activation in CF results in a large Ca\(^{2+}\) \(^{2+}\) mobilization, accounting for the large CaCC-mediated anion secretion compared with normal epithelium. Similar to basolateral P2Y\(_2\)-R–dependent signal transduction, apical receptor stimulation–activated PKC does not couple to anion transport in CF because of the absence of functional CFTR.

**Role of Apical and Basolateral P2Y\(_2\)-R in Airway Epithelial Function**

The protocols using Na\(^+\)-free/low Cl\(^-\) luminal solutions were designed to allow us to use anion secretion as a sensitive read-out of P2Y\(_2\)-R–mediated signal transduction at apical and basolateral barriers. This strategy allowed us to discover that airway epithelia appear to be able to functionally confine Ca\(^{2+}\)-regulated signaling to the barrier ipsilateral to receptor stimulation, whereas other pathways (e.g., PKC) are not. Thus, both normal and CF airways may be able to respond selectively to nucleotide (and perhaps other agonists) stimulation at the apical or basolateral barriers.

These data also could have implications for regulation of net transepithelial ion transport rates. Although not explored in this study, extracellular nucleotides inhibit epithelial Na\(^+\) channel (Devor and Pilewski, 1999) and limit Na\(^+\) absorption in airway epithelia. Under these conditions, driving forces for Cl\(^-\) secretion across epithelia exist. In normal airways, anion transport resulting from P2Y\(_2\)-R stimulation at either barrier, via regulation of CFTR and/or CaCC, could play a vital role in the composition, pH (e.g., secreted HCO\(_3\)\(^-\)), and depth of airway surface liquids, facilitating maintenance of the mucociliary clearance mechanism and, thus, airway homeostasis. On the other hand, in CF airways, there is a predicted failure to respond to nucleotides released in the vicinity of the basolateral barrier, e.g., from inflammatory cells or autocrine release. The failure to respond could limit the ability of the CF epithelium to respond to submucosal stresses. Conversely, although CaCC, under resting circumstances, appears to be inactive, the large Ca\(^{2+}\)-releasable stores and apparent upregulation of CaCC make activation of the pathway to initiate anion secretion to restore volume on airway surfaces by luminal nucleotides an attractive therapeutic strategy.

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