

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+}_i) 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+}_i 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+}_i 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+}_i . 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+}_i ; and (3) Ca^{2+}_i regulation of the Ca^{2+} -sensitive anion conductance (via CaCC) is compartmentalized in both CF and normal airway epithelia, with basolaterally released Ca^{2+}_i 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 (Snouwaert 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+}_i -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 ($\text{P2Y}_2\text{-R}$; Parr et al., 1994). In human airway epithelia, $\text{P2Y}_2\text{-R}$ is linked to PLC-generated inositol(1,4,5) trisphosphate (IP_3)-mediated release of Ca^{2+}_i (Brown et al., 1991). However, the transduction pathways that link occupancy of airway $\text{P2Y}_2\text{-Rs}$ 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; $\text{P2Y}_2\text{-R}$, P2Y_2 purinergic receptor.

servations from electrophysiologic (Cl^- secretion) and imaging studies (ΔCa^{2+}_i), illustrate these questions.

First, whereas activation of apical P2Y_2 -Rs by nucleotides evoked smaller changes in Ca^{2+}_i 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+}_i concentration, are important for Cl^- secretion in airway epithelia. Second, in cultured normal airway epithelial cells prestimulated with ionomycin to maximally raise Ca^{2+}_i 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 without a further rise in Ca^{2+}_i (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+}_i (Mason et al., 1991), raising the possibility of compartmentalization of Ca^{2+}_i signaling in columnar airway epithelial cells (Paradiso, 1997).

In the present study, we sought to test the relative roles of polarized Ca^{2+}_i 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+}_i 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+}_i 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.

Chemicals and Solutions

Acetoxymethyl ester (AM) of Fura-2, 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid (H_2DIDS), and BAPTA were purchased from Molecular Probes. Chelerythrine chloride and PMA were purchased from Alexis Biochemical. ATP and UTP were obtained from Boehringer Mannheim Biochemical. Ionomycin and thapsigargin were obtained from Calbiochem-Novabiochem. All other chemicals were obtained from Sigma-Aldrich.

Stock solutions of Fura-2/AM, BAPTA-AM, ionomycin, PMA, chelerythrine, and thapsigargin, all at 1 mM, were dissolved in DMSO and stored up to 30 d at -20°C without a loss of potency of the drugs. Stock solutions of *N*-methyl-D-glucamine (NMG) gluconate and NMG-Cl (both at 0.5 M, pH 7.0) were prepared by mixing NMG base with equimolar concentrations of D-gluconic acid lactone and HCl, respectively, and stored up to 1 wk at 4°C . For Na^+ -free, HCO_3^- -containing solutions, a stock solution of NMG- HCO_3^- (1 M, pH 7.0) was prepared by bubbling NMG base (1 M) with 100% CO_2 .

The standard Krebs's bicarbonate Ringer (KBR) solution contained (in mM): 125 NaCl, 2.5 K_2HPO_4 , 1.3 CaCl_2 , 1.3 MgCl_2 , 25 NaHCO_3 , and 5 D-glucose (5% $\text{CO}_2/95\%$ O_2). For NMG-Cl Ringer solution, NaCl and NaHCO_3 were replaced mole for mole by NMG-Cl and NMG- HCO_3^- , respectively. For NMG-gluconate (low Cl^-) Ringer, NaCl and NaHCO_3 were replaced mole for mole with NMG-gluconate and NMG- HCO_3^- , respectively (final $\text{Cl}^- = 2.6$ mM) and 2 mM CaSO_4 was added to the solution to compensate for Ca^{2+} chelation by gluconate, as previously reported (Clarke and Boucher, 1992).

Cell Culture, Perfusion Chamber, and Bioelectric Measurements

Nasal epithelial cells were harvested from polyps by enzymatic digestion (Protease XIV [Sigma-Aldrich] for 24–48 h at 4°C) as previously described (Wu et al., 1985). Nasal epithelial cells were plated on porous Transwell Col filters (pore diam = 0.4 μm ; Costar) affixed to O-rings and maintained in serum-free Ham's F12 medium supplemented with insulin (10 $\mu\text{g}/\text{ml}$), transferrin (5 $\mu\text{g}/\text{ml}$), 3,3',5-triiodothyronine (3×10^{-8} M), endothelial cell growth supplement (3.75 $\mu\text{g}/\text{ml}$), hydrocortisone (5×10^{-9} M), and CaCl_2 (10^{-3} M). Polarized monolayers were studied 7–12 d after seeding, a time coincident for the development of the maximal transepithelial potential difference (V_t). For normal nasal epithelium $V_t = -9.8 \pm 1.2$ mV mucosal side negative and chord resistance (R_T) = $408 \pm 32 \Omega\text{-cm}^2$ ($n = 90$; 10 different individuals); for CF $V_t = -21.7 \pm 1.4$ mV, and $R_T = 396 \pm 38 \Omega\text{-cm}^2$ ($n = 85$; 8 different individuals).

After confluency was achieved, polarized monolayers of normal or CF epithelium were loaded with Fura-2 (5 μM at 37°C for 25 min) or coloaded with 100 μM BAPTA-AM $\pm 1 \mu\text{M}$ chelerythrine chloride before being mounted in a miniature Ussing chamber over an objective (Zeiss LD Achroplan 40 \times , NA 0.6; working distance 1.8 mm) of a Zeiss Axiovert 35 microscope. The chamber is similar in design to that described by Larsen et al. (1990) for sweat duct epithelial cells in primary culture. In brief, a perfusion chamber was constructed in which solutions bathing apical and basolateral surfaces could be changed rapidly and independently. The chamber consists of top (mucosal) and bottom (serosal) half-chambers (volume = 250 μl each) made from light-absorbing polyacetal. The Transwell wafer containing the cell monolayer was mounted apical surface up, between the two half-chambers. Effective sealing is achieved by means of rubber O-rings embedded in grooves of the two half-chambers, which are screwed tightly together. A glass coverslip is affixed to the bottom of the serosal chamber with dental sticking wax (model Deiberit-502; Ludwig Bohme). The mucosal chamber is open to

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 containing 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 $\sim 42^\circ\text{C}$ with a small heater and fan. The prewarmed Ringer solutions are delivered to the chamber 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 transepithelial 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+}_i

Measurements of Ca^{2+}_i 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 corresponding signal measured in Fura-2-loaded cells before taking the ratio (340/380). The corrected ratio was converted to Ca^{2+}_i by using external Ca^{2+} standards as described previously (Paradiso et al., 1995).

Data Analysis

Where applicable, data are presented as the mean \pm SEM for a given experimental condition. All of the data presented in summary form are expressed as the absolute change (Δ) in Ca^{2+}_i 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.

RESULTS

Simultaneous Measurements of Cell Ca^{2+}_i and Anion Transport in Nasal Monolayers

To compare the effects of apical versus basolateral triphosphate nucleotides on changes in Ca^{2+}_i and an-

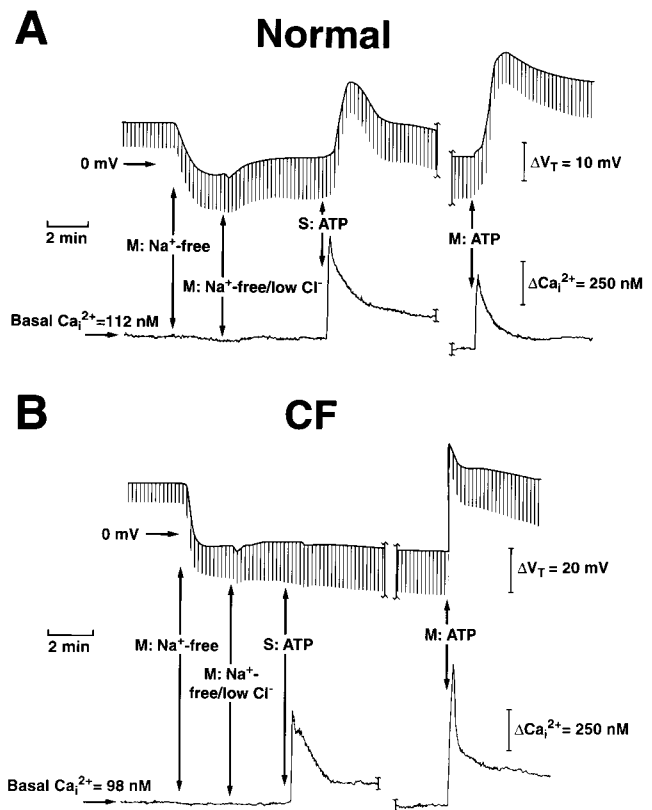


FIGURE 1. Simultaneous measurements of intracellular Ca^{2+}_i (Ca^{2+}_i) and transepithelial potential difference (V_t) in normal (A) and cystic fibrosis (CF; B) nasal epithelia. Monolayers of nasal epithelial cells were initially perfused bilaterally in Krebs' bicarbonate Ringer before changing to mucosal Na^+ -free (*N*-methyl-D-glucamine [NMG] Cl^-), and then Na^+ -free/low Cl^- (2.6 mM; NMG gluconate) Ringer. 100 μM ATP was added to the serosal (S) or mucosal (M) bath as indicated. The break in the traces represents a washout period (~ 30 min) for serosal ATP before adding mucosal ATP to the perfusate. At 100 μM ATP, each trace is representative of at least 85 independent experiments (eight or more different individuals).

ion secretion, we chose experimental conditions that maximized the signal generated by anion movement through the apical membrane by bathing the preparations with Na^+ -free, low Cl^- Ringer. Representative tracings depicting simultaneous measurements of anion secretion and changes in Ca^{2+}_i induced by serosal or mucosal administration of ATP (100 μM) in normal and CF epithelial cell preparations are shown in Fig. 1 (A and B). The downward deflections shown in each tracing (Fig. 1, A and B) were produced by passing a current pulse across the tissue as described in MATERIALS AND METHODS, and the break in the tracings represents a 30-min washout period after serosal ATP treatment before exposing monolayers to mucosal ATP.

Under these conditions, there exist chemical gradients across the intercellular shunt for Na^+ and Cl^- . These gradients generate electromotive forces that contribute to basal V_t , but previous equivalent circuit analy-

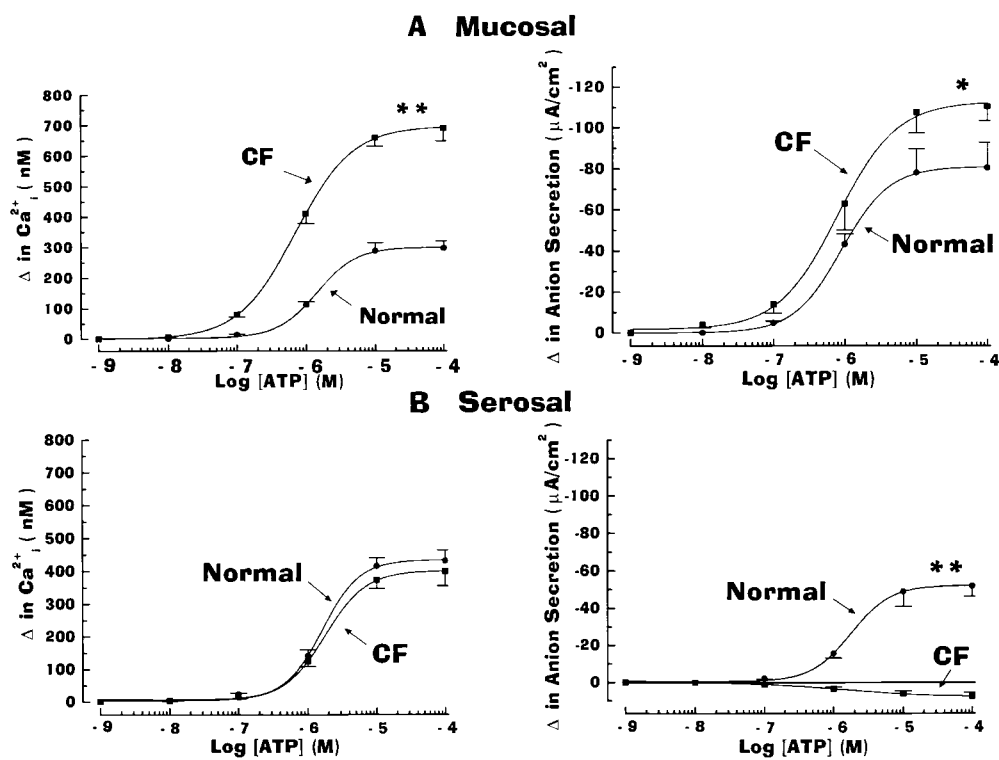


FIGURE 2. Dose-response relationship for the effects of ATP on the change in anion secretion and intracellular Ca^{2+} (Ca^{2+}_i) in normal and cystic fibrosis (CF) nasal epithelia. In monolayers perfused in serosal Krebs bicarbonate Ringer and luminal Na^+ -free/low Cl^- Ringer, ATP (10^{-9} – 10^{-4} M) was added to either mucosal (A) or serosal (B) bathing solution. The change in anion transport and Ca^{2+}_i was calculated as the absolute difference between maximal ATP-stimulated anion transport and Ca^{2+}_i and steady-state equivalent current and Ca^{2+}_i before ATP addition. Each data point represents mean \pm SEM for at least six independent experiments (six or more different individuals). Normal and CF values are significantly different (* $P < 0.05$ and ** $P < 0.01$) from each other. For basolateral changes

in Ca^{2+}_i , values were not significantly different ($P > 0.05$; symbol not shown) between the normal and CF epithelia. For normal airway, the change in anion secretion and Ca^{2+}_i in response to apically applied ATP was significantly different ($P < 0.05$; symbol not shown) from basolaterally applied ATP.

ses 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 V_t 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, HCO_3^- has been implicated as a relevant anion during transepithelial ion transport (Illek et al., 1997; Clarke and Harline, 1998). Moreover, CFTR has a

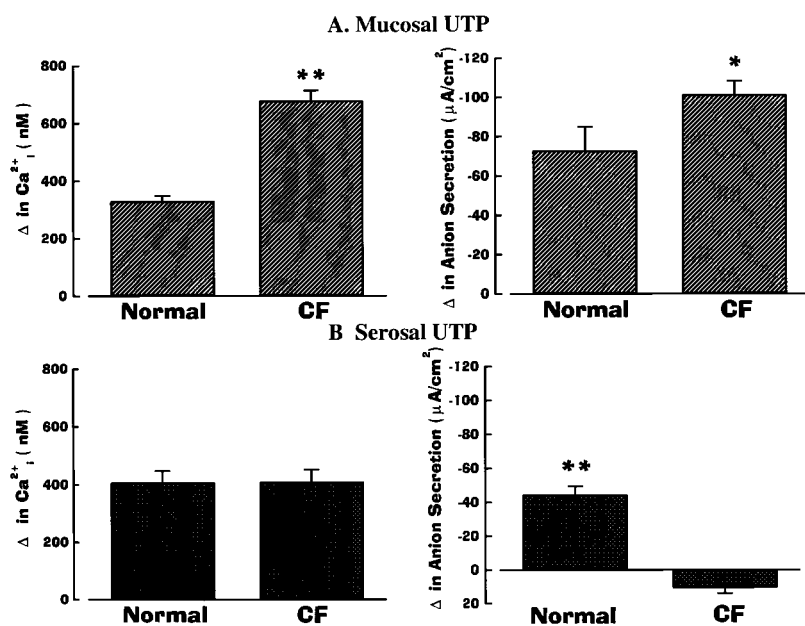


FIGURE 3. Summary data on anion secretion and intracellular Ca^{2+} (Ca^{2+}_i) in normal and cystic fibrosis (CF) nasal epithelia exposed to 100 μM of mucosal (A) or serosal (B) UTP. Normal and CF values are significantly different (* $P < 0.05$ and ** $P < 0.01$) from each other. For basolateral changes in Ca^{2+}_i , values were not significantly different ($P > 0.05$; symbol not shown) between normal and CF epithelia. Each bar represents the mean \pm SEM for eight independent experiments (five different individuals).

finite, but small, permselectivity for HCO_3^- (Linsdell et al., 1999), and CaCC has been reported to conduct 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 Ca^{2+}_i 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 Ca^{2+}_i (spike), followed by a relaxation of Ca^{2+}_i levels to a sustained plateau over the next 2–3 min. We have previously shown in normal nasal monolayers that the initial spike in Ca^{2+}_i , in response to serosal ATP, was entirely due to an internal Ca^{2+} release that was functionally confined to the basolateral domain of cells, whereas the sustained plateau phase results from influx of Ca^{2+} solely across the basolateral membrane of cells (Paradiso et al., 1995). Concomitant with these changes of Ca^{2+}_i , serosal ATP elicited changes in V_t , reaching peak hyperpolarizing values more slowly (i.e., ΔV_t in which the luminal side becomes more negative with respect to the serosal bath) than the Ca^{2+}_i 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 Ca^{2+}_i in normal airway epithelia (Fig. 1 A; also see Fig. 2). We have previously demonstrated (Paradiso et al., 1995) that the initial spike of Ca^{2+}_i in response to a mucosal ATP challenge results from internal Ca^{2+} release from stores in the apical domain of cells, and the sustained plateau results from 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 triphosphate nucleotide.

For CF nasal epithelium, ATP added to the basolateral surface of cells elicited an identical pattern of change of Ca^{2+}_i , 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., ΔV_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 Ca^{2+}_i in CF compared with normal nasal cells. These changes in Ca^{2+}_i 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 Ca^{2+}_i 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 Ca^{2+}_i and anion secretion in CF as compared with normal airway epithelium. For both normal and CF, maximal responses of Ca^{2+}_i and anion secretion were obtained at 10^{-5} – 10^{-4} M. In both tissues, ATP was equipotent for ΔCa^{2+}_i and Δ anion secretion: for the normal airway, the ED₅₀ for Ca^{2+}_i mobilization and anion transport were 1.04×10^{-6} M and 1.02×10^{-6} M, respectively; for CF preparations, the ED₅₀ for Ca^{2+}_i and anion transport were 0.69×10^{-6} M and 0.78×10^{-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 Ca^{2+}_i in normal and CF tissues, with the maximal efficacy obtained at 10^{-5} – 10^{-4} M. In terms of potencies, the ED₅₀ for Ca^{2+}_i in normal and CF were 1.44×10^{-6} M and 1.58×10^{-6} M, respectively. These potency values of nucleotide-mobilized Ca^{2+}_i 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 ΔCa^{2+}_i 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 Ca^{2+}_i 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 Ca^{2+}_i 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 Ca^{2+} and anion secretion as ATP in normal and CF airway epithelia. Mucosal additions of UTP (Fig. 3 A) were effective in mobilizing Ca^{2+}_i 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 Ca^{2+}_i to the same extent in normal and CF airways (Fig. 3 B). Like the ATP-induced anion

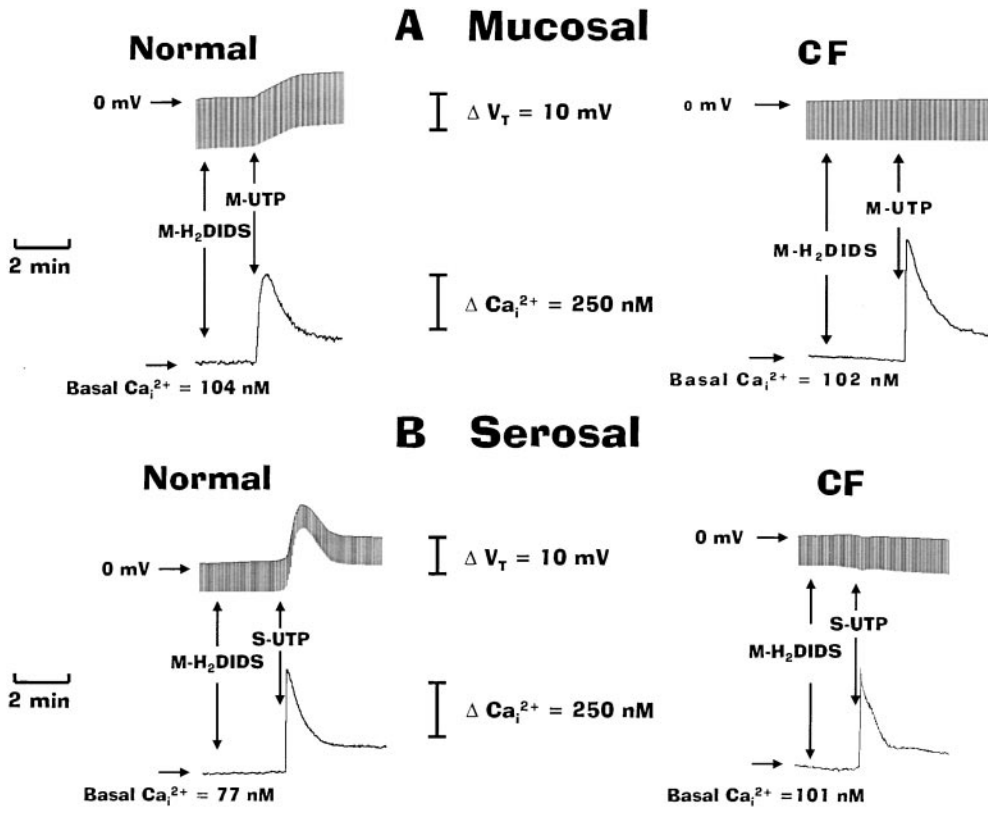


FIGURE 4. Effects of H₂DIDS and UTP applied to the mucosal (A) or serosal (B) compartments on the transepithelial potential difference (V_t) and intracellular Ca^{2+} (Ca_i^{2+}) in normal and cystic fibrosis (CF) nasal monolayers. For these protocols, monolayers were perfused serosally with Krebs's bicarbonate Ringer and mucosally with Na⁺-free/low Cl⁻ Ringer before adding H₂DIDS (1.5 mM) to the mucosal perfusate as indicated in the traces. Serosal (S) or mucosal (M) UTP (100 μ M) was applied to the apical or basolateral membrane as shown. Each trace is representative of four or more independent experiments (three different individuals).

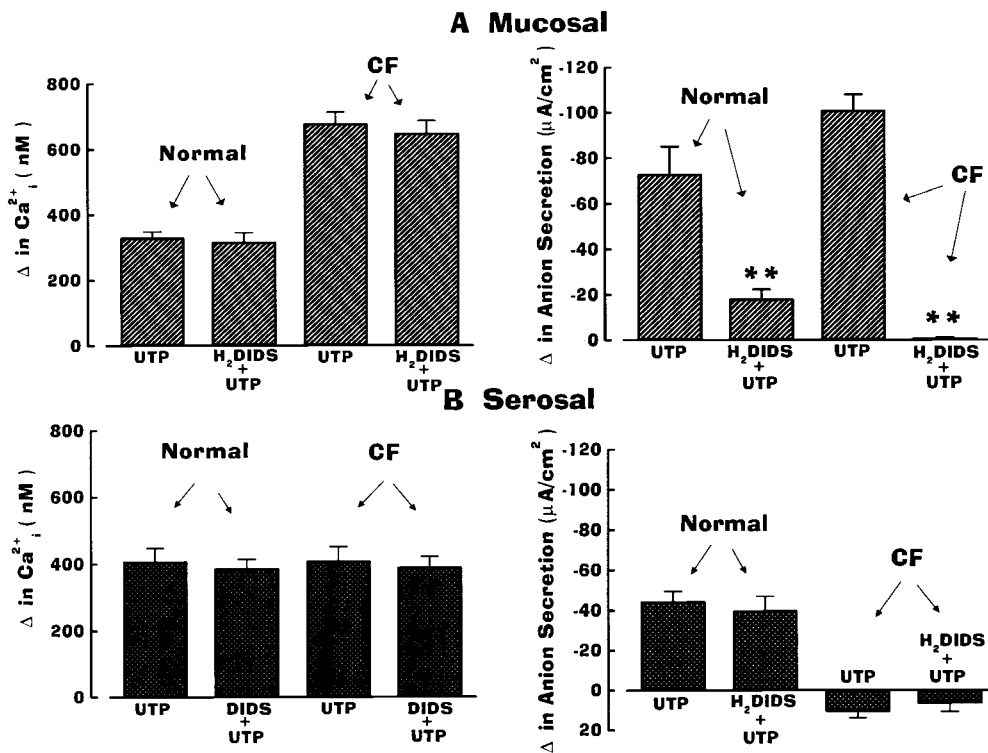


FIGURE 5. Summary data on anion secretion and intracellular Ca^{2+} (Ca_i^{2+}) in normal and cystic fibrosis (CF) nasal epithelia pretreated with luminal H₂DIDS (1.5 mM) and exposed to a single concentration (100 μ M) of mucosal (A) or serosal (B) UTP. Data are included (from Fig. 3) for tissues not treated with H₂DIDS for comparison with tissues treated with the disulfonic stilbene. Values for H₂DIDS-treated tissues and tissues not treated with H₂DIDS are significantly different (** $P < 0.01$) or otherwise not different ($P > 0.05$; symbol not shown) from each other. For H₂DIDS-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 ΔCa^{2+}_i and Δ anion secretion. For mucosal addition of UTP, the ED_{50} for Ca^{2+}_i mobilization and anion transport was 0.98×10^{-6} M and 1.06×10^{-6} M, respectively; for serosal application of UTP, the ED_{50} for Ca^{2+}_i and anion transport was 1.12×10^{-6} M and 1.08×10^{-6} M, respectively.

Protocols Using H_2DIDS 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_2DIDS . 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_2DIDS (1.5 mM) before UTP (100 μM) additions. In the normal human airway, H_2DIDS markedly inhibited anion secretion ($\sim 75\%$) in response to mucosal UTP, and H_2DIDS 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_2DIDS 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_2DIDS , similar to that detected in H_2DIDS -free experiments (Fig. 4 B). Importantly, mucosal H_2DIDS 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 $\text{P2Y}_2\text{-R}$ -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_2DIDS (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

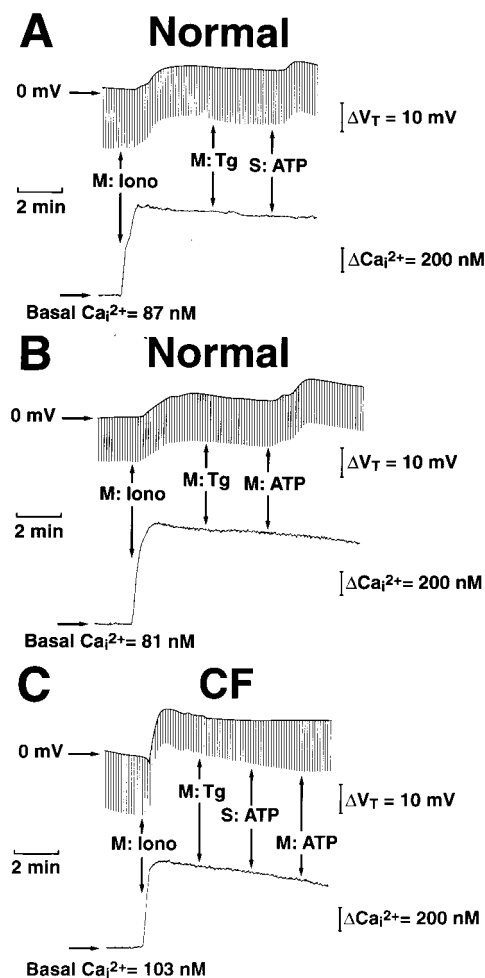


FIGURE 6. Separation between intracellular Ca^{2+} (Ca^{2+}_i) and the transepithelial potential difference (V_t) in response to ATP in normal (A and B) and cystic fibrosis (CF; C) nasal epithelia. For these protocols, monolayers were perfused serosally with Krebs's bicarbonate Ringer and mucosally with 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).

H_2DIDS pretreatment suggested that triphosphate nucleotides can modulate CFTR via a signal transduction pathway that may or may not involve changes in Ca^{2+}_i . Thus, an important aspect of this study was to evaluate whether regulation of anion secretion by extracellular ATP/UTP is tightly coupled to Ca^{2+}_i in normal and CF human airway epithelia. To address this issue, two different maneuvers were used in an attempt to test whether ATP/UTP-regulated Ca^{2+}_i could be dissociated from anion secretion.

As illustrated in Fig. 6, the first approach involved maximal elevation of Ca^{2+}_i by exposing cells to ionomycin and the endoplasmic reticulum Ca^{2+} pump blocker thapsigargin, followed by ATP. In these studies, normal

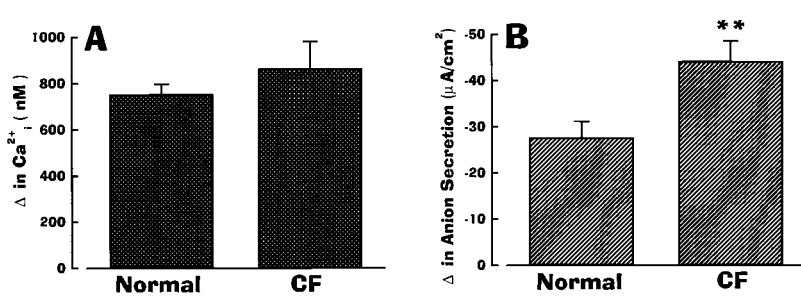


FIGURE 7. Summary data showing changes in intracellular Ca^{2+} (Ca^{2+}_i ; A) and anion secretion (B) in normal and cystic fibrosis (CF) monolayers exposed to mucosal ionomycin (300 nM). Each bar represents mean \pm SEM for 40 or more independent experiments (three different individuals). For Ca^{2+}_i , normal and CF values are not significantly different ($P > 0.05$; symbol not shown) from each other; for anion secretion, normal and CF values are significantly different (** $P < 0.01$) from each other.

(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^{2+} (1.3 mM), resulted in a maximal increase of Ca^{2+}_i ; subsequent addition of serosal ionomycin (300 nM; not shown) or mucosal thapsigargin (500 nM; Fig. 6) caused no additional change in V_t and cell Ca^{2+} . We next tested the effects of unilaterally applied triphosphate nucleotides on changes of Ca^{2+}_i and V_t . In the normal airway, serosal (Fig. 6 A) and mucosal (Fig. 6 B) ATP (100 μM) induced an additional increase in V_t without an apparent additional change in Ca^{2+}_i . In contrast to normal nasal tissues, neither serosal nor mucosal administration of ATP elicited changes in V_t 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^{2+}_i and anion secretion

(see Fig. 7) and, second, for the effects of mucosal and serosal ATP/UTP on Ca^{2+}_i and anion transport (see Fig. 8) after pretreatment with ionomycin/thapsigargin in normal and CF airway preparations.

As shown in Fig. 7, Ca^{2+}_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^{2+}_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^{2+} was clamped at high levels with ionomycin/thapsigargin pretreatment,

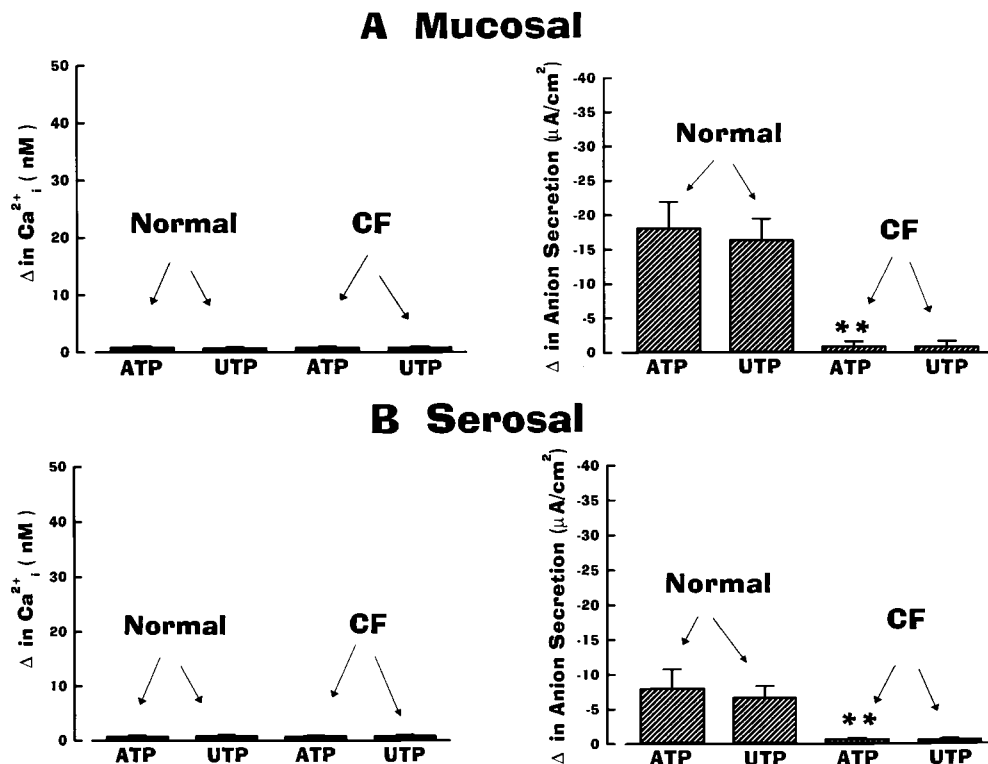


FIGURE 8. Summary data showing changes in anion secretion and intracellular Ca^{2+} (Ca^{2+}_i) in normal and cystic fibrosis (CF) nasal epithelia exposed to mucosal (A) or serosal (B) ATP (100 μM) or UTP (100 μM) under high Ca^{2+}_i conditions. Monolayers were pretreated with 300 nM ionomycin + 500 nM thapsigargin before ATP/UTP additions (see Fig. 6). Each bar represents mean \pm SEM for four or more independent experiments (three different individuals). Mucosal ATP/UTP-stimulated anion transport activities were significantly (** $P < 0.01$) larger than serosal ATP/UTP equivalent currents. No significant change ($P > 0.05$; symbol not shown) in Ca^{2+}_i was detected with mucosal/serosal addition of agonists.

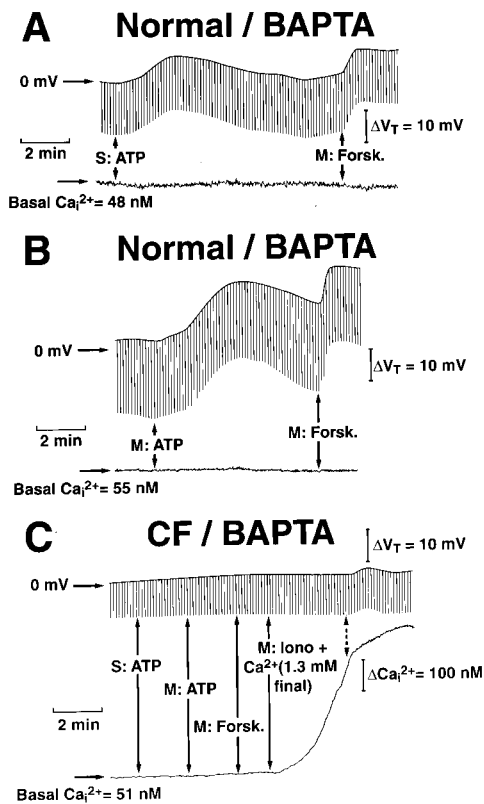


FIGURE 9. Changes in the transepithelial potential difference (V_t) and intracellular Ca^{2+} (Ca^{2+}_i) activities in response to serosal (S) or mucosal (M) ATP (100 μ M) in normal (A and B) or cystic fibrosis (CF; C) BAPTA-treated nasal epithelial preparations. Monolayers were initially perfused with serosal Krebs bicarbonate Ringer/mucosal Na^+ -free/low Cl^- Ringer and low bilateral extracellular Ca^{2+} (300 μ M) before the addition of ATP, as indicated in the traces. For normal and CF nasal preparations, forskolin (Forsk; 10 μ M) was added to the mucosal perfusate at the times indicated. In CF preparations, ionomycin (Iono; 300 nM) + extracellular Ca^{2+} (1.3 mM final concentration) was added to the mucosal bath as shown in the trace. Each trace is representative of at least five independent experiments (four or more different individuals).

ATP and UTP were equally effective in inducing anion secretion without affecting Ca^{2+}_i 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 additions 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^{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^{2+}_i to low levels. To reduce Ca^{2+} influx that would result from $P2Y_2$ -R activation (Paradiso et al., 1995), nasal monolayers were exposed to bilateral low Ca^{2+} (300 μ M) solutions. As illustrated in Fig. 9, the addition of serosal (Fig. 9 A) or mucosal (Fig. 9 B) ATP (100 μ M) failed to

raise Ca^{2+}_i , but increased V_t , 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 μ M) evoked an additional increase in V_t 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 V_t in CF (Fig. 9 C). However, when monolayers were exposed bilaterally to high extracellular Ca^{2+} (1.3 mM) plus mucosal ionomycin (300 nM), Ca^{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^{2+} . To detect a change in cell Ca^{2+} in BAPTA-loaded cells, sufficient Ca^{2+} influx must occur to overwhelm the buffering capacity of BAPTA, thus, giving rise to the slow increase in Ca^{2+}_i (Fig. 9 C). The small magnitude of change in V_t shown in Fig. 9 C may be related not only to the magnitude change in Ca^{2+}_i , but also to the rate of change of cell Ca^{2+} (which is not a focus of our study). Data for the mucosal and serosal ATP/UTP on changes in anion transport rates and Ca^{2+}_i levels in BAPTA-treated normal and CF airway epithelia are summarized in Fig. 10 (A and B).

There are several relevant observations arising from the Ca^{2+} clamp experiments that should be of note. First, in normal nasal monolayers, the magnitude of the anion secretory response to apically applied ATP/UTP was identical under experimental conditions when cell Ca^{2+} was clamped at high levels with ionomycin/thapsigargin treatment or low levels with BAPTA treatment (Figs. 8 and 10, respectively), strongly indicating that the signaling pathway underlying triphosphate nucleotide stimulation of anion secretion has a molecular component that is independent of Ca^{2+}_i , likely via CFTR, during $P2Y_2$ -R activation. Second, in normal airway cells, serosal ATP/UTP-stimulated anion transport rates under both Ca^{2+}_i -clamped conditions were smaller than those observed for mucosal additions of ATP/UTP. These data suggest that molecular mechanism(s) underlying the signaling pathway for the stimulation of anion transport by ATP/UTP under Ca^{2+}_i -clamped conditions is less efficient for serosal compared with mucosal addition of these triphosphate nucleotides. Finally, in CF nasal monolayers, mucosal addition of ATP/UTP failed to elicit an anion secretory response in Ca^{2+}_i -clamped cells, suggesting that CaCC is the sole Cl^- channel mediating anion transport activity in CF nasal epithelia.

Effects of PKC Activation and Inhibition on UTP-stimulated Anion Secretion in Ca^{2+}_i -clamped Normal Nasal Tissue

We have previously reported (Boucher et al., 1989) no detectable difference in total PKC activity in normal and

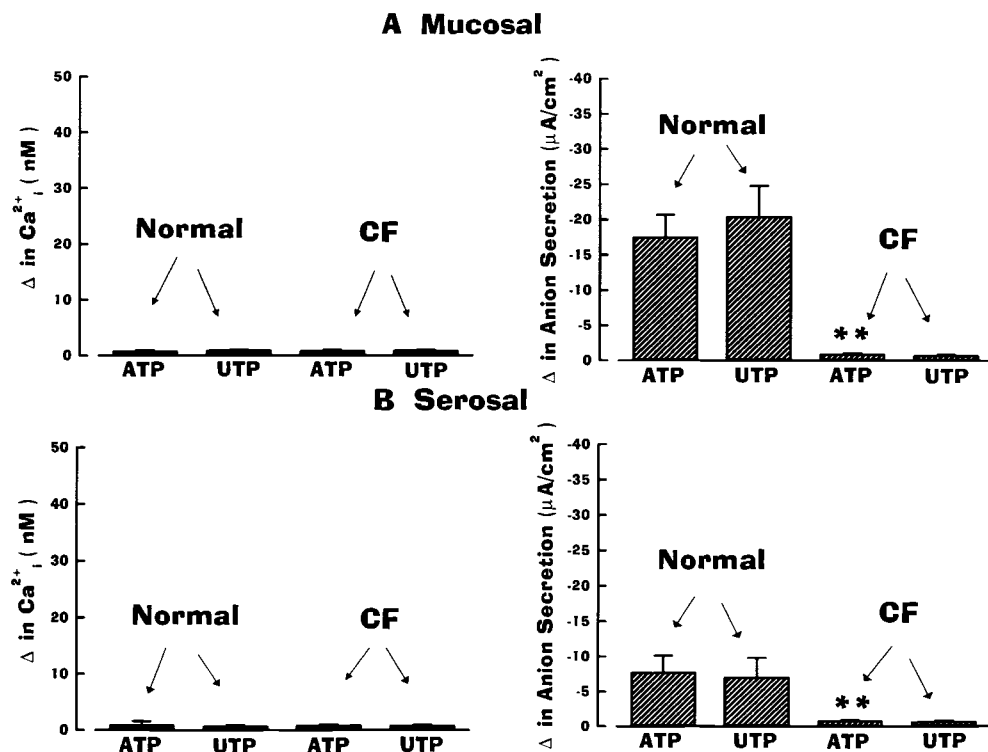


FIGURE 10. Summary data showing changes in anion transport and intracellular Ca^{2+} (Ca^{2+}_i) activities in normal and cystic fibrosis (CF) nasal epithelium exposed to mucosal (A) or serosal (B) ATP (100 μM) or UTP (100 μM) in BAPTA pretreated cells. Monolayers were perfused with serosal Krebs bicarbonate Ringer/mucosal Na^+ -free/low Cl^- Ringer and low bilateral extracellular Ca^{2+} (300 μM) before triphosphate nucleotide addition. Each bar represents mean \pm SEM for at least five independent experiments (four or more different individuals). Mucosal ATP/UTP-stimulated equivalent currents were significantly ($**P < 0.01$) larger than serosal ATP/UTP transport activities. No significant change ($P > 0.05$; symbol not shown) in Ca^{2+}_i was detected with mucosal/serosal addition of agonists.

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^{2+}_i (Figs. 8 and 10), we tested whether a Ca^{2+} -independent PKC was a functional regulator of CFTR-mediated anion secretion after P2Y_2 -R activation. In these studies, monolayers of normal nasal epithelia were Ca^{2+}_i -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_t with no change in Ca^{2+}_i (Fig. 11 A). Importantly, subsequent exposure of nasal cells to serosal and mucosal UTP (100 μM) failed to cause an additional increase in V_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^{2+}_i -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

Purinoreceptors 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 ΔCa^{2+}_i (Fig. 2 and associated text of Fig. 3) argues that the P2Y_2 -R, and not P2X -Rs, mediated the nucleotide responses on both the apical and basolateral surfaces of our airway preparations.

P2Y_2 purinoreceptors activate $\text{PLC}\beta$ in a heterotrimeric G protein-dependent manner (Boeynaems et al., 1998) and increase Ca^{2+}_i through two distinct, but related pathways. Initially, IP_3 is formed from PLC-induced breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) and activates IP_3 channel receptors in the endoplasmic reticulum, resulting in channel opening and release of stored calcium into the cytoplasm. Subsequently, depletion of intracellular Ca^{2+} stores activates a pathway for Ca^{2+} influx across the plasma membrane,

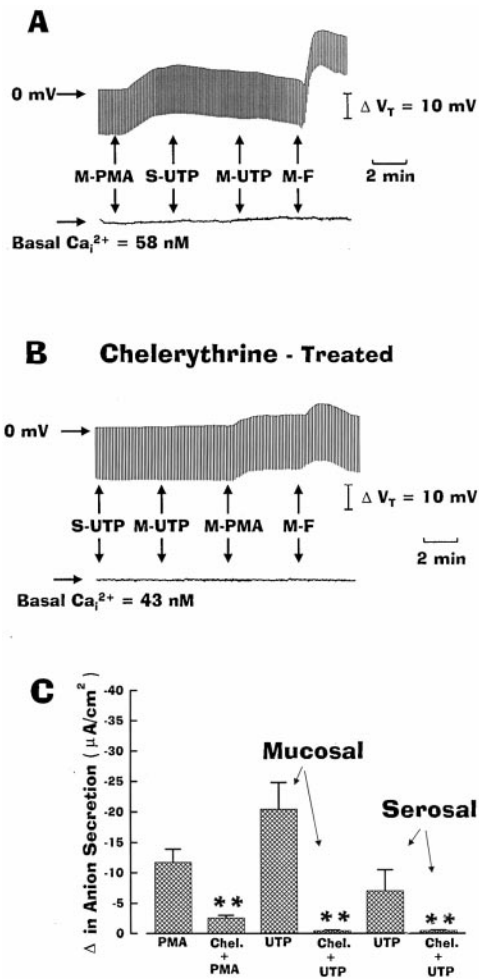


FIGURE 11. Effect of PMA and chelerythrine on UTP-stimulated anion secretion in intracellular Ca²⁺ (Ca²⁺_i)-clamped normal nasal monolayers. In the absence (A) or presence (B) of chelerythrine (1 μM), monolayers were perfused serosally with Krebs' bicarbonate Ringer and mucosally with Na⁺-free/low Cl⁻ Ringer before adding serosal (S) or mucosal (M) PMA (100 nM), UTP (100 μM), or forskolin (F; 10 μM) as indicated. C shows summary data generated from four or more independent experiments (three different individuals). Chelerythrine significantly (**P < 0.01) blocked PMA and UTP-induced anion transport activities.

which was originally termed "capacitative Ca²⁺ entry" or, more recently, "store-operated calcium entry" (Clapham, 1995; Putney, 1986, 1990). Both phases of Ca²⁺ mobilization can act in concert for the subsequent modulation of multiple plasma membrane transport processes, including the Ca²⁺-activated Cl⁻ channel (i.e., CaCC) in airway epithelia.

Besides PLC-mediated changes of Ca²⁺_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 Ca²⁺-independent manner, and their findings agree with more recent studies by Liedtke and Cole (1998), who reported that Ca²⁺-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 Ca²⁺_i mediated by extracellular triphosphate nucleotides. For example, Hwang et al. (1996) reported that triphosphate nucleotides activated multiple types of Ca²⁺-sensitive and Ca²⁺-insensitive Cl⁻ conductances in rat tracheal epithelial cells. A dissociation between the regulation of Cl⁻ conductance and Ca²⁺_i 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 Ca²⁺_i (either directly or indirectly) in airway epithelial preparations. To better quantitate the role of intracellular Ca²⁺ in modulating anion transport activity in polarized tissues, we used a unique experimental technique that allowed us to measure simultaneous changes of Ca²⁺_i and anion secretion induced by P2Y₂-R activation 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 P2Y₂-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 Ca²⁺_i and anion secretion. Contrasting maneuvers were used to clamp Ca²⁺_i at different levels in CF nasal tissues. The first approach involved maximally elevating Ca²⁺_i by the addition of ion-

omycin, followed by thapsigargin. CF nasal tissue pretreated with these agents exhibited no additional change in Ca^{2+}_i 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^{2+}_i to low levels. These experiments revealed that the addition of mucosal ATP/UTP to BAPTA-treated CF cells caused no change in Ca^{2+}_i , and again failed to increase anion secretion in CF epithelium (Figs. 9 and 10). The data showing that Ca^{2+}_i -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_2DIDS (Figs. 4 and 5), argue that anion secretion in CF epithelia in response to ATP/UTP is mediated exclusively via Ca^{2+}_i regulation of CaCC.

A key observation of this study was the markedly larger Ca^{2+}_i response of CF as compared with normal tissues to mucosal ATP/UTP (Figs. 2 and 3), raising the possibility that regulation of Ca^{2+}_i metabolism is different in CF. Based on our functional data alone, it is not apparent how mucosal ATP/UTP activated a larger Ca^{2+}_i 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_3 -releasable Ca^{2+} stores) markers, calreticulin, and IP_3 receptors, which show that endoplasmic reticulum Ca^{2+} stores are preferentially distributed to the apical domain, and that CF cells exhibit a greater expression of apical ER Ca^{2+} stores (Ribeiro et al., 1999).

Basolateral P2Y₂-R Regulation of Anion Secretion in CF Airway Epithelia

A central observation of this study was that basolateral activation of P2Y₂-R with ATP or UTP raised Ca^{2+}_i 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^{2+} , mobilized by activation of the basolateral P2Y₂-R, is functionally confined to that barrier, i.e., the Ca^{2+}_i released from basolateral stores cannot activate apical Ca^{2+} -sensitive Cl^- channels. Our data in normal tissues imply that this compartmentalized release/regulation of Ca^{2+}_i 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^{2+} -activated K^+ channels (Clarke and Boucher, 1992), suggesting that this compartmentalization is symmetric. The mechanism for compartmentalization of Ca^{2+} signaling in airway epithelia is unknown, but it may involve the actions of mitochondria

acting as a “ Ca^{2+} fence” (Tinel et al., 1999) and/or Ca^{2+} binding proteins (Nomiya et al., 1998).

Apical Membrane P2Y₂-R Regulation of Anion Secretion in Normal Airway Epithelia

For apical P2Y₂-R-regulated anion secretion in normal airway tissue, we propose that two distinct Cl^- channels (e.g., CaCC and CFTR) mediate anion efflux across the apical membrane, and that the linkage coupling activation of apical P2Y₂-R to the induction of anion transport involves both Ca^{2+}_i -regulated CaCC and Ca^{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 P2Y₂-Rs reflects Ca^{2+}_i -regulated activation of CaCC. In support of this notion, anion transport activity was substantially blocked (~75%) by luminal H_2DIDS 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^{2+}_i -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_2DIDS or Ca^{2+}_i -clamping agents indicates that apical P2Y₂-R activation modulates multiple Cl^- channels via Ca^{2+} -dependent (i.e., CaCC) and Ca^{2+} -independent signal transduction pathways. We propose that the Ca^{2+} -independent signaling pathway linking apical P2Y₂-R activation to anion secretion reflects Ca^{2+} -independent PKC regulation of CFTR-mediated anion secretion for several reasons. First, as noted above, activation of the apical P2Y₂-Rs by ATP/UTP increased anion secretory activity in a Ca^{2+} -independent manner (Figs. 8 and 10). Second, the PMA-stimulated anion secretion abolished UTP-regulated anion secretion (Fig. 11) in Ca^{2+}_i -clamped monolayers, 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 apically applied UTP-regulated anion secretion under Ca^{2+}_i -clamped conditions (Fig. 11), which is consistent with the notion that a Ca^{2+} -independent PKC is the intracellular second messenger relevant to CFTR-mediated anion secretion in normal airway epithelia.

Basolateral Membrane P2Y₂-R Activation of Anion Transport in Normal Airway Epithelia

For basolateral P2Y₂-R-regulated secretion, we propose that (1) only a single Cl^- channel (i.e., CFTR) mediates anion efflux across the apical membrane, and (2) that the linkage between P2Y₂-R activation and CFTR in-

Apical

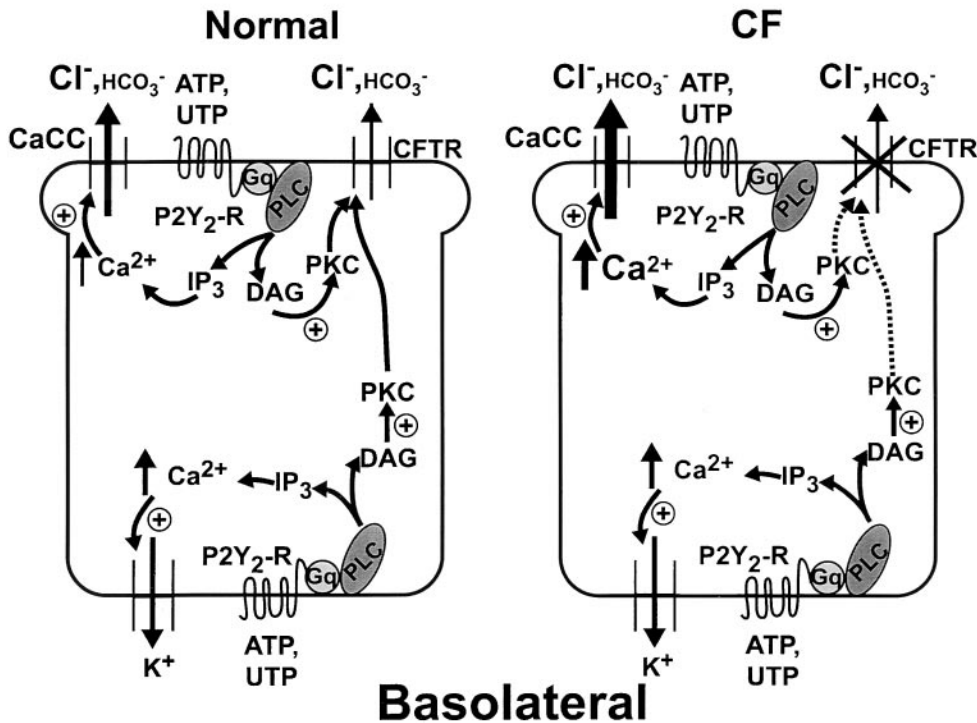


FIGURE 12. Model for basolateral and apical P₂Y₂ receptor (P₂Y₂-R) activation of anion secretion in normal and cystic fibrosis (CF) human airway epithelia. ATP; UTP; P₂Y₂-R (P₂Y₂ purinergic receptor); Gq (heterotrimeric GTP-binding protein); PLC (phospholipase C); IP₃ (inositol 1,4,5-triphosphate); Ca²⁺_i (intracellular Ca²⁺); DAG (diacylglycerol); PKC; CFTR (cystic fibrosis transmembrane conductance regulator); and CaCC (Ca²⁺-activated Cl⁻ channel).

volves regulation via PKC rather than Ca²⁺_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 P₂Y₂-R activation is further supported by data that show that mucosal H₂DIDS 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 Ca²⁺_i-independent PKC is based on several interrelated observations derived from the Ca²⁺_i-clamped studies. First, as noted above, activation of the basolateral P₂Y₂-Rs by ATP/UTP increased anion transport activity in a Ca²⁺_i-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 Ca²⁺_i-clamped monolayers, again suggesting that the PMA- and UTP-regulated signaling pathways occur via the same cellular mechanism, i.e., Ca²⁺_i-independent PKC. Finally, PKC inhibition by chelerythrine completely abolished serosally applied UTP-

regulated anion secretion under Ca²⁺_i clamp conditions (Fig. 11), which is again consistent with the notion that a Ca²⁺_i-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 P₂Y₂-R activation couples to apical anion secretion through two related pathways. First, the Ca²⁺_i signal resulting from PLC-generated IP₃ activates Ca²⁺_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 Ca²⁺_i-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 Ca²⁺_i signal generated by basolateral nucleotide application is restricted to the basolateral compartment. In contrast, apical P₂Y₂-R activation in normal epithelium increases anion secretion as a result of Ca²⁺_i-dependent activation of CaCC as well as CFTR regulation by a Ca²⁺_i-independent PKC.

In CF epithelium, although basolateral P₂Y₂-R activation stimulates PLC to the same extent as in normal ep-

ithelium, 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+}_i 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 the 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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