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CFTR Regulation of Intracellular Calcium in Normal and Cystic Fibrosis Human Airway Epithelia

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Abstract. In cystic fibrosis airway epithelia, mutation of the CFTR protein causes a reduced response of Cl^- secretion to secretagogues acting via cAMP. Using a Ca^{2+} imaging system, the hypothesis that CFTR activation may permit ATP release and regulate $[Ca^{2+}]_i$ via a receptor-mediated mechanism, is tested in this study. Application of external nucleotides produced a significant increase in $[Ca^{2+}]_i$ in normal (16HBE14o$^-$ cell line and primary lung culture) and in cystic fibrosis (CFTE29o$^-$ cell line) human airway epithelia. The potency order of nucleotides on $[Ca^{2+}]_i$ variation was UTP $\gg$ ATP > UDP > ADP > AMP > adenosine in both cell types. The nucleotide $[Ca^{2+}]_i$ response could be mimicked by activation of CFTR with forskolin (20 μM) in a temperature-dependent manner. In 16HBE14o$^-$ cells, the forskolin-induced $[Ca^{2+}]_i$ response increased with increasing temperature. In CFTE29o$^-$ cells, forskolin had no effect on $[Ca^{2+}]_i$ at body temperature-forskolin-induced $[Ca^{2+}]_i$ response in CF cells could only be observed at low experimental temperature (14°C) or when cells were cultured at 26°C instead of 37°C. Pretreatment with CFTR channel blockers glibenclamide (100 μM) and DPC (100 μM), with hexokinase (0.5 U/mg), and with the purinoceptor antagonist suramin (100 μM), inhibited the forskolin $[Ca^{2+}]_i$ response. Together, these results demonstrate that once activated, CFTR regulates $[Ca^{2+}]_i$ by mediating nucleotide release and activating cell surface purinoceptors in normal and CF human airway epithelia.

Key words: CFTR — Cystic fibrosis — Intracellular Ca^{2+} — Airway epithelium — Forskolin — Purinoceptors

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Introduction

The inherited disease CF is caused by mutation of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. It is characterized by a defect in ion transport in both secretory and absorptive epithelia. In addition to its Cl^- channel activity, there is increasing evidence suggesting that CFTR has a regulatory function, and is also involved in epithelial ion transport via interactions with other Cl^--selective and cation-selective channels (Kunzelman & Schreiber, 1999). Several studies have identified a role for CFTR as a protein channel regulator controlling the activity of an outwardly rectifying chloride channel (ORCC) (Egan et al., 1992; Gabriel et al., 1993; Schwiebert et al., 1995), and an amiloride-sensitive Na^+ channel (Johnson et al., 1995; Kunzelmann, Kathofer & Greger, 1995; Stutts et al., 1995; Ismailov et al., 1996; Mall et al., 1996, 1998). However, the mechanism by which CFTR exerts its regulatory role over other epithelial ion channels is still not completely understood, though several hypothesis including direct intramembrane or cytoplasmic protein-protein interaction, or indirect regulation are currently being explored. More specifically, it has been proposed that the CFTR may regulate ORCC activity via autocrine activation of luminal purinoceptors involving ATP (Schwiebert et al., 1995), and it has recently been demonstrated that in adenovirus-CFTR infected Chinese Hamster Ovary cells, CFTR plays a central role in the regulation of $[Ca^{2+}]_i$ (Urbach & Harvey, 1999).

In normal and CF human airway epithelia, external ATP has been shown to regulate $[Ca^{2+}]_i$ (Mason, Paradiso & Boucher, 1991; Paradiso et al., 1995) and Cl^- secretion (Knowles, Clarke & Boucher, 1991; Stutts et al., 1992; Galietta et al., 1992). Excised inside-out patch clamp studies on normal human airway epithelial cells demonstrated that increasing $[Ca^{2+}]_i$ (>100 nM) by addi-

tion of extracellular nucleotides, activates an ORCC pathway (Urbach et al., 1994). Studies focusing on the external control of ORCC activity observed that addition of extracellular ATP to both normal and CF airway epithelia, mediates ORCC channel activity via activation of luminal P2 receptors (Stutts et al., 1992). In addition, experimental maneuvers designed to cause an increase in $[Ca^{2+}]_i$ have been shown to inhibit Na^+ absorption in sheep trachea and human bronchus (Graham et al., 1992). Extracellular nucleotides may therefore play a central role in determining the balance between secretion and absorption via effects on $[Ca^{2+}]_i$ in airway epithelial cells. If, as we demonstrate in this study, nucleotide release is under CFTR control, this would confer an autocrine role for CFTR in controlling Cl^- and Na^+ transport in airway epithelial cells.

Using fura-2 fluorescence spectrometry, we investigate the autocrine function of the CFTR in regulating $[Ca^{2+}]_i$ by mediating ATP release from the cell and activating luminal purinoceptors in human airway epithelia. Activation of CFTR is achieved by the use of the cAMP agonist, forskolin. Nucleotide and forskolin regulation of $[Ca^{2+}]_i$ is examined in primary epithelial cell cultures of normal human primary lung (NHPL), in a CFTR-expressing human bronchial epithelial cell line ($16HBE14o^-$) and in a ΔF508-CFTR human tracheal epithelial cell line ($CFTE29o^-$). Based on the hypothesis that temperature reduction facilitates expression of the mutant ΔF508-CFTR protein in the plasma membrane (Denning et al., 1992), and should therefore restore autocrine functionality of the CFTR in $[Ca^{2+}]_i$ regulation, temperature sensitivity of the forskolin-$Ca^{2+}{}_i$ response is determined in normal and CF airway epithelia. For the first time, evidence is provided for a regulatory role of $[Ca^{2+}]_i$ by CFTR in airway epithelia. Regulation of $[Ca^{2+}]_i$ by the CFTR may in turn normalize NaCl transport in CF airway epithelia by stimulating Ca^{2+}-dependent Cl^- secretion (Boucher et al., 1989), and simultaneously downregulating Na^+ hyperabsorption (Graham et al., 1992).

Materials and Methods

Cell Culture

Primary cultures of normal human nonmetastatic proximal lung epithelia (NHPL) were initiated from biopsies of secondary bronchus (local medical ethics committee approved). The tissue was trimmed and chopped finely into 1 mm^2 pieces and plated into troughs cut into the base of tissue culture flasks and maintained in Ham's F12 medium (Biowhittaker, Walkersville, MD) supplemented with 10% FBS, 3% L-glutamine, 10,000 μg/ml streptomycin, 10,000 U/ml penicillin G, 200 μl epidermal growth factor, 20 μl Vitamin A and 10 μl hydrocortisone. Epithelial cells grew around the explant as a monolayer. The normal $16HBE14o^-$ cell line is derived from surface epithelium of mainstream, second-generation bronchi (Cozens et al., 1994). This cell line expresses normal levels of CFTR mRNA and CFTR protein (Cozens et al., 1992). The $CFTE29o^-$ cell line is an immortalized cell line derived from tracheal epithelium homozygous for the ΔF508 mutation. This post-crisis cell line is defective in cAMP-dependent ion transport, but secretes Cl^- in response to treatment with Ca^{2+} ionophores (Kunzelman et al., 1993). $CFTE29o^-$ cells also express CFTR mRNA and CFTR protein but do not have the capacity to form tight junctions and polarized monolayers after the early stages of transformation (Gruenert et al., 1995). Both normal and CF airway epithelial cells were grown on fibronectin/collagen/BSA coated tissue culture flasks in Eagle's Minimal Essential Medium (EMEM, Biowhittaker) supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin (Cozens et al., 1994). Cells were initially cultured at 37°C. Those required for higher incubation experiments were allowed to reach confluency at 37°C, but cells required for lower incubation experiments were removed from the 37°C incubator before confluency was attained and cultured for two days at 26°C. Experiments were performed at either 14°C, room temperature (22°C) or 32°C to mimic body temperature without dye leakage. For experiments at 14°C, the cells were stored on ice for 30 min after the fura-2/AM loading procedure and before the start of each experiment.

Calcium Spectrofluorescence

Intracellular Ca^{2+} concentration was determined in cells isolated by trypsin or in confluent cell monolayers grown on fibronectin/collagen/BSA treated glass coverslips. Epithelial cells were loaded with 5 μM of the Ca^{2+}-sensitive fluorescent probe fura-2 acetoxy-methyl ester (fura-2/AM) for 30 min, in the dark, at room temperature (22°C). The cells were washed twice in Hepes buffered Kreb's-Heinsleit solution (1,500 rpm for 5 min) (NaCl 140 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, HEPES 10 mM, Tris-HCl 10 mM, glucose 10 mM, pH 7.4, Osmol 280–290 mosmol). Isolated cells were transferred to glass coverslips treated with a 1:10 dilution of poly-L-lysine. Isolated and monolayer coverslips were mounted on a temperature-controlled Peltier perfusion chamber (Medical System, model TC 202, Hertfordshire, UK) on an inverted microscope equipped for epifluorescence (Diaphot 200, Nikon, Netherlands). The light from a Xenon lamp (OSRAM, Germany) was filtered through alternating 340- and 380-nm interference filters (10-nm bandwidth, Nikon), mounted on a chopper motorized under computer control (STARWISE FLUO system, IMSTAR, France). The emission fluorescence produced after fura-2 excitation, was passed through a 400-nm dichroic mirror, filtered at 510-nm. The transmitted light image was detected using an intensified CCD video camera (Darkstar, Photonics Sciences, UK) coupled to the microscope. The fluorescence obtained at each excitation wavelength (F_{340} and F_{380}) depends upon the level of Ca^{2+} binding to fura-2, according to an in vivo calibration of the dye performed using a range of EGTA-buffered Ca^{2+} solutions of the fura-2 free acid. The $[Ca^{2+}]_i$ was calculated automatically by the computer using the Grynkiewicz equation (Grynkiewicz, Peonie & Tsein, 1985): $[Ca^{2+}] = K' (R - R_{min}/R_{max} - R)$, where K' is the product of the dissociation constant of Ca^{2+} binding with fura-2, R is the experimental ratio of F_{340} and F_{380} from which the background fluorescence has been subtracted, and R_{max} and R_{min} are the values of R under saturating and Ca^{2+}-free conditions, respectively.

Suramin was obtained from Calbiochem (Nottingham, UK), 2,2′-iminodibenzoic acid (DPC) from Aldrich and the other compounds from SIGMA (Dorset, UK). All results are presented as mean values ± SE mean of n independent experiments. Statistical significant differences were determined by a Students t-test. A one-way ANOVA was used to show that there were no significant differences in basal and in ATP-induced $Ca^{2+}{}_i$ responses measured at the various incubation and experimental temperatures (Fig. 2). Differences were deemed significant if $P \leq 0.05$.

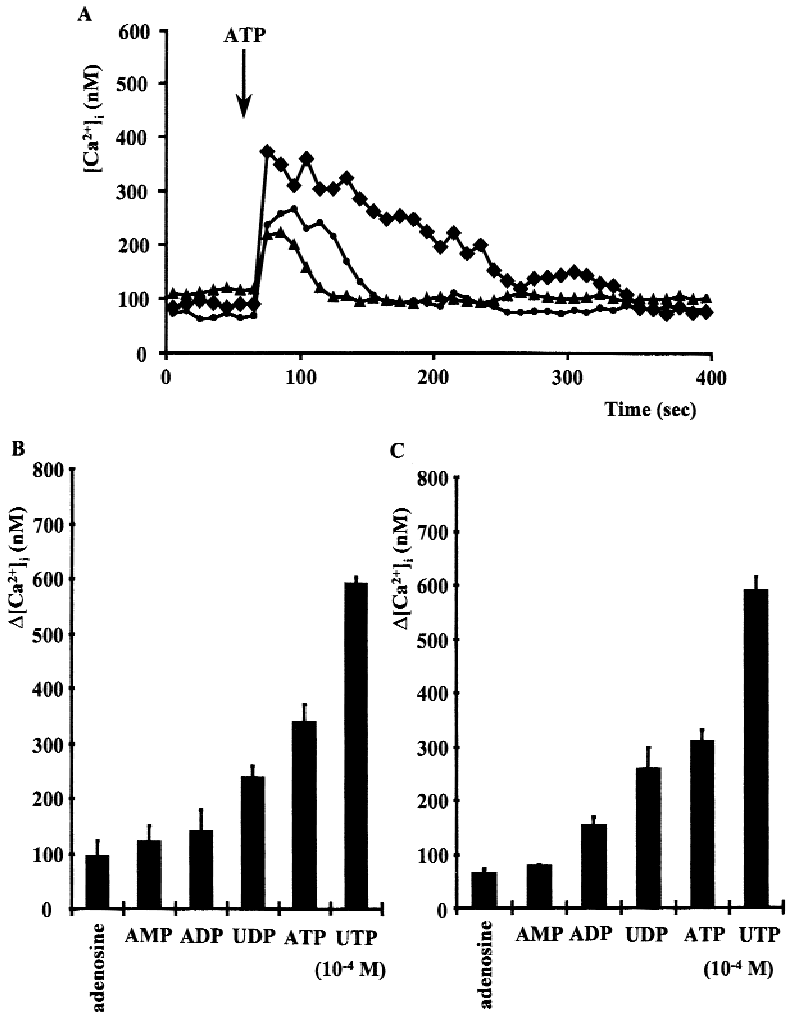

Fig 1. Typical $[Ca^{2+}]_i$ responses to apical exposure to ATP in 16HBE14o$^-$ monolayers at 10^{-4} M (♦), 10^{-6} M (●) 10^{-8} M (▲) (*A*). Sensitivity of $[Ca^{2+}]_i$ changes ($\Delta[Ca^{2+}]_i$) to extracellular nucleotides each added at a final concentration of 10^{-4} M to isolated 16HBE14o$^-$ cells (*B*) and CFTE29o$^-$ cells (*C*). For both cell types, $n = 4$ for adenosine and AMP, and $n = 6$ for ADP, UDP, ATP and UTP.

Results

External Nucleotide Effect on Intracellular $[Ca^{2+}]$ at Room Temperature

Exposure of 16HBE14o$^-$ and CFTE29o$^-$ cells to external nucleotides produced a rapid and transient increase in $[Ca^{2+}]_i$. The magnitude of the $[Ca^{2+}]_i$ response was dependent upon external nucleotide concentration. In 16HBE14o$^-$ cell monolayers, exposure to apical ATP 10^{-4} M stimulated a sixfold increase in $[Ca^{2+}]_i$, while exposure to ATP 10^{-8} M stimulated a twofold increase (Fig. 1*A*). ATP 10^{-4} M peak $[Ca^{2+}]_i$ was reached in 81 ± 10 sec ($P < 0.001$, $n = 6$), followed by a recovery towards basal value over a 5 min period. A similar $Ca^{2+}{}_i$ response to extracellular ATP was achieved in isolated 16HBE14o$^-$ and CFTE29o$^-$ cells, producing half maximum responses at 2.2 ± 3.32 μM ($n = 6$) and 2.2 ± 3.87 μM ($n = 6$), respectively. The effect of other external nucleotides (10^{-4} M) on $[Ca^{2+}]_i$ were also tested in isolated 16HBE14o$^-$ and CFTE29o$^-$ cells, and both cell types produced a similar nucleotide potency order of UTP > ATP > UDP > ADP > AMP > adenosine (Fig. 1*B* and *C*), thereby indicating that the same nucleotide receptor subtypes and Ca^{2+} signaling mechanism are present in normal (16HBE14o$^-$) and CF (CFTE29o$^-$) airway epithelial cells.

Temperature Sensitivity of Basal- and ATP-$[Ca^{2+}]_i$ Response

The temperature sensitivity of the basal $[Ca^{2+}]_i$, and the ATP induced $[Ca^{2+}]_i$ response, was determined in 16HBE14o$^-$ and CFTE29o$^-$ cells. 16HBE14o$^-$ monolayer and isolated cells and CFTE29o$^-$ isolated cells were cultured at either 26°C or 37°C and experiments performed at 14, 22 or 32°C. Although it has previously been observed that increasing temperature, decreases the effective dissociation constant of fura-2 for Ca^{2+} (Paltauf-Doburzynska & Graier, 1997), the results presented

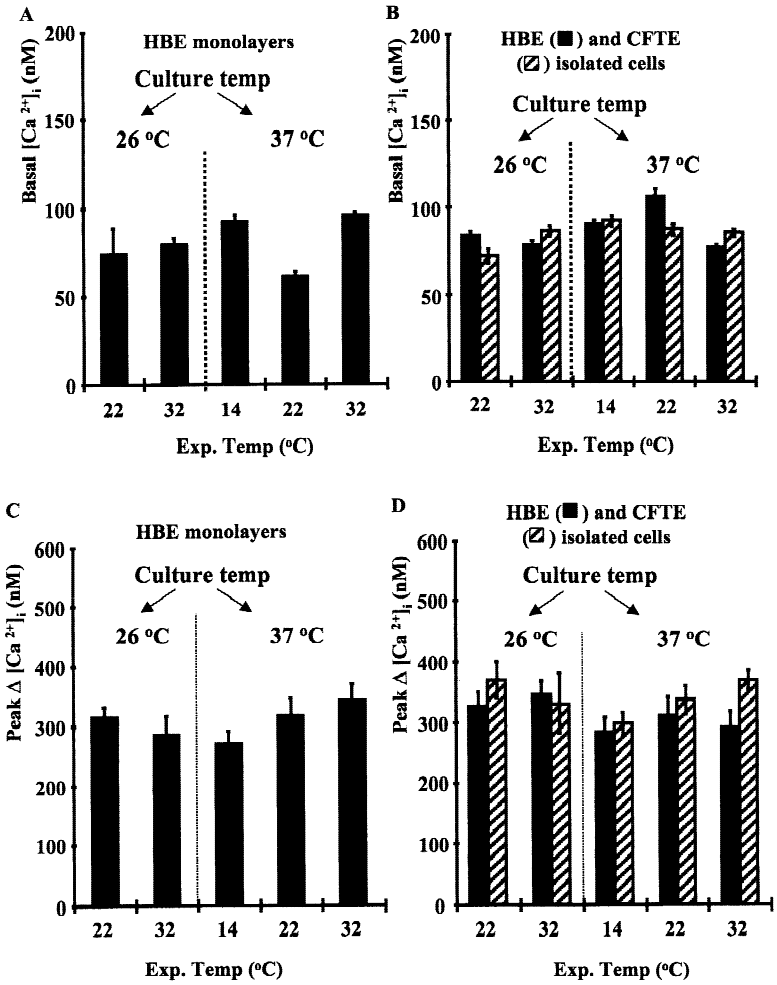

Fig 2. Basal $[Ca^{2+}]_i$ at various culture (*C*) and experimental (*E*) temperatures in 16HBE14o$^-$ monolayer cells (*A*) and in 16HBE14o$^-$ and CFTE29o$^-$ isolated cells (*B*). Temperature dependency of the peak $\Delta[Ca^{2+}]_i$ in response to ATP (10^{-4} M) in 16HBE14o$^-$ monolayers (*C*) and 16HBE14o$^-$ and CFTE29o$^-$ isolated cells (*D*). For all experiments, $n = 4$ for basal $[Ca^{2+}]_i$ measurements, and $n = 6$ for ATP $[Ca^{2+}]_i$ measurements.

in Fig. 2 illustrate that neither resting levels of $[Ca^{2+}]_i$ ($P > 0.05$) nor the ATP-induced $[Ca^{2+}]_i$ response ($P > 0.1$) in 16HBE14o$^-$ and CFTE29o$^-$ cells show any significant temperature sensitivity between 14 and 32°C.

Forskolin Effect on $[Ca^{2+}]_i$

Activation of CFTR and its possible role in regulating $[Ca^{2+}]_i$ was examined using the cAMP agonist forskolin (20 μM). In confluent monolayers of 16HBE14o$^-$ cells cultured at 37°C and tested at 32°C, apical forskolin (20 μM) stimulated a sixfold increase in $[Ca^{2+}]_i$ ($P < 0.001$, $n = 6$) after 110 ± 13 sec, followed by a recovery towards basal level over a 10 min period (Fig. 3*A*). Using the same culturing and experimental temperatures, a similar forskolin-$Ca^{2+}{}_i$ response was also observed in isolated 16HBE14o$^-$ and NHPL cells. Application of forskolin (20 μM) mediated a $\Delta[Ca^{2+}]_i$ to a maximum of 455 ± 25 nM ($P < 0.0009$, $n = 6$) and 619 ± 14 nM ($P < 0.0009$, $n = 4$) in 16HBE14o$^-$ and NHPL cells respectively. However, forskolin (20 μM) failed to stimulate an increase in $[Ca^{2+}]_i$ in isolated CFTE29o$^-$ cells cultured at 37°C and tested at 32°C ($\Delta[Ca^{2+}]_i = 18 \pm 3$ nM, $P > 0.47$; $n = 6$). Failure of forskolin to mediate a $Ca^{2+}{}_i$ response in CFTE29o$^-$ cells at this temperature may be explained by the absence of functional CFTR in the membrane of CF tissue under normal conditions (Denning et al., 1992).

Tempterature Sensitivity of Forskolin-$[Ca^{2+}]_i$ Response

Using a range of culturing and experimental temperature conditions, temperature sensitivity of the ΔF508-CFTR mutation, and temperature dependence of the forskolin-mediated $[Ca^{2+}]_i$ response was tested in 16HBE14o$^-$ monolayer and isolated cells and CFTE29o$^-$ isolated cells. In 16HBE14o$^-$ cells the forskolin-$Ca^{2+}{}_i$ response was temperature sensitive in such a way that increasing temperature increased $Ca^{2+}{}_i$ mobilization accordingly. For both monolayer and isolated 16HBE14o$^-$ cells, the largest response was observed for cells cultured at 37°C

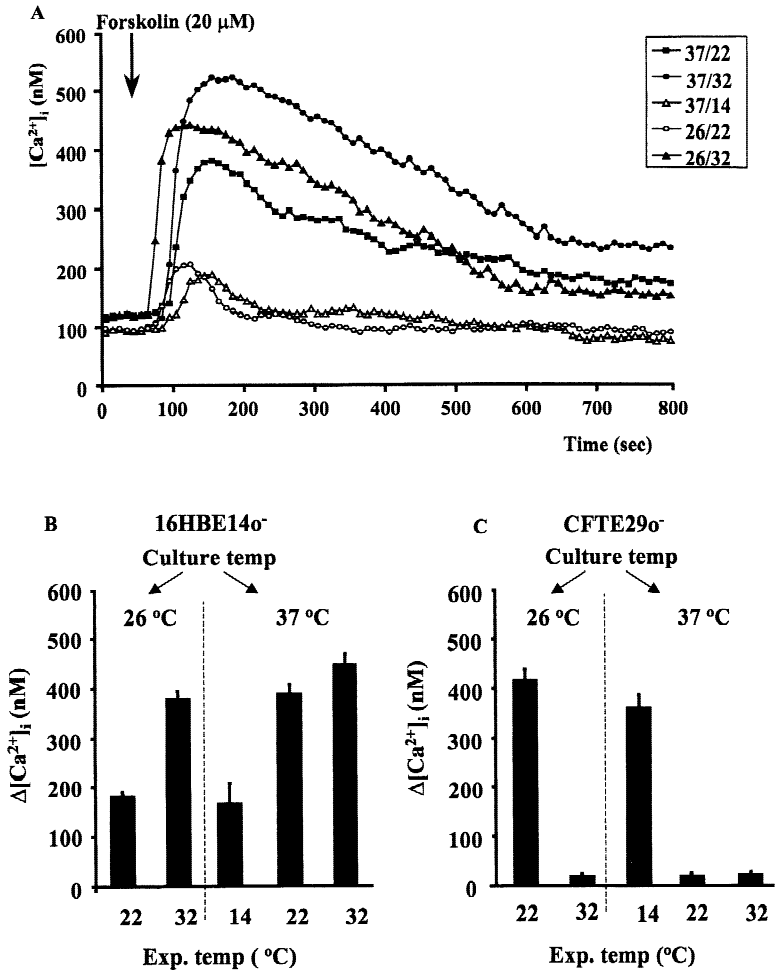

Fig 3. Temperature dependency of the forskolin (20 μM) effect on $[Ca^{2+}]_i$ in 16HBE14o$^-$ monolayers (*A*), in isolated 16HBE14o$^-$ cells (*B*) and in isolated CFTE29o$^-$ cells (*C*).

and tested at 32°C. The amplitude of the $[Ca^{2+}]_i$ rise was significantly decreased upon lowering the temperature at which cells were either cultured or tested (Fig. 3*A* and *B*). On the other hand, opposite temperature sensitivity of the forskolin-mediated $[Ca^{2+}]_i$ response was observed for CF cells when compared with normal cells (Fig. 3*C*). When forskolin (20 μM) was applied to CFTE29o$^-$ cells cultured at body temperature and tested at 22°C and 32°C, no significant change in $[Ca^{2+}]_i$ was observed. However, upon decreasing the temperature at which cells were cultured and tested, forskolin-mediated an increase in $[Ca^{2+}]_i$ (Fig. 3*C*). When CFTE29o$^-$ cells were cultured at 37°C, and then exposed to forskolin (20 μM) at the lower acute experimental temperature of 14°C, a threefold increase in $[Ca^{2+}]_i$ was observed ($P < 0.001$, $n = 6$). Similarly, forskolin had no effect on $[Ca^{2+}]_i$ when the culturing temperature of CFTE29o$^-$ cells was reduced to 26°C, and experiments performed at 32°C. For CF cells cultured at 26°C and tested at 22°C however, forskolin (20 μM) produced its maximum increase in $[Ca^{2+}]_i$, stimulating a greater than sixfold response, ($P < 0.001$; $n = 6$).

Opposite sensitivity of the $[Ca^{2+}]_i$ response to forskolin in 16HBE14o$^-$ and CFTE29o$^-$ cells, and the absence of temperature sensitivity on basal $[Ca^{2+}]_i$ and on the ATP-induced $[Ca^{2+}]_i$ response, is indicative of both defective insertion of the ΔF508-CFTR into the plasma membrane at body temperature, corrected by either acute, (14°C) or more gradual, (26°C) temperature reduction. In addition, these experiments also demonstrate CFTRs role in the forskolin-$Ca^{2+}{}_i$ response.

Glibenclamide and DPC Inhibition of Forskolin $[Ca^{2+}]_i$ Response

To assess the role of functional CFTR in the release of ATP and regulation of $[Ca^{2+}]_i$, cells were pretreated with the CFTR channel blockers glibenclamide and DPC prior to forskolin application. Incubation of 16HBE14o$^-$ and CFTE29o$^-$ cells with either of the CFTR channel blockers (100 μM for 5 min), had no effect on basal $[Ca^{2+}]_i$, but significantly downregulated the forskolin-mediated $Ca^{2+}{}_i$ response previously observed in both cell types

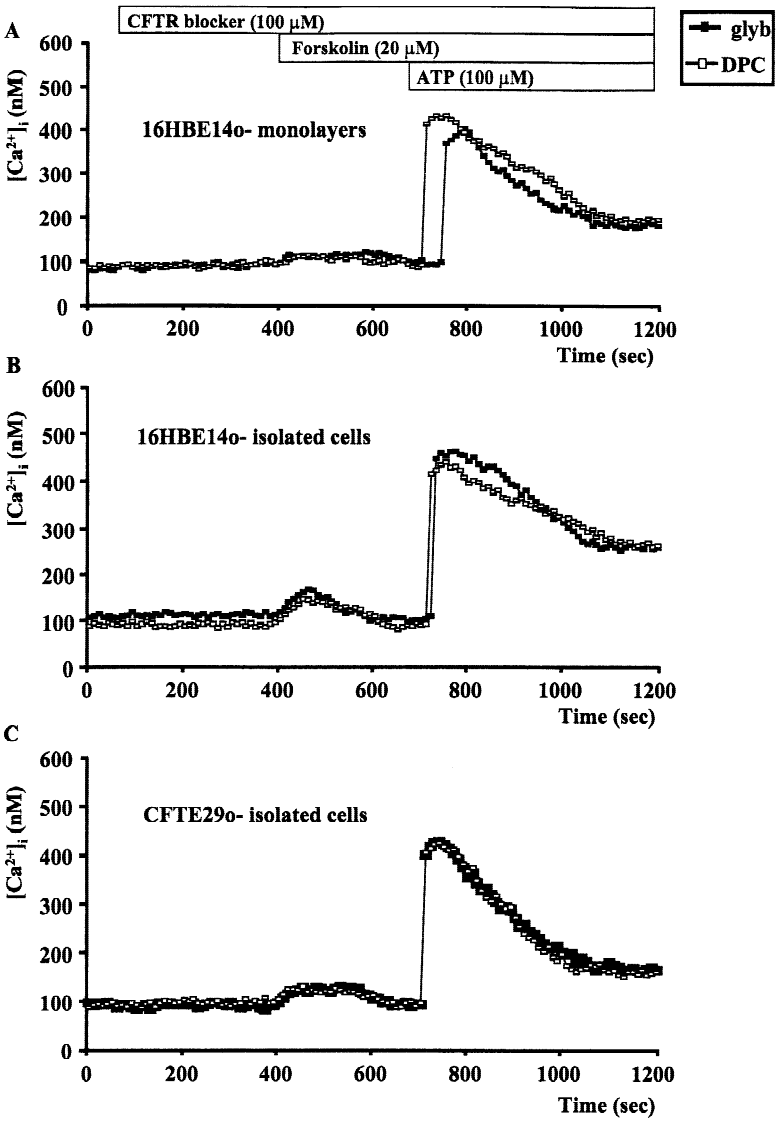

Fig 4. Representative tracings showing the effects of glibenclamide (100 μM) and DPC (100 μM) pre-treatment on forskolin (20 μM) and ATP (10^{-4} M) responses to $[Ca^{2+}]_i$ in 16HBE14o$^-$ monolayers (*A*), 16HBE14o$^-$ isolated cells (*B*) and CFTE29o$^-$ isolated cells (*C*). 16HBE14o$^-$ cells were incubated at 37°C and experiments performed at 32°C, and CFTE29o$^-$ cells were incubated at 26°C and experiments performed at 22°C.

(Fig. 4). To validate the site of action of the CFTR channel inhibitors, and to demonstrate their failure to inhibit purinoceptor activation by external nucleotides, their effect on ATP-induced $Ca^{2+}{}_i$ response was also determined. Following pretreatment with either glibenclamide or DPC (100 μM for 5 min), and after apical application of forskolin (20 μM), addition of apical ATP (10^{-4} M) stimulated a fourfold increase in $[Ca^{2+}]_i$ in both cell types (16HBE14o$^-$ monolayers: $P < 0.005$, $n = 6$; Fig. 4*A*), (16HBE14o$^-$ isolated cells: $P < 0.001$, $n = 6$; Fig. 4*B*), (CFTE29o$^-$ cells: $P < 0.002$, $n = 6$; Fig. 4*C*). Taken together, these results demonstrate that inhibition of CFTR, inhibits the forskolin-induced $Ca^{2+}{}_i$ response at a step prior to purinoceptor activation.

Hexokinase Inhibition of Forskolin $[Ca^{2+}]_i$ Response

To substantiate the hypothesis that cAMP activation of CFTR mediates ATP release, and to justify the role of ATP in the forskolin-Ca^{2+} response, the inhibitory effect of hexokinase on the forskolin-induced $[Ca^{2+}]_i$ response was tested. Hexokinase behaves as an ATP scavanger, trapping ATP in solution, cleaving the γ-phosphate and donating it to glucose, creating glucose-6-phosphate. Thus for all hexokinase experiments, glucose (5 μM) was included in the cell bathing solution.

Pretreatment of 16HBE14o$^-$ cell monolayers and isolated 16HBE14o$^-$ and CFTE29o$^-$ cells with hexokinase (0.5 U/mg for 10 min), had no significant effect on basal $[Ca^{2+}]_i$ but completely abolished the $Ca^{2+}{}_i$ response induced by forskolin (20 μM) (Fig. 5). As a control experiment and to illustrate the specific inhibitory action of hexokinase/glucose on the ATP-Ca^{2+} response, the effect of hexokinase pretreatment on ATP- and UTP-induced $[Ca^{2+}]_i$ response was tested in 16HBE14o$^-$ cell monolayers. As expected, ATP stimulated a transient increase in $[Ca^{2+}]_i$ before being consumed by hexokinase/glucose,

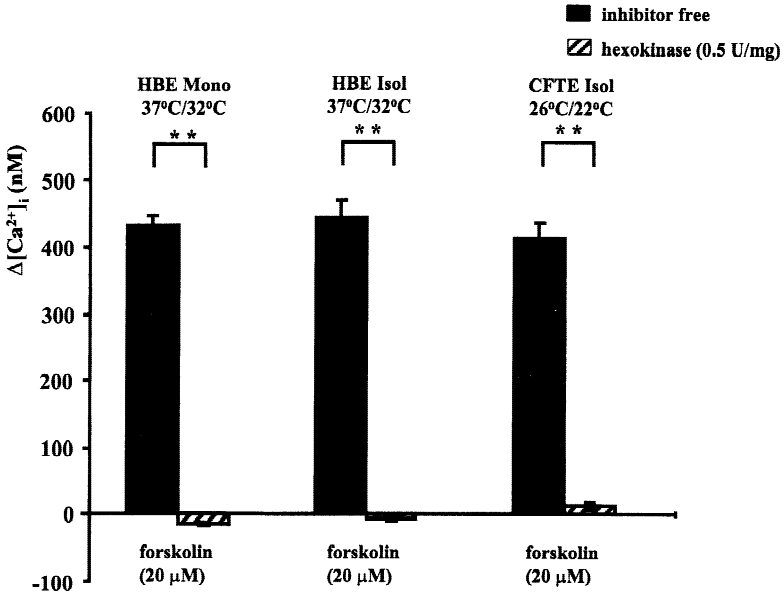

Fig 5. Inhibition of the $[Ca^{2+}]_i$ response to forskolin (20 μM) following pre-treatment with hexokinase (0.5 U/mg for 10 min). 16HBE14o$^-$ cells were incubated at 37°C and experiments performed at 32°C, and CFTE29o$^-$ isolated cells were incubated at 26°C and experiments performed at 22°C. (**$P > 0.001$, $n = 6$).

but the UTP-$Ca^{2+}{}_i$ response was unaffected by pretreatment with this scavenger (*results not shown*).

Taken together, these results demonstrate the role of ATP in the forskolin-Ca^{2+} response. Consumption of extracellular ATP by hexokinase/glucose inhibits forskolin activation and CFTR regulation of $[Ca^{2+}]_i$ at a step prior to purinoceptor activation, thereby substantiating the hypothesis that cAMP activation of CFTR mediates endogenous release of ATP.

Suramin Inhibition of Forskolin $[Ca^{2+}]_i$ Response

The role of purinoceptors in the $[Ca^{2+}]_i$ response to forskolin was investigated by pretreating 16HBE14o$^-$ monolayer and isolated cells and CFTE29o$^-$ isolated cells with the competitive purinoceptor antagonist, suramin (100 μM for 5 min). Suramin (100 μM) had no effect on basal $[Ca^{2+}]_i$ level, but significantly abolished the forskolin (20 μM) mediated $[Ca^{2+}]_i$ responses (Fig. 6). As a control it was demonstrated that 16HBE14o$^-$ cell monolayers pretreated with apical suramin (100 μM for 5 min), failed to mediate an ATP-$Ca^{2+}{}_i$ increase (*results not shown*). Together, these results demonstrate the selectivity of suramin for ATP-sensitive purinoceptors, and verify that ATP release following CFTR activation by forskolin, regulates $[Ca^{2+}]_i$ via a receptor-mediated mechanism.

Discussion

The hypothesis that CFTR mediates an autocrine function in normal and CF human airway epithelial cells by releasing ATP and regulating $[Ca^{2+}]_i$ via purinoceptor activation, was tested in this study. Given the central role for nucleotides in this response, the first step involved identification of purinoceptor existence in airway epithelia by determining the effect of a wide range of external nucleotides on $[Ca^{2+}]_i$. Purinoceptors which respond to extracellular nucleotides by increasing $[Ca^{2+}]_i$ and/or activating Cl^- secretion, have previously been identified in human airway epithelia (Brown et al., 1991; Knowles et al., 1991; Mason et al., 1991; Clarke and Boucher, 1992; Stutts et al., 1992; Parr et al., 1994; Hwang, Schwiebert & Guggino, 1996). Prior to this present study, a P_{2U} ($P2Y_2$) receptor was identified in 16HBE14o$^-$ cells that responded equipotently to ATP and UTP to stimulate Ca^{2+}-dependent Cl^- channel (CaCC) activity (Koslowsky et al., 1994). In this study, the similarities obtained between the nucleotide-Ca^{2+} responses and potency orders in 16HBE14o$^-$ and CFTE29o$^-$ cells, indicate that the purinoceptor subtypes and signaling pathways involved in Ca^{2+} homeostasis are unaffected by the ΔF508 mutation in cultured airway epithelia.

Activation of CFTR, and investigation of its proposed role in $[Ca^{2+}]_i$ regulation was achieved using the potent secretagogue forskolin. Contradictory effects of forskolin on $[Ca^{2+}]_i$ have previously been described: forskolin is reported to have no effect on $[Ca^{2+}]_i$ in human nasal epithelial cells (Verbeek et al., 1990; Paradiso, Cheng & Boucher, 1991) or in human tracheal epithelial cells (Yamaya et al., 1993), but has been shown to produce an increase in $[Ca^{2+}]_i$ in murine tracheal epithelium (Grubb, Paradiso & Boucher, 1994) and murine nasal epithelium (MacVinish et al., 1998). Incubation of murine tracheal epithelium with BAPTA-AM or application of ionomycin prior to forskolin, significantly decreased

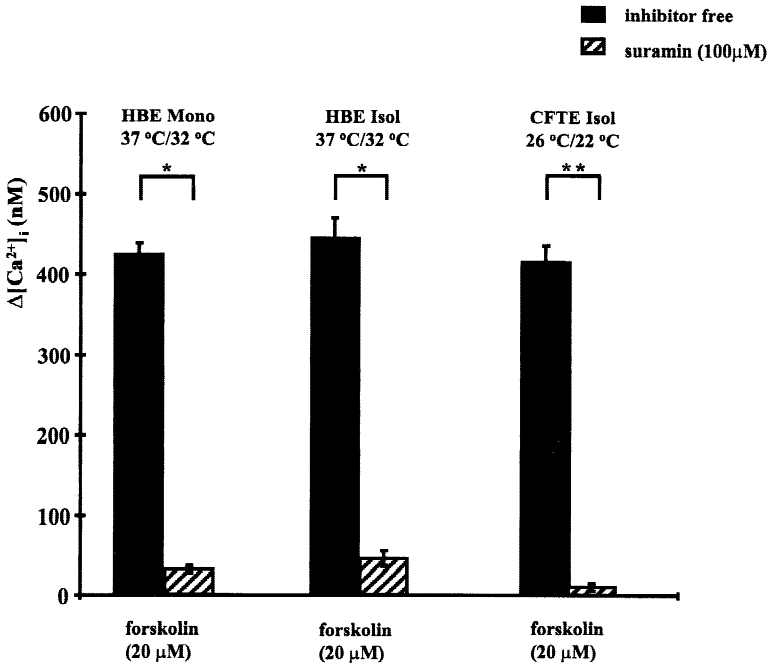

Fig 6. Inhibition of the $[Ca^{2+}]_i$ response to forskolin (20 μM) following pretreatment with suramin (100 μM for 5 min). The 16HBE14o$^-$ cells were incubated at 37°C and experiments performed at 32°C, and CFTE29o$^-$ isolated cells were incubated at 26°C and experiments performed at 22°C. (*$P < 0.005$, **$P < 0.001$, $n = 6$).

the forskolin-induced Cl^- secretory response (Grubb et al., 1994). These results indicate that by increasing $[Ca^{2+}]_i$, forskolin induces Cl^- secretion by upregulating Ca^{2+}-dependent Cl^- channel activity. In murine nasal epithelium release of $Ca^{2+}{}_i$ by the Ca^{2+}-releasing agents TBHQ-ionomycin, attenuated the ability of forskolin to cause further release, thereby indicating that at least some of the Ca^{2+} storage sites are common to both agents (MacVinish et al., 1998).

In this study, forskolin (20 μM) stimulated an increase in $[Ca^{2+}]_i$ at temperatures close to body temperature in epithelial cells expressing normal levels of CFTR protein-NHPL and 16HBE14o$^-$ cells, and also in the colonic carcinoma cell line, T84 (*unpublished observation*). On the contrary, in ΔF508-CFTR expressing cells (CFTE29o$^-$ cells) cultured at body temperature, forskolin failed to stimulate an increase in $[Ca^{2+}]_i$ at temperatures between 22 and 32°C. This is in accordance with a similar result obtained using adenovirus-CFTR infected CHO cells, where forskolin induced an increase in $[Ca^{2+}]_i$, but failed to stimulate Ca^{2+} mobilization in native CHO cells which do not spontaneously express CFTR protein (Urbach & Harvey, 1999). However, in this study, when CFTE29o$^-$ cells were either cultured or tested at a lower temperature a forskolin-mediated $[Ca^{2+}]_i$ response was observed. These results are consistent with the fact that processing of the mutant ΔF508-CFTR protein is temperature sensitive, and at reduced temperature some of the mutant protein escapes from the ER, is fully glycosylated in the Golgi complex, and delivered to the cell membrane (Denning et al., 1992; French et al., 1996).

CFTE29o$^-$ cells cultured at 37 and 26°C, and tested at 14 and 22°C respectively, produced a $Ca^{2+}{}_i$ response to forskolin that was equivalent to 80% of the largest response obtained at body temperature in normal cells. French et al. (1996) previously reported an increase of ΔF508-CFTR expression to 16% of normal levels when cells were grown at 27°C. Denning et al. (1992) reported that when the culture temperature was decreased to 23°C, 30% of the mutated CFTR was found in the glycosylated form. There is no report comparing the rate of insertion of ΔF508-CFTR and CFTR at lower temperature (14°C). In normal airway epithelia, temperature dependence of the forskolin response was opposite to CF airway epithelia in such a way that increasing temperature increased the $Ca^{2+}{}_i$ response. The possibility of a temperature dependency on the Ca^{2+} signaling pathway was dismissed following the demonstration that neither basal nor ATP-induced $[Ca^{2+}]_i$ were affected over the tested temperature range of 14–32°C. Therefore, the opposite temperature dependency of CFTR and ΔF508-CFTR expression may explain the opposite temperature dependency of the forskolin-induced $Ca^{2+}{}_i$ mobilization observed for 16HBE14o$^-$ and CFTE29o$^-$ cells. Together, these results demonstrate that the autocrine functionality of CFTR in regulating $[Ca^{2+}]_i$ is intact in ΔF508 cells at reduced temperature.

Further evidence for the role of CFTR activity in mediating the forskolin-induced $[Ca^{2+}]_i$ response was achieved by demonstrating the inhibitory effect of the CFTR channel blockers glibenclamide (Sheppard & Welsh, 1993) and DPC (McCarty et al., 1993). Since these drugs do not affect the $[Ca^{2+}]_i$ response to exog-

enous nucleotides, their site of action must be proximal to purinoceptor activation. Evidence for stimulation of purinoceptors by endogenous ATP released in response to forskolin treatment in normal and CF airway epithelia, is supported by the inhibitory effect of the ATP scavenger, hexokinase and the purinoceptor antagonist, suramin. Specificity of hexokinase and suramin inhibition on external ATP during the forskolin-mediated $[Ca^{2+}]_i$ response in airway epithelial cells was verified by demonstrating that in 16HBE14o$^-$ monolayers, hexokinase down-regulates the $Ca^{2+}{}_i$ response induced by external ATP but not by external UTP, and that suramin selectively blocks the ATP mediated $[Ca^{2+}]_i$ response. Thus, the opposite temperature dependence of forskolin on $[Ca^{2+}]_i$ in normal and CF airway epithelial cells observed in this study, as well as the abolition of this response by inhibitors acting at steps proximal to the $Ca^{2+}{}_i$ signaling cascade, argue in favor of an autocrine role for CFTR in regulating $[Ca^{2+}]_i$ via nucleotide release. Taken together, the results of this study indicate involvement of the CFTR protein in the forskolin-induced $Ca^{2+}{}_i$ response in human airway epithelia via an autocrine mechanism involving purinoceptor activation by endogenous release of nucleotides from the cell. Moreover, the data from CF airway epithelia indicate that once inserted into the membrane, ΔF508-CFTR can support an autocrine mobilization of $Ca^{2+}{}_i$.

The evidence provided in this study supporting an autocrine function of the CFTR channel in mediating ATP release and regulating $[Ca^{2+}]_i$, appears to reconcile the known channel function of CFTR with emerging data supporting a role for CFTR as a regulator of other ion channels. However, much controversy exists regarding the relationship between CFTR and ATP, fueled by the uncertainty of whether the CFTR conducts ATP directly or regulates its conductance through an associated channel. The simplest explanation for CFTR regulation of ATP release and $Ca^{2+}{}_i$ mobilization is to suggest direct ATP mediation via the intrinsic CFTR Cl^- channel. Although several studies have demonstrated an ATP conductance of the CFTR channel (Abraham et al., 1992; Reisin et al., 1992, 1994; Cantiello et al., 1994; Paysk and Foskett, 1995; Schwiebert et al., 1995; Prat et al., 1996; Cantiello et al., 1998), this theory is currently rather controversial because it has not been observed by all investigators (Grygorczyk, Tabcharani & Hanrahan, 1996; Li et al., 1996; Reddy et al., 1996; Grygorczyk & Hanrahan, 1997). However, many of these studies do not dismiss the possibility that the CFTR may be closely associated with separate ATP permeation pathways. Thus another explanation for the connection between CFTR and ATP release may be that ATP is transported via a heterologous pathway that is activated by CFTR, independently of its Cl^- channel activity. In a recent study (Schwiebert et al., 1998) it was demonstrated that the ability of CFTR to function as a Cl^- channel and as a conductance regulator are not mutually exclusive: one function could be eliminated while the other was preserved. In support of this hypothesis, Taylor et al. (1998) recently demonstrated that under severe hypotonic challenge, the ATP release mechanism is expressed by CF epithelia, and may be an entity separate from but regulated by the CFTR. In this present study, the $Ca^{2+}{}_i$ response to CFTR activation was inhibited by two CFTR channel blockers: glibenclamide and DPC. These results therefore favor the hypothesis that ATP is trafficked via CFTR, or at least that Cl^- conductance through CFTR is necessary for ATP release.

The possibility of vesicle trafficking whereby CFTR facilitates insertion of additional channel proteins into the membrane has also been proposed (Bradbury et al., 1992; Takahasi et al., 1996). Alternatively, CFTR-mediated ATP release may involve intermediate proteins such as cytoskeletal components (Cantiello, 1996). Recent studies have identified the presence of a PDZ-binding domain at the extreme COOH terminus of the CFTR that may be associated with anchoring proteins that link transmembrane glycoproteins to actin-binding proteins and the actin cytoskeleton (Short et al., 1998; Wang et al., 1998). These studies reinforce the hypothesis concerning regulatory cofactors that may mediate CFTR-regulated ATP conductance and confer CFTR regulation on separate yet closely associated ion channel proteins (Sugita et al., 1998).

To conclude, the involvement of CFTR in the regulation of $[Ca^{2+}]_i$ may explain its key role in regulating the activity of other ion channel transporters. In airway epithelial cells, it has been demonstrated that cAMP-stimulated and CFTR-dependent release of ATP out of the cell is essential to CFTRs regulatory interaction with the ORCC (Schwiebert et al., 1995). In addition, stimulation of ORCC activity by ATP was shown to involve $P2Y_2$ purinergic receptor activation, and possibly $Ca^{2+}{}_i$ mobilization. In a comparable study using tracheal epithelium, Hwang et al., (1996) demonstrated that apical ATP increased CaCC activity via a Ca^{2+}-dependent signaling pathway involving activation of luminal $P2Y_2$ receptors. As a corollary to our study, increasing $[Ca^{2+}]_i$ via activation of the CFTR autocrine pathway, may in turn activate an alternative CaCC conductance which is apparently preserved in CF airway epithelia (Boucher et al., 1989), thereby compensating at least in part, the defective CFTR channel. In addition, the effect of $Ca^{2+}{}_i$ on Cl^- secretion is reinforced through the activation of Ca^{2+}-dependent basolateral K^+ channels. Increasing $[Ca^{2+}]_i$ in normal and CF airway epithelia, increases basolateral K^+ secretion, thereby hyperpolarizing the apical membrane and increasing the driving force for Cl^- exit through CaCCs as well as apical Cl^- channels that are open under basal conditions (Welsh & McCann, 1985;

Welsh & Liedtke, 1986). CFTR expression is also linked to the cAMP-dependent regulation of amiloride-sensitive Na^+ channels (Boucher et al., 1988). Consequently, Na^+ permeability is abnormal in CF affected airway epithelia (Stutts et al., 1995). Preliminary data in other cell systems support the hypothesis that CFTR regulation of Na^+ conductance is linked to the role of CFTR as a mediator of ATP release (Ma et al., 1996). Together, these results suggest that regulation of $[Ca^{2+}]_i$ by the CFTR protein may control the balance between secretion and absorption in airway epithelia, by concurrently stimulating Cl^- secretion while inhibiting excess Na^+ absorption.

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