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Cystic Fibrosis Transmembrane Regulator-independent Release of ATP

ITS IMPLICATIONS FOR THE REGULATION OF $P2Y_2$ RECEPTORS IN AIRWAY EPITHELIA*

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The cystic fibrosis (CF) transmembrane regulator (CFTR) is a cyclic AMP-dependent Cl^- channel that is defective in CF cells. It has been hypothesized that CFTR exhibits an ATP release function that controls the airway surface ATP concentrations. In airway epithelial cells, CFTR-independent Ca^{2+}-activated Cl^- conductance is regulated by the $P2Y_2$ receptor. Thus, ATP may function as an autocrine signaling factor promoting Cl^- secretion in normal but not CF epithelia if ATP release is defective. We have tested for CFTR-dependent ATP release using four independent detection systems. First, a luciferase assay detected no differences in ATP concentrations in the medium from control *versus* cyclic AMP-stimulated primary normal human nasal epithelial (HNE) cells. A marked accumulation of extracellular ATP resulted from mechanical stimulation effected by a medium displacement. Second, high pressure liquid chromatography analysis of ^3H-labeled species released from [^3H]adenine-loaded HNE cells revealed no differences between basal and cyclic AMP-stimulated cells. Mechanical stimulation of HNE cells again resulted in enhanced accumulation of extracellular [^3H]ATP and [^3H]ADP. Third, when measuring ATP concentrations via nucleoside diphosphokinase-catalyzed phosphorylation of [α-^{33}P]dADP, equivalent formation of [^{33}P]dATP was observed in the media of control and cyclic AMP-stimulated HNE cells and nasal epithelial cells from wild-type and CF mice. Mechanically stimulated [^{33}P]dATP formation was similar in both cell types. Fourth, 1321N1 cells stably expressing the human $P2Y_2$ receptor were used as a reporter system for detection of ATP via $P2Y_2$ receptor-promoted formation of [^3H]inositol phosphates. Basal [^3H]inositol phosphate accumulation was of the same magnitude in control and CFTR-transduced cells, and no change was observed following addition of forskolin and isoproterenol. In both cell types, mechanical stimulation resulted in hexokinase-attenuable [^3H]inositol phosphate formation. In summary, our data suggest that ATP release may be triggered by mechanical stimulation of cell surfaces. No evidence was found supporting a role for CFTR in the release of ATP.

The wide distribution of cell surface P2 receptors (1, 2) and the presence in most tissues of ectoenzymes that rapidly degrade extracellular nucleotides (3) support the notion that regulated process(es) for the cellular release of ATP may exist. Indeed, ATP has been found in an extracellular location in many tissues (4–10), and ATP secretion from intracellular granules during platelet activation as well as nerve transmission are well described events where physiological release of nucleotides occurs (4). However, in most tissues where significant ATP concentrations in the extracellular space have been detected, the mechanism(s) of ATP release have not been identified.

In the airways, the volume and composition of the liquid secretions may be regulated by extracellular nucleotides (11–14). In cystic fibrosis (CF)[1] airway epithelia, the $P2Y_2$ receptor is linked to a Ca^{2+}-dependent chloride channel that provides an alternative Cl^- secretory pathway to the CF transmembrane regulator (CFTR) Cl^- channel (11). This alternative chloride channel (Cl_a) has been identified as a potential target for therapy of CF lung disease. The localization of $P2Y_2$ receptors on the apical surface of airway epithelia suggests the possibility that these receptors are regulated endogenously by the release of ATP onto the lumen.

It has been recently proposed that CFTR itself modulates the composition of airway surface liquids by acting as a channel for ATP, regulating Cl_a pathways via activation of $P2Y_2$ receptors (15). In CF with defective CFTR, an implication of this hypothesis is that resting levels of ATP would be reduced, resulting in reduced Cl_a as well as CFTR activation. The notion that CFTR mediates the release of ATP evolved from studies showing that protein kinase A stimulated a single channel (CFTR) current in CFTR-expressing cells when 100 mM ATP was present in the intracellular compartment (15, 16). In addition, CFTR was shown to regulate the activity of a second chloride channel in excised patches, an activity thought to reflect ATP release and activation of outwardly rectifying Cl^- channels by $P2Y_2$ receptors (15). However, other studies found either no evidence for CFTR-mediated ATP conductance (17–19) or that some but not all CFTR Cl^- channels could be associated with an ATP permeability (20). Measurements of ATP released from cells have also produced results that either support (15, 21) or do not support (22, 23) a role for CFTR in the regulation of ATP release. The current debate (24–26) reflects the uncertainty that prevails on this issue (reviewed in Ref. 27). In this study, we tested the function of CFTR as a pathway for ATP release

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[1] The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; Cl_a, alternative chloride channel; HNE, human nasal epithelial; DMEM-H, Dulbecco's modified high glucose Eagle's medium; HPLC, high pressure liquid chromatography; NDPK, nucleoside diphosphokinase.

using both biochemical methods for directly measuring ATP release in intact cells and an assay utilizing the human $P2Y_2$ receptor as a biological reporter for ATP release within the relevant cellular biophase (receptor domain).

MATERIALS AND METHODS

Cell Culture—Primary cultures of human nasal epithelial (HNE) cells and immortalized nasal epithelial cells from either normal or CF mice (28) were grown as polarized epithelia on 12.5-mm porous Transwell Col filters (Costar) as reported previously (29). Assays with HNE cells were carried out 7–10 days after seeding, a time coincident with the development of the maximal ion transport activity (29). T84 human colonic carcinoma cells, a cell line expressing high levels of endogenous CFTR (30), were grown as a polarized epithelium onto cross-linked collagen supports as described previously (30). Control and CFTR-transduced NIH-3T3 fibroblasts were grown on 12-well plastic plates as described (31). HP2U-1321N1 cells, a clonal cell line derived from human astrocytoma cells stably expressing the human $P2Y_2$ receptor (32), were grown on 12-well plastic plates (inositol phosphate assay) or on 6-well plates ($^{36}Cl^-$ efflux measurement and Western blotting) in DMEM-H containing 5% fetal bovine serum and antibiotics as described previously (9).

Luciferin/Luciferase Assay—HNE cells were preincubated for 1 h in a Krebs-Ringer solution (2.4 mM K_2HPO_4, 0.4 mM KH_2PO_4, 115 mM NaCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 25 mM NaH_2CO_3, and 5 mM glucose) at 37 °C and 5% CO_2. The cells were exposed for the indicated times to the designated drug. The mucosal medium (0.3 ml) was collected, centrifuged to remove potentially detached cells, and boiled for 1 min. A 100-μl sample aliquot was diluted with 200 μl of H_2O prior to measurements. The luciferin/luciferase mixture (300 μM luciferin, 5 μg/ml luciferase, 25 mM HEPES (pH 7.8), 6.25 mM $MgCl_2$, 0.63 mM EDTA, 75 μM dithiothreitol, and 1 mg/ml bovine serum albumin) was added to samples via an LB953 AutoLumat luminometer (Berthold GmbH, Wildbad, Germany), and the sample luminescence was compared with an ATP standard curve performed for each individual experiment. To assess intracellular ATP content, cells were lysed with 5% trichloroacetic acid followed by ethyl ether extraction and neutralization.

Release of [^{3}H]Adenine-labeled Nucleotides—The cells were labeled for 3 h with 10 μCi/ml [^{3}H]adenine as described previously (33). Labeled cells were exposed to the indicated drug without washing away the label to avoid unnecessary agitation of the cells. The mucosal medium was collected, and ^{3}H-labeled species were resolved by high pressure liquid chromatography (HPLC).

Nucleoside Diphosphokinase (NDPK)-catalyzed Formation of [α-^{33}P]dATP—NDPK catalyzes the phosphorylation of nucleoside diphosphates utilizing ATP as the γ-phosphate donor molecule (34). We used [α-^{33}P]dADP, obtained as described previously (29), as the acceptor molecule to quantitatively determine the formation of [α-^{33}P]dATP as a function of ATP concentration. HNE cells and nasal epithelial cells from either wild-type or CF mice were preincubated for 1 h in 0.3 ml of (mucosal) HEPES (pH 7.4)-buffered DMEM-H (HEPES/DMEM). Incubations were in the presence of 0.5 units/ml NDPK and 10 nM [α-^{33}P]dADP (0.2 μCi) added to the mucosal bath. ^{33}P-Labeled species were resolved by HPLC.

Quantification of Nucleotides by HPLC—Nucleotides were separated by HPLC (Shimadzu) via a strong anion-exchange column (Rainin Instrument Co. Inc.) with a mobile phase developed from 0.45 M NH_4COOH (pH 4.8) to 0.5 M $Na_2H_2PO_4$ (pH 2.7) over a 30-min period (9). Radioactivity was measured on line with a Radiomatic 500TR analyzer (Packard Instrument Co.). Species were identified and quantified as described previously (9).

Release of ^{51}Cr from HNE Cells—Confluent polarized HNE cells were incubated for 3 h in HEPES/DMEM containing 10 μCi of [^{51}Cr]Na_2CrO_3 added to the mucosal bath. The cells were washed (four times) and preincubated for 1 h in HEPES/DMEM. The mucosal solution (0.5 ml) was removed at the times indicated, and the radioactivity was quantified with a Cobra Autogamma counter (Packard Instrument Co.).

Expression of CFTR in HP2U-1321N1 Astrocytoma Cells—Subconfluent HP2U-1321N1 cells grown on 12-well plastic plates were infected with adenoviral vector (5×10^9 particles/well) containing DNA encoding either the CFTR gene (AdCFTR) or *LacZ* gene (AdLacZ) as described (31). Cells were assayed for CFTR expression 48 h after infection. For Western blot analysis, CFTR was localized with an antiserum raised against a carboxyl-terminal peptide of CFTR (35). [^{36}Cl]Chloride efflux was quantified as described previously (31).

Measurement of [^{3}H]Inositol Phosphates—HP2U-1321N1 cells were labeled overnight in 0.5 ml of inositol-free DMEM-H containing 4 μCi/ml *myo*-[^{3}H]inositol. At the time of assay, 10 mM LiCl was added to the cells for 15 min, followed by a further 15-min incubation in the presence of the indicated drugs. Incubations were terminated by the addition of 5% trichloroacetic acid, and the resulting [^{3}H]inositol phosphates were separated and quantified by chromatography on Dowex columns as described previously (9).

Reagents—ATP and dATP were purchased from Pharmacia (Uppsala, Sweden). Hexokinase, NDPK, forskolin, and dADP were from Boehringer Mannheim. Luciferin, luciferase, and isoproterenol were obtained from Sigma. [^{3}H]Adenine (17 Ci/mmol) and *myo*-[^{3}H]inositol (20 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO). [α-^{33}P]dATP (3000 Ci/mmol), [^{51}Cr]Na_2CrO_3 (300–500 mCi/mg cromium), and [^{36}Cl]NaCl (3 mCi/g chloride) were from Amersham Pharmacia Biotech.

Expression of the Results—Except where stated otherwise, pooled data are expressed as means ± S.E. and are representative of at least three independent experiments performed with duplicate or triplicate samples. For statistical comparisons, unpaired *t* test was used, and $p < 0.05$ was considered significant.

RESULTS

Release of ATP from HNE Cells—ATP was quantified in the mucosal medium bathing HNE cells utilizing the luciferin/luciferase method. Under resting conditions, *i.e.* the cells were kept undisturbed for 1 h prior to the assay, accumulation of ATP was 18.5 ± 3.4 pmol/10^6 cells (3.3 ± 0.6 nM in 0.3 ml of medium; $n = 9$). Cell monolayers incubated with forskolin and isoproterenol (20 μM each, 10 min), a drug combination that is maximally effective in initiating cyclic AMP-dependent Cl^- secretion via CFTR (33), exhibited a concentration of (mucosal) ATP of 4.2 ± 0.7 nM (25.1 ± 4.1 pmol/10^6 cells; $n = 9$), a value not significantly different from control incubations. In contrast, perturbing the cell surface by a medium change (data not shown) or by gently pipetting the mucosal medium up and down twice resulted in a marked accumulation of mucosal ATP (49.5 ± 8 nM, 297 ± 61 pmol/10^6 cells). Mechanically released ATP by a medium displacement represented 0.9–1.6% of the cellular ATP content, and it was unaffected by exposing the cells to the cyclic AMP mixture (51 ± 12 nM, 306 ± 79 pmol/10^6 cells). The possibility that cell lysis occurred during mechanical stimulation was investigated with ^{51}Cr-loaded HNE cells. No changes in ^{51}Cr base-line levels were observed during a 10-min period following a medium displacement (Table I). Moreover, base-line radioactivity remained unchanged after five repetitive medium displacements (data not shown). Thus, although we cannot rule out the possibility that a small number of damaged cells contributed to ^{51}Cr base-line levels, our results suggest that ATP was released from intact (not damaged) cells. Consistent with this, we have recently shown that no cell lysis occurred during mechanically promoted ATP release from 1321N1 cells (36).

Release of [^{3}H]Adenine-labeled Nucleotides—We loaded the intracellular pool of ATP with [^{3}H]adenine, and the release of ^{3}H-labeled species was quantified by HPLC. Because the relatively large release of [^{3}H]ATP during medium changes (data not shown and Ref. 9) could mask a potential contribution by CFTR, a protocol was utilized that avoided cell washes. HPLC analysis of (mucosal) medium bathing resting [^{3}H]adenine-labeled HNE cells demonstrated a small peak of [^{3}H]ADP, barely distinguishable from background levels (Fig. 1*A*) (nonincorporated [^{3}H]adenine eluted at 5–8 min). No increase in [^{3}H]ATP or [^{3}H]ADP accumulation was observed in forskolin/isoproterenol-stimulated HNE cells (Fig. 1*B*). Incubation of cells with ionomycin to induce elevation of intracellular Ca^{2+} resulted in a small accumulation of extracellular [^{3}H]ATP and greater accumulation of [^{3}H]ADP (Fig. 1*C*). A marked accumulation of [^{3}H]ATP and [^{3}H]ADP was observed after a medium displacement (Fig. 1*D*). This protocol was repeated with T84 cells and with NIH-3T3 cells stably expressing either normal CFTR or the α-subunit of the interleukin-2 receptor as a control

TABLE I
Release of ^{51}Cr from HNE cells

Mechanical stimulation was applied to [^{51}Cr]Na_2CrO_3-labeled HNE cells by gently pipetting up and down 0.25 ml of the mucosal medium twice. Undisturbed and stimulated cells were incubated for the times indicated, and the radioactivity released into the mucosal medium was quantified. The results are expressed as the means ± S.E. from triplicate samples, and they are representative of two independent experiments performed under similar conditions.

	Control	Mechanical stimulation
	cpm	*cpm*
0 min	775 ± 52	752 ± 30
1 min	806 ± 124	850 ± 172
5 min	772 ± 209	730 ± 182
10 min	780 ± 234	722 ± 75
SDS	17,043 ± 3995	19,117 ± 2556

(31). In all cases, addition of forskolin/isoproterenol (2–10 min) promoted no release of [^{3}H]adenine-labeled species, but a large accumulation of ^{3}H-labeled nucleotides was observed after a medium displacement irrespective of the presence of CFTR (data not shown).

ATP-dependent Conversion of [α-^{33}P]dADP to [α-^{33}P]dATP—The possibility that CFTR mediates the release of ATP from a nucleotide pool not accessed by [^{3}H]adenine (37) was investigated by trapping released ATP with NDPK and [α-^{33}P]dADP. The NDPK-catalyzed conversion of [α-^{33}P]dADP to [α-^{33}P]dATP was measured by HPLC as a function of ATP concentration (Fig. 2). Moreover, since exogenous NDPK activity greatly exceeds endogenous ecto-ATPase activity, the assay resulted in a system that effectively locks the γ-phosphate of ATP onto the [α-^{33}P]dADP molecule upon release. Fig. 3 illustrates the conversion of [α-^{33}P]dADP to [α-^{33}P]dATP in the mucosal HNE cell baths under various conditions. A basal conversion (16 ± 3%) of [α-^{33}P]dADP to [α-^{33}P]dATP was observed with cells that had not been treated with agonists (Fig. 2*A*). Addition of forskolin and isoproterenol (20 μM each) did not result in increased conversion (14 ± 4%) of [α-^{33}P]dADP to [α-^{33}P]dATP, indicating that elevation of cellular cyclic AMP did not promote the release of ATP (Fig. 2*B*). However, an ~2-fold greater conversion (26 ± 3%) of [α-^{33}P]dADP to [α-^{33}P]dATP was observed following addition of 1 μM ionomycin, consistent with a Ca^{2+}-promoted ATP release (Fig. 2*C*). A 4-fold increased formation (71 ± 6%) of [α-^{33}P]dATP occurred following mechanical stimulation of the cells (Fig. 2*D*). The calculated ATP concentrations were 6 ± 1, 5 ± 1, 17 ± 3, and 97 ± 11 nM for control cells, cAMP-stimulated cells, ionomycin-treated cells, and mechanically stimulated cells, respectively.

This protocol was repeated with immortalized nasal epithelial cells derived from either normal or CF mice. The results, summarized in Fig. 4, showed no differences between normal and CF cells under basal or forskolin/isoproterenol-stimulated conditions. The increased [^{33}P]dATP formation observed after mechanical stimulation was also similar in control and CF mouse cells. The muscarinic-cholinergic agonist carbachol promoted a small increase in [^{33}P]dATP formation, although this was not statistically significant.

The $P2Y_2$ Receptor as a Biosensor for ATP—Finally, in an attempt to assay for CFTR-dependent ATP release in the relevant physiological environment, *i.e.* the liquid layer associated with the cell surface, we used the $P2Y_2$ receptor as a biosensor for ATP to investigate the role of CFTR in the activation of $P2Y_2$ receptors in intact cells. As such, we took advantage of a cell line (1321N1 human astrocytoma cells) that is null for expression of P2 receptors (32). First, we achieved a high expression level of $P2Y_2$ receptors by retroviral infection of 1321N1 cells with the cDNA encoding the human $P2Y_2$ receptor (32). 1321N1 cells stably expressing the human $P2Y_2$ receptor (HP2U-1321N1 cells) exhibit a marked ATP-stimulated formation of inositol phosphates, whereas control cells are unresponsive (9). Overexpression of the $P2Y_2$ receptor in 1321N1 cells resulted in large receptor reserve, and consequently, ATP potency in HP2U-1321N1 cells (EC_{50} = 180 nM (9)) is increased 30-fold relative to ATP potency observed with the $P2Y_2$ receptor natively expressed in airway epithelial cells (38). More important, significant accumulation of inositol phosphates in HP2U-1321N1 cells was detectable at concentrations of ATP as low as 10 nM (see Fig. 6 and Ref. 9). Second, we infected the HP2U-1321N1 cells with an adenoviral vector containing the CFTR cDNA (AdCFTR) or with adenoviral vectors encoding the *LacZ* gene (AdLacZ) as a control and assayed for the effect of CFTR expression on the accumulation of inositol phosphates.

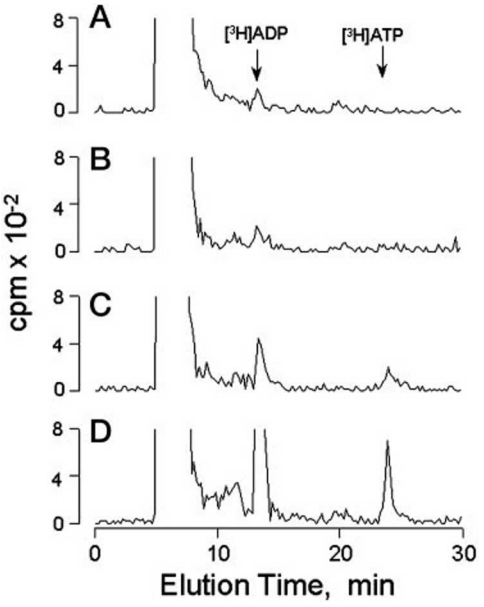

FIG. 1. **Release of ^{3}H-labeled species from [^{3}H]adenine-labeled HNE cells.** [^{3}H]Adenine-loaded HNE cells were incubated (300 μl of mucosal bath) for 10 min with vehicle (*A*), 20 μM isoproterenol and 20 μM forskolin (*B*), or 1 μM ionomycin (*C*), or they were subjected to a gentle medium displacement by withdrawing and placing back twice 150 μl of the mucosal medium (*D*). The [^{3}H]adenine-labeled species present in the mucosal medium were analyzed by HPLC as detailed under "Materials and Methods."

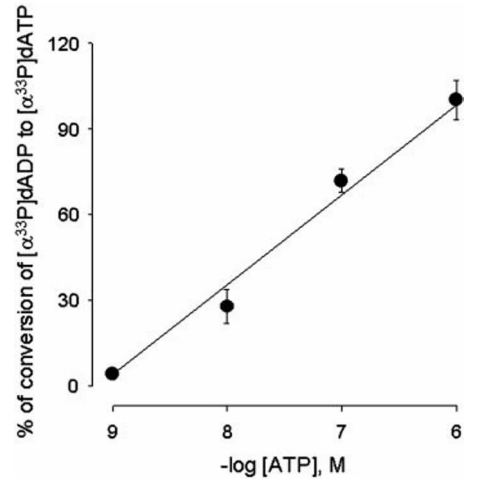

FIG. 2. **ATP-dependent conversion of [α-^{33}P]dADP to [α-^{33}P]dATP.** Incubations were for 10 min in 0.5 ml of DMEM-H containing 0.5 units/ml NDPK, 10 nM [α-^{33}P]dADP, and the indicated concentrations of ATP. The formation of [α-^{33}P]dATP was quantified by HPLC as indicated under "Materials and Methods." The data are the means ± S.E. from four experiments performed in duplicate.

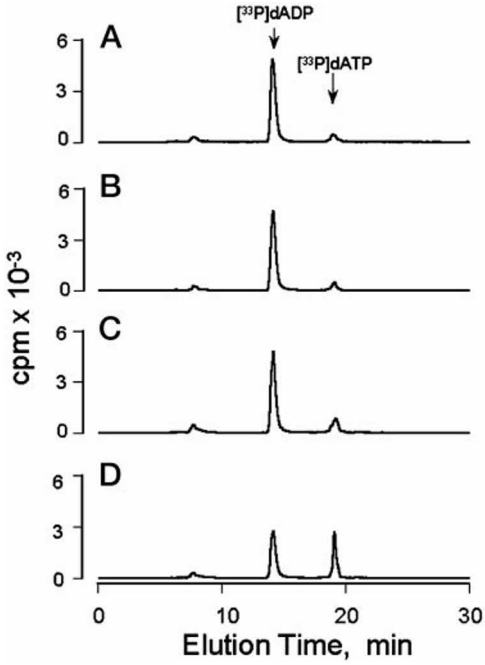

FIG. 3. **Conversion of [α-^{33}P]dADP to [α-^{33}P]dATP in the medium bathing HNE cells.** The cells were incubated with vehicle (*A*), forskolin and isoproterenol (*B*), or ionomycin (*C*), or they were subjected to a medium displacement (*D*) as detailed in the legend of Fig. 1. Incubations were for 10 min in the presence of 0.5 units/ml NDPK and 10 nM [α-^{33}P]dADP, and the resulting α-^{33}P-labeled species were quantified by HPLC as described under "Materials and Methods." All additions were to the mucosal medium. The results are representative of three experiments performed in duplicate.

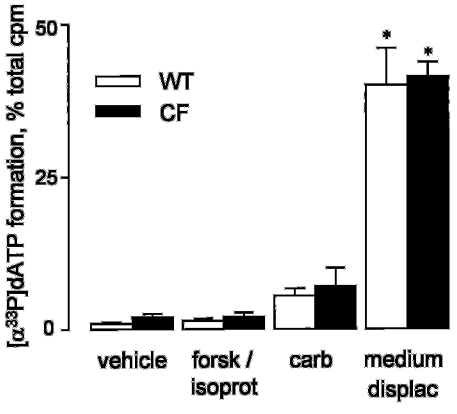

FIG. 4. **Conversion of [α-^{33}P]dADP to [α-^{33}P]dATP in the medium bathing mouse nasal epithelial cells.** Cells from wild-type (*WT*; *white bars*) or CF (*black bars*) mice were incubated with vehicle, 20 μM forskolin and 20 μM isoproterenol (*forsk/isoprot*), or 1 mM carbachol (*carb*), or they were subjected to a medium displacement (*medium displac*). Incubations were for 10 min in the presence of 0.5 units/ml NDPK and 10 nM [α-^{33}P]dADP, and the resulting α-^{33}P-labeled species were quantified by HPLC as descried under "Materials and Methods." All additions were to the mucosal medium. The results are expressed as the percent conversion of [α-^{33}P]dADP to [α-^{33}P]dATP, and the data represent the means ± S.E. from three experiments performed in duplicate. *, $p < 0.05$.

Fig. 5*A* shows a Western blot for CFTR of lysates from AdLacZ- or AdCFTR-infected cells. A band (~180 kDa), revealed by an anti-CFTR antiserum (antiserum 858 (35)), was present in AdCFTR-infected cells, but not in cells infected with the *LacZ* vector. A functional cyclic AMP-activated Cl^- permeability was demonstrated in AdCFTR-infected HP2U-1321N1 cells, but not in AdLacZ-infected cells, as indicated by forskolin- and isoproterenol-stimulated $^{36}Cl^-$ efflux (Fig. 5*B*).

Fig. 6 illustrates the effect of CFTR expression and cyclic

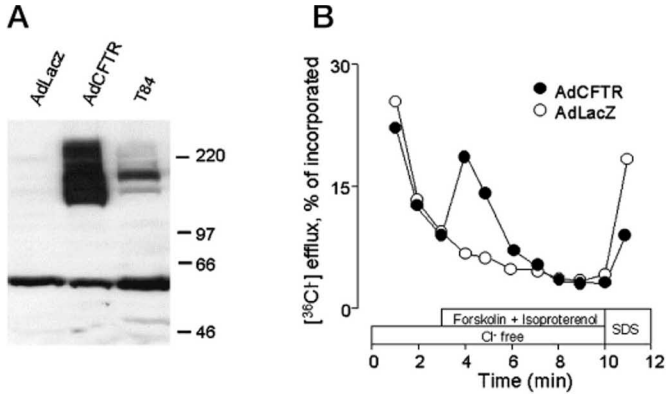

FIG. 5. **Expression of CFTR in HP2U-1321N1 cells.** Subconfluent HP2U-1321N1 cells grown on 12-well (*A*) or 6-well (*B*) plastic plates were infected with adenovirus harboring the CFTR (AdCFTR) or *LacZ* (AdLacZ) plasmid (31). *A*, AdLacZ-infected cells, AdCFTR-infected cells, and (control) T84 cells were lysed with urea lysis buffer, and proteins (50 mg/lane) were separated by SDS-polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose membrane. CFTR was localized with antiserum 858 (35). *B*, shown is released ^{36}Cl from ^{36}Cl-loaded cells. Forskolin and isoproterenol (20 μM each) were included following collection of the 3-min sample. The results are expressed as percent of the initial intracellular radioactivity and are representative of two independent experiments performed in triplicate that differed <10% from the mean. Error bars were omitted for clarity.

AMP-dependent activation on $P2Y_2$ receptor-promoted accumulation of inositol phosphates in HP2U-1321N1 cells. Basal accumulation of inositol phosphates was indistinguishable among control HP2U-1321N1 cells (*i.e.* cells that were not infected with adenovirus) and AdLacZ- and AdCFTR-infected HP2U-1321N1 cells, indicating that the mere expression of CFTR did not result in basal ATP-mediated activation of $P2Y_2$ receptors. To test the effect of cyclic AMP-activated CFTR, inositol phosphates were measured following addition of forskolin and isoproterenol. No increase in the total formation of inositol phosphates was observed with any cell type in response to this maneuver (Fig. 6). As a control for the sensitivity of the cells used in this assay, the cells were exposed to 10 nM exogenous ATP, and similar 3–4-fold increases in inositol phosphate accumulation were observed in the three cell types. We also tested for expression of a Ca^{2+}_i regulation of ATP release in astrocytoma cells by addition of ionomycin, which resulted in a significant accumulation of inositol phosphates in all three cell types (Fig. 6) that was abolished when hexokinase was included in the assay (data not shown). We have previously shown that wild-type as well as HP2U-1321N1 cells release considerable amounts of ATP into the medium during a medium change (9, 32). Consistent with these previous reports, displacement of the medium bathing HP2U-1321N1 cells resulted in a marked accumulation of inositol phosphates (Fig. 6) that was sensitive to hexokinase and that was similar in magnitude to accumulation stimulated by 100–300 nM ATP (data not shown). No difference in response to this maneuver was observed between control cells or cells expressing CFTR (Fig. 6). We conclude from these experiments that CFTR has no role in ATP release, whereas elevation of intracellular calcium and/or mechanical stimulation of HP2U-1321N1 human astrocytoma cells results in the release of ATP by a CFTR-independent mechanism.

DISCUSSION

This study demonstrates the presence of ATP in the mucosal surface liquid of nonstimulated airway epithelial cells and that accumulation of extracellular ATP increases substantially when cells are subjected to mechanical stimuli. Unlike previous studies in which CFTR was reported to act as an ATP channel

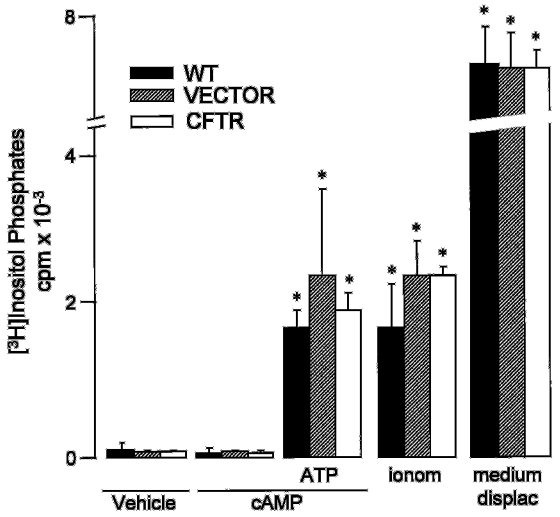

FIG. 6. **Effect of CFTR expression on $P2Y_2$ receptor-mediated formation of [^{3}H]inositol phosphates.** [^{3}H]Inositol-labeled HP2U-1321N1 cells and cells infected with AdLacZ or AdCFTR were incubated for 15 min under the indicated conditions. Forskolin and isoproterenol (*cAMP*) were added at 20 μM final concentrations, ATP at 10 nM, and ionomycin (*ionom*) at 1 μM. *medium displac* indicates withdrawing and gently replacing the medium twice. The results represent the means ± S.E. from four experiments performed in triplicate. *WT*, wild-type. *, $p < 0.05$.

(15, 16, 21), we found no evidence involving CFTR in the release of ATP from intact epithelial and non-epithelial cells. The cause of this discrepancy may reside, at least in part, in the methodological approaches.

Previously, studies implicating CFTR as an ATP channel have employed permeabilized cells, membrane patches, and/or repetitive changes of cell media and in general have subjected the cells to stresses not consistent with physiological conditions. We have used both classical methods for extracellular detection of ATP, *e.g.* the luciferase assay, and HPLC separation of ^{3}H-labeled nucleotides released from [^{3}H]adenine-preloaded cells as well as newly developed approaches for trapping ATP with NDPK and have adopted conditions in which sampling for ATP release was carefully controlled. Although we were able to detect ATP accumulation in the liquids bathing the surface of resting normal HNE cells, we observed no differences after incubating the cells with agents that promoted elevation of intracellular cyclic AMP. Moreover, no differences were found in extracellular ATP accumulation with nasal epithelial cells from normal or CF mice under basal conditions or as a function of cyclic AMP pathway stimulation. We have shown here that a marked release of ATP occurred after mechanical stimulation of airway epithelial cell surfaces by a medium displacement. However, mechanically stimulated release of ATP was of the same magnitude in normal or CF airway epithelial cells as well as in control or CFTR-expressing non-epithelial cells. A CFTR-independent mechanically stimulated ATP release was also recently reported with colonic and human airway epithelial cell lines (23).

To directly test the potential regulation of $P2Y_2$ receptors by CFTR, we have used a functional assay in which the $P2Y_2$ receptor acted as a biosensor for releasable ATP. We were able to couple the advantage of the high expression level of recombinant $P2Y_2$ receptors attainable by retroviral infection of 1321N1 cells with adenovirus-mediated expression of CFTR in these cells to test the effect of CFTR activation on $P2Y_2$ receptor-promoted inositol phosphate formation. Our hypothesis was that local accumulation of CFTR-releasable ATP in the unstirred and consequently difficult to sample liquid layer on the cell surface would be detected by coexpression of these two recombinant proteins on the surface of 1321N1 cells. Consistent with our previous reports showing mechanically promoted release of ATP from 1321N1 cells (9, 36), a sustained accumulation of inositol phosphates was observed following a medium displacement (Fig. 6). More important, inositol phosphate accumulation in 1321N1 cells was of the same magnitude regardless of CFTR expression, indicating that CFTR was not involved in mechanically induced ATP release. Although HP2U-1321N1 cells were sensitive to a 10 nM concentration of exogenously added ATP, no effect on inositol phosphates was observed following addition of forskolin and isoproterenol to CFTR-transduced HP2U-1321N1 cells as compared with control cells. Thus, activation of CFTR in 1321N1 cells did not result in accumulation of extracellular ATP in concentrations high enough to promote $P2Y_2$ receptor activation. In summary, by functionally expressing both CFTR and $P2Y_2$ receptors in a null cell line, we were able to directly test the hypothesis that CFTR regulates the activity of $P2Y_2$ receptors. We have found no evidence to support this hypothesis.

The presence of $P2Y_2$ receptors in the airway epithelia (32, 38) and their coupling to a Cl^- secretory pathway on the mucosal surface of HNE cells (11) suggest that extracellular accumulation of nucleotides might be important for the regulation of ion permeabilities. The recent identification of an ectonucleotidase activity on HNE cells (29) suggests one mechanism for regulating extracellular accumulation of nucleotides. Our present study indicates that ~20 pmol of ATP/10^6 cells accumulate at steady state on the mucosal surface of resting normal HNE cells. This closely resembles the ATP concentration observed in the lumen of normal human airways *in vivo* (39). Furthermore, ATP transiently accumulates following mechanical stimulation of cells in concentrations capable of activating $P2Y_2$ receptors. Previously, mechanically induced nonlytic release of ATP had been described in a variety of tissues. For example, changes in blood fluxes were shown to cause the release of ATP from endothelial cells (10, 40); shear forces promoted ATP release from mouse fibroblasts (6); and ATP release secondary to mechanical stimulation was also described with rat basophilic cells (7), 1321N1 human astrocytoma cells (9, 36), and rat hepatocytes (8). Stretch-promoted ATP release was reported with rabbit urinary bladder epithelial cells (41) and human lung adenocarcinoma Calu-3 cells (40), and ATP release triggered by bath turbulence was observed with T84 cells and with immortalized human tracheal epithelial cell lines (23, 26). Our current study extends these observations to primary human airway epithelial cells. It is not clear whether mechanical stimulation of airway epithelial cells and release across the epithelial surface occurs *in vivo* and what its functional significance might be. Mechanical forces applied on the airways by air fluxes may represent a primary mechanism for autocrine and paracrine regulation of electrolyte homeostasis in airway epithelia.

In conclusion, the techniques used in this study for detecting extracellular ATP are thought to closely approximate physiological conditions, creating a system in which pertinent and reliable information is generated. We found that ATP is released from each of the studied cell types in response to mechanical stimulation and/or secondary to elevation of intracellular calcium levels. No evidence was found indicating that CFTR was involved in ATP release in airway epithelia from two species. This study suggests that CFTR itself does not directly effect ATP or regulate other possible ATP release pathways in the cells we have studied, but our data do not rule out possible CFTR-dependent regulation of ATP release pathways in other cell types. The occurrence of ATP release independent of acti-

vation of CFTR raises new possibilities for treatment of CF. For potential therapeutic targeting, it may important to identify, at the molecular level, the mechanism involved in nucleotide release from airway epithelia.

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