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 \(^3\)H-labeled species released from \(^3\)H 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 \(^3\)H ATP and \(^[33P]\)dATP. Third, when measuring ATP concentrations via nucleoside diphosphokinase-catalyzed phosphorylation of \([\alpha-\beta^3P]\)dADP, equivalent formation of \([\beta^3P]\)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 \([\beta^3P]\)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 \(^3\)H inositol phosphates. Basal \(^3\)H 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-attenuatable \(^3\)H 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) 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 CI\(^-\) 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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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; NDKP, nucleoside diphosphokinase.
using both biochemical methods for directly measuring ATP release in intact cells and an assay utilizing the human P2Y2 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-well plastic plates as described (31). HP2U-1321N1 cells, a clonal cell line derived from human astrocytoma cells stably expressing the human P2Y2 receptor (32), were grown on 12-well plastic plates (inositol phosphate assay) or on 6-well plates (3H- 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-Henseleit buffer (2.4 mM KH2PO4, 0.4 mM KH2PO4, 115 mM NaCl, 1.2 mM MgCl2, 1.2 mM CaCl2, 25 mM NaHCO3, and 5 mM glucose) at 37°C and 5% CO2. The cells were exposed for the indicated times to tic acid followed by ethyl ether extraction and neutralization. ATP standard curve performed for each individual experiment. To assess intracellular ATP content, cells were lysed with 5% trichloroacetic acid (TCA) and 5% trichloroacetic acid (TCA) was added to the resulting [3H]inositol phosphates were separated and quantified by chromatography on Dowex columns as described previously (9).

Results—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. [3H]Adenine (17 Ci/mmol) and [3H]-inositol (20 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO). [3-33P]dATP (3000 Ci/mmol), [3H]Na2CrO4 (300–500 mCi/mg chromium), and [36Cl]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/106 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/106 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 twelve resulted in a marked accumulation of mucosal ATP (49.5 ± 8 nM, 297 ± 61 pmol/106 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/106 cells). The possibility that cell lysis occurred during mechanical stimulation was investigated with 51Cr-loaded HNE cells. No changes in 51Cr 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). This, although we cannot rule out the possibility that a small number of damaged cells contributed to 51Cr 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 3H-Adenine-labeled Nucleotides—We loaded the intracellular pool of ATP with [3H]adenine, and the release of 3H-labeled species was quantified by HPLC. Because the relatively large release of [3H]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 [3H]adenine-labeled HNE cells demonstrated a small peak of [3H]ADP, barely distinguishable from background levels (Fig. 1A) (non- incorporated [3H]adenine eluted at 5–5 min). No increase in [3H]ATP or [3H]ADP accumulation was observed in forskolin/ isoproterenol-stimulated HNE cells (Fig. 1B). Incubation of HP2U-1321N1 cells with 250 μM ATP to induce elevation of intracellular Ca2+ resulted in a small accumulation of extracellular [3H]ATP and greater accumulation of [3H]ADP (Fig. 1C). A marked accumulation of [3H]ATP and [3H]ADP was observed after a medium displacement (Fig. 1D). 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 32Cr from HNE cells

Mechanical stimulation was applied to [32Cr]Na2CrO4-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 |
|---------|------------------------|
| 0 min   | 775 ± 52              |
| 1 min   | 806 ± 124             |
| 5 min   | 772 ± 209             |
| 10 min  | 780 ± 234             |
| SDS     | 17,043 ± 3965         |

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

**ATP-dependent Conversion of [α-32P]dADP to [α-32P]dATP—**

The possibility that CFTR mediates the release of ATP from a nucleotide pool not accessed by [3H]adenine (37) was investigated by trapping released ATP with NDPK and [α-32P]dADP. The NDPK-catalyzed conversion of [α-32P]dADP to [α-32P]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-32P]dADP molecule upon release. Fig. 3 illustrates the conversion of [α-32P]dADP to [α-32P]dATP in the mucosal HNE cell baths under various conditions. A basal conversion (16 ± 3%) of [α-32P]dADP to [α-32P]dATP was observed with cells that had not been treated with agonists (Fig. 2A). Addition of forskolin and isoproterenol (20 μM each) did not result in increased conversion (14 ± 4%) of [α-32P]dADP to [α-32P]dATP, indicating that elevation of cellular cyclic AMP did not promote the release of ATP (Fig. 2B). However, an ~2-fold greater conversion (26 ± 3%) of [α-32P]dADP to [α-32P]dATP was observed following addition of 1 μM ionomycin, consistent with a Ca2+-promoted ATP release (Fig. 2C). A 4-fold increased formation (71 ± 6%) of [α-32P]dATP occurred following mechanical stimulation of the cells (Fig. 2D). 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 [32P]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 [32P]dATP formation, although this was not statistically significant.

The P2Y2 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 P2Y2 receptor as a biosensor for ATP to investigate the role of CFTR in the activation of P2Y2 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 P2Y2 receptors by retroviral infection of 1321N1 cells with the cDNA encoding the human P2Y2 receptor (32). 1321N1 cells stably expressing the human P2Y2 re-
The percent conversion of all additions were to the mucosal medium. The results are expressed as described under “Materials and Methods.” All additions were to the mucosal medium bathing mouse nasal epithelial cells. Cells from wild-type and isoformycin (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 [32P]dADP, and the resulting [32P]-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.

**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.
S.E. from four experiments performed in triplicate. gently replacing the medium twice. The results represent the means

\[ cAMP \] were added at 20 pmol 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 separa
tion of [3H]labeled nucleotides released from [3H]adenine-pre-
loaded cells as well as newly developed approaches for trapping ATP with NDPK and have adopted conditions in which sam-
ping 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 differ-
ences after incubating the cells with agents that promoted
elevation of intracellular cyclic AMP. Moreover, no differences
were found in extracellular ATP accumulation with nasal ep-
ithelial 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 me-
chanical stimulation of airway epithelial cell surfaces by a
medium displacement. However, mechanically stimulated re-
lease 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 stimu-
lated ATP release was also recently reported with rabbit uri-
mary 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 func-
tional 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 me-
chanical stimulation and/or secondary to elevation of intracel-
lular 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-
viation 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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