
Adenosine and its nucleotides activate wild-type and R117H CFTR through an A2B receptor-coupled pathway. Am. J. Physiol. 276 (Cell Physiol.: 45): C361–C369, 1999.—ATP and its metabolites stimulate Cl− secretion in human epithelium in vitro and in vivo. The specific purinergic receptor subtypes that govern these effects have been difficult to separate, in part due to multiple parallel pathways for Cl− secretion in respiratory and intestinal epithelia. In a simplified model using COS-7 cells, we demonstrate acquisition of an ATP-, ADP-, AMP-, and adenosine (ADO)-regulated halide permeability specifically following expression of wild-type (wt) cystic fibrosis transmembrane conductance regulator (CFTR). This halide permeability is blocked by the P1 purinergic receptor antagonist 8-phenyl theophylline, sensitive to the protein kinase A inhibitor H-89, and associated with a modest, dose-dependent increase in cellular cAMP concentration. Phorbol esters poorly activate halide permeability compared with ADO, and ADO-stimulated efflux was not affected by treatment with the protein kinase C inhibitor bisindolylmaleimide I. The A2A AR agonist 5′-N-ethylcarboxamidine adenosine and ADO were strong activators, whereas the A2B AR agonist R-phenylisopropyladenosine failed to activate halide permeability. Metabolic conversion of ADO nucleotides by surface ecto-5′-nucleotidase to more active (less phosphorylated) forms contributes to anion transport activation in these cells. Immunoprecipitation with anti-A2B AR antibody identified a 31-kDa protein in both COS-7 and human bronchial epithelial cells. Together, these findings indicate that ADO and its nucleotides are capable of activating wtCFTR-dependent halide permeability through A2B AR and that this AR subtype is present in human bronchial epithelium. We also present data showing that this pathway can activate clinically significant mutant CFTR molecules such as R117H.

Surface receptors represent physiological and potentially accessible routes to modulate CFTR activity and Cl− secretion in vivo. For example, vasoactive intestinal peptide is capable of CFTR activation in certain epithelia (26, 42). β2-Adrenergic receptor stimulation is a well-established receptor-coupled method to activate CFTR-dependent Cl− secretion in the mammalian airway and is an important mechanism by which to discriminate between cystic fibrosis (CF) and non-CF phenotypes in vivo (22). Study of receptor-coupled CFTR activation is somewhat difficult, however, due in part to the many Cl−-secretory pathways that may be influenced by surface receptor activation. Furthermore, the specific purinergic receptor subtypes and diverse Cl−-secretory pathways described, those responsible for signaling through CFTR are not known with certainty. ADO receptor (AR) activation by ADO and ADO analogs has been shown to be a potent apical stimulus to elevate cellular cAMP concentration and activate cAMP-dependent Cl− secretion in CFTR-expressing epithelia, including human and canine airway cell monolayers (21, 23, 31) and the T84 human colonic cell line (2–4, 25, 41). Although ADO-dependent activation of luminal Cl− secretion in each of these epithelia appears to be at least in part CFTR dependent, a mechanistic understanding of AR-coupled Cl−secretion has been limited by the numerous purinergic receptor subtypes and diverse Cl−-secretory pathways inherent in these epithelia. Adenosine (ADO) nucleotides appear to be important regulators of Cl− secretion through surface receptors. However, stimulation of Cl− secretion in airway and intestinal epithelia following purinergic receptor activation regulates Cl−-conductive pathways by both CFTR-dependent and CFTR-independent mechanisms (2–4, 12, 23, 27, 31, 37, 38, 40, 41). Moreover, of the many purinergic receptors and pathways described, those responsible for signaling through CFTR are not known with certainty. ADO receptor (AR) activation by ADO and ADO analogs has been shown to be a potent apical stimulus to elevate cellular cAMP concentration and activate cAMP-dependent Cl− secretion in CFTR-expressing epithelia, including human and canine airway cell monolayers (21, 23, 31) and the T84 human colonic cell line (2–4, 25, 41). Although ADO-dependent activation of luminal Cl− secretion in each of these epithelia appears to be at least in part CFTR-dependent, a mechanistic understanding of AR-coupled Cl−secretion has been limited by the numerous purinergic receptor subtypes and diverse Cl−-secretory pathways inherent in these epithelia. Additional studies of purinergic receptor subtypes capable of signaling CFTR are not known with certainty. ADO receptor (AR) activation by ADO and ADO analogs has been shown to be a potent apical stimulus to elevate cellular cAMP concentration and activate cAMP-dependent Cl− secretion in CFTR-expressing epithelia, including human and canine airway cell monolayers (21, 23, 31) and the T84 human colonic cell line (2–4, 25, 41). Although ADO-dependent activation of luminal Cl− secretion in each of these epithelia appears to be at least in part CFTR dependent, a mechanistic understanding of AR-coupled Cl−secretion has been limited by the numerous purinergic receptor subtypes and diverse Cl−-secretory pathways inherent in these epithelia. Additional studies of purinergic receptor subtypes capable of signaling CFTR are not known with certainty. ADO receptor (AR) activation by ADO and ADO analogs has been shown to be a potent apical stimulus to elevate cellular cAMP concentration and activate cAMP-dependent Cl− secretion in CFTR-expressing epithelia, including human and canine airway cell monolayers (21, 23, 31) and the T84 human colonic cell line (2–4, 25, 41). Although ADO-dependent activation of luminal Cl− secretion in each of these epithelia appears to be at least in part CFTR dependent, a mechanistic understanding of AR-coupled Cl−secretion has been limited by the numerous purinergic receptor subtypes and diverse Cl−-secretory pathways inherent in these epithelia. Additional studies of purinergic receptor subtypes capable of signaling CFTR are not known with certainty.
cally important CFTR mutation (R117H) through this receptor-coupled pathway.

**METHODS**

Transient CFTR expression. CFTR was transiently expressed in COS-7 and HeLa cells using a vaccinia-based expression system. This system is a well established method to study the function and processing of wtCFTR and mutant CFTR proteins (9). Cells grown on Vectabond-treated glass coverslips (for fluorescence measurements), 100-mm tissue culture-treated petri dishes, or tissue culture-treated six-well trays (Costar) were infected with vaccinia virus containing the T7 polymerase (vTF7-3; generous gift of Dr. B. Moss, National Institutes of Health; hereafter referred to as vT7) at a multiplicity of infection of 10 for 30 min. After vaccinia infection, wtCFTR or R117H CFTR under control of the T7 promoter in the pTM-1 vector was introduced into cells in complex with 1,2-dioleoyl-3-trimethylammonium-propane/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOTAP/DOPE; dioleoylphosphatidylethanolamine/DOPA; from Avanti Polar Lipids, Birmingham, AL). Cells were then washed in PBS, returned to DMEM plus 10% fetal bovine serum, and studied 18–24 h postinfection.

Functional CFTR assay. CFTR function in individual cells was assayed using the halide-quenched dye 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ). Briefly, cells were loaded for 10 min with SPQ (10 μM) by hypotonic shock and then mounted in a specially designed perfusion chamber for fluorescence measurements. Fluorescence of single cells was measured with a Zeiss inverted microscope, a PTI imaging system, and a Hamamatsu camera. Excitation was at 340 nm, and emission was measured at >410 nm. All functional studies were at 37°C. Cells were bathed in a quenching buffer (NaI) at the beginning of the experiments and were switched after establishment of a stable baseline to a halide-free (NO3) dequenching buffer at 200 s. The SPQ assay used in these studies was configured to detect I- efflux through CFTR or other permeability pathways as described previously (10). Cells were stimulated with agonist, unless otherwise indicated, at 5 μM and then returned to the quenching NaI buffer. Fluorescence was normalized to the baseline (quenched) value (average fluorescence from 100–200 s), with increases presented as percent increase in fluorescence over basal. Values used to generate curves are from all dequenching cells (~90% of all cells studied) in paired experiments performed over 1–2 days. Values are means ± SE. The buffers used in the SPQ assay were 1) NaI buffer (in mM: 130 NaI, 5 KNO3, 5 Ca(NO3)2, 2.5 Mg(NO3)2, 10 d-glucose, and 10 HEPES (pH 7.30)) and 2) NaNO3 buffer (identical to NaI buffer except that 130 mM NaNO3 replaced NaI). Relative permeability coefficients for maximal I- exit from COS-7 cells were measured by SPQ as the rate of dequench after agonist stimulation (mean fluorescence/s for the 100 s of maximal fluorescence change). To maximize intracellular inhibitor activity for certain experiments (see Fig. 3), cells were loaded with inhibitors at concentrations above the inhibition constant values reported in cell-free systems. Specifically, the staurosporine concentration used (see Fig. 3) was 100- to 1,000-fold above the IC50 values for purified kinases including PKA, PKC, and protein kinase G (IC50 values for these enzymes are 0.5–10 nM). H-89 (PKA inhibitor) and bisindolylmaleimide I (BIM-I; PKC inhibitor) were used (see Fig. 3) at concentrations 100- to 200-fold above those known to inhibit the respective purified enzymes in cell-free systems (per manufacturer’s protocol).

Human primary airway cell tissue culture. Bronchial tissue from surgical specimens was obtained through the University of Alabama at Birmingham Tissue Procurement Office. Cells were isolated and grown as explant cultures in plastic tissue culture dishes (15), except that serum-free growth medium was modified as described in Ref. 44. Cells were fed serum-free growth medium on alternate days and studied at wk postseed.

CAMP levels. Cellular CAMP levels were measured using a standardized fluorometric CAMP assay kit (Cayman Chemicals, Ann Arbor, MI); 1 × 106 cells were stimulated with agonist for 10 min and extracted with ice-cold ethanol, and the CAMP levels from the supernatant were measured with a Zeiss inverted microscope, a PTI imaging system, and a Hamamatsu camera. Excitation was at 340 nm, and emission was measured at >410 nm. All functional studies were at 37°C. Cells were bathed in a quenching buffer (NaI) at the beginning of the experiments and were switched after establishment of a stable baseline to a halide-free (NO3) dequenching buffer at 200 s. The SPQ assay used in these studies was configured to detect I- efflux through CFTR or other permeability pathways as described previously (10). Cells were stimulated with agonist, unless otherwise indicated, at 5 μM and then returned to the quenching NaI buffer. Fluorescence was normalized to the baseline (quenched) value (average fluorescence from 100–200 s), with increases presented as percent increase in fluorescence over basal. Values used to generate curves are from all dequenching cells (~90% of all cells studied) in paired experiments performed over 1–2 days. Values are means ± SE. The buffers used in the SPQ assay were 1) NaI buffer (in mM: 130 NaI, 5 KNO3, 5 Ca(NO3)2, 2.5 Mg(NO3)2, 10 d-glucose, and 10 HEPES (pH 7.30)) and 2) NaNO3 buffer (identical to NaI buffer except that 130 mM NaN3 replaced NaI). Relative permeability coefficients for maximal I- exit from COS-7 cells were measured by SPQ as the rate of dequench after agonist stimulation (mean fluorescence/s for the 100 s of maximal fluorescence change). To maximize intracellular inhibitor activity for certain experiments (see Fig. 3), cells were loaded with inhibitors at concentrations above the inhibition constant values reported in cell-free systems. Specifically, the staurosporine concentration used (see Fig. 3) was 100- to 1,000-fold above the IC50 values for purified kinases including PKA, PKC, and protein kinase G (IC50 values for these enzymes are 0.5–10 nM). H-89 (PKA inhibitor) and bisindolylmaleimide I (BIM-I; PKC inhibitor) were used (see Fig. 3) at concentrations 100- to 200-fold above those known to inhibit the respective purified enzymes in cell-free systems (per manufacturer’s protocol).
mide (200 µM) had minimal effect on halide efflux stimulated either by forskolin plus IBMX or by ADO, suggesting that the cotransporter is not a substantial participant in these two activation pathways. Maximal activation by either forskolin plus IBMX or by ADO was decreased by 10% in the presence of furosemide (data not shown).

Stimulatory effects were also observed in T84 cells expressing endogenous wtCFTR, although ADO nucleotide-dependent responses were frequently more rapid in onset and of greater magnitude than pharmacologic stimulation of adenyl cyclase (Fig. 1C). Interpretation of these effects in T84 cells is complicated by the presence of multiple endogenous halide permeability pathways activated by both CFTR-dependent and CFTR-independent nucleotides (2–4, 25, 38, 40, 41). ADO nucleotides may augment halide efflux in T84 cells by stimulation of these additional pathways. In contrast, ADO and its nucleotides failed to activate halide permeability in HeLa cells expressing wtCFTR compared with direct adenyl cyclase stimulation (Fig. 1D). Thus CFTR is necessary but not sufficient to confer ADO- and ADO nucleotide-regulated halide per-

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**Fig. 1.** Activation of halide permeability in COS-7 (A and B), T84 (C), and HeLa (D) cells. F, fluorescence. A: COS-7 cells were stimulated with various agonists. Cystic fibrosis transmembrane conductance regulator (CFTR)-expressing cells were stimulated to raise cellular cAMP with 20 µM forskolin + 100 µM IBMX [relative permeability coefficient for maximal I~Cl~ exit from cells (PI) = 2.25], ionomycin (2 µM; PI = 0.09), inactive forskolin analog dideoxyforskolin (ddforskolin; 20 µM; PI = 0.10), or UTP (200 µM; PI = 0.03). Control COS-7 cells (after vT7 infection) not expressing CFTR were stimulated with ATP (200 µM; PI = 0.05) or adenosine (ADO; 200 µM; PI = 0.06). B: CFTR-expressing COS-7 cells were stimulated with ADO (200 µM, PI = 2.05), with its nucleotides AMP (200 µM, PI = 1.88), ADP (200 µM, PI = 0.93), or ATP (200 µM, PI = 0.42), or with no agonist (NO3 control; PI = 0.08). C: T84 cells were stimulated with ADO (200 µM), ADP (200 µM), ATP (200 µM), or cAMP (20 µM forskolin and 100 µM IBMX). D: HeLa cells after CFTR expression and ATP (200 µM) followed by cAMP (20 µM forskolin + 100 µM IBMX) at 800 s or ADO (200 µM) followed by cAMP (20 µM forskolin + 100 µM IBMX) at 800 s. Numbers in parentheses are total numbers of cells included in curves (means ± SE). CFTR expression levels in COS-7 and HeLa cells were approximately equivalent, as determined by immunoprecipitation studies (data not shown).
meability in the COS-7 and HeLa cell lines following transient expression.

Figure 1 suggests that ADO and its nucleotides activate CFTR in COS-7 cells by a purinergic receptor pathway. To differentiate between ADO (P₁)- and ATP (P₂)-selective receptors, we studied the effects of the methylxanthine AR (P₁) antagonist 8-PT on ADO nucleotide-activated halide permeability. The results in COS-7 cells expressing wtCFTR (Fig. 2, A–D) indicate that ADO nucleotide-activated halide permeability is sensitive to 8-PT at concentrations known to elicit AR blockade (29). These findings support AR (P₁) rather than ATP receptor (P₂) activation of CFTR in COS-7 cells.

The effects of kinase inhibition are shown in Fig. 3. In the first set of experiments (Fig. 3, A and B), the broad-spectrum kinase inhibitor staurosporine reduced halide permeability after stimulation by 20 µM forskolin plus 100 µM IBMX (to raise cellular cAMP) and ADO (200 µM) in a similar, dose-dependent manner. These results suggest that the effects of ADO are indirect and include kinase activation. Because CFTR has been shown to be regulated by adenyl cyclase, PKA, and PKC, we investigated the effects of a PKA-specific inhibitor (H-89) and a PKC-specific inhibitor (BIM-I) on ADO- and forskolin-stimulated efflux. As is shown in Fig. 3C, inhibition of the PKA pathway by H-89 (4 µM; 2-h incubation) blocked both ADO- and forskolin-stimulated halide effluxes. In contrast, treatment with the PKC inhibitor BIM-I had no effect on ADO-stimulated halide efflux (Fig. 3D), whereas similar exposure to H-89 (8 µM) plus the adenyl cyclase inhibitor SQ-22536 (40 µM) completely abolished the response to ADO (data not shown). Furthermore, stimulation of cells with the PKC activator PMA (100 nM; Fig. 3E), at a concentration shown previously to acutely activate halide efflux in primary canine airway cells (24) and CFTR-dependent halide efflux in C127i cells (13), had very little effect on halide efflux compared with ADO. Together, these results indicate that ADO activation of halide permeability in CFTR-expressing COS-7 cells is sensitive to adenylyl cyclase and PKA inhibition and suggest that ADO activates wtCFTR in COS-7 cells through a cAMP- and PKA-dependent AR pathway.

We next investigated ADO nucleotide potency in COS-7 cells expressing wtCFTR. Determining an analog activity series is a useful way to functionally identify AR subtypes, for example, in human respiratory cell monolayers (23). Figure 4 indicates that NECA and ADO are strong activators of halide permeability, whereas R-PIA is not (5 nM to 5 µM). Together, the results support AR-COUPLED ACTIVATION OF WILD-TYPE AND R117H CFTR in COS-7 cells.

Fig. 3. Effect of inhibitors on ADO and forskolin-activated halide permeability. Cells were incubated with inhibitors [0.5 and 5 µM staurosporine and 2 µM bisindolylmaleimide I (BIM-I) for A, B, and D, respectively] during hypotonic 6-methoxy-N-(3-sulfopropyl)quino-linium (SPQ) load (15 min) and then perfused with inhibitors during experiments. For C, cells were incubated with 20 mM SPQ + H-89 (4 µM) in isotonic medium for 2 h previous to study as described below. A and B: dose-dependent inhibition of ADO- and cAMP-stimulated halide efflux in wild-type (wt) CFTR-expressing cells by broad-spectrum kinase inhibitor staurosporine. A: cAMP-activated halide permeability (20 µM forskolin + 100 µM IBMX). Cells were stimulated at 400 s by cAMP only or cAMP + 0.5 or 5 µM staurosporine. B: ADO-activated halide permeability (200 µM). Cells were stimulated at 400 s by ADO only or ADO + 0.5 or 5 µM staurosporine. C: effect of specific inhibitors on ADO-activated halide permeability. Cells were stimulated with ADO (0.2 µM) or forskolin (5 µM) at 400 s. H-89, a protein kinase A inhibitor (4 µM), blocked both ADO and forskolin activation. D: effect of BIM-I on ADO-activated halide permeability. Protein kinase C inhibitor BIM-I (2 µM) did not block stimulation with ADO (0.2 µM). Forskolin-stimulated cells (20 µM) are included for comparison. E: effect of phorbol 12-myristate 13-acetate (PMA) compared with ADO-activated halide permeability. Cells were stimulated with ADO (200 µM) or PMA (100 nM) as shown. Numbers in parentheses are total numbers of cells included in curves (means ± SE).
shown in Fig. 6 do not account for desensitization of cell permeability measurements by SPQ such as those induced by acute addition of agonists. The cumulative halide concentrations of 200 µM. The results in Fig. 5 demonstrate that ADO and three of its nucleotides elevate cellular cAMP levels above control values. Interestingly, ADO and its nucleotides (ADO, AMP, ADP, and ATP; each at concentrations of 0.005, 0.5, and 5 µM) of agonists were 0.22, 0.57, and 0.55 for NECA, 0.16, 0.28, and 0.21 for ADO, and 0.114, 0.053, and 0.032 for R-PIA, respectively.

selectivity series NECA > ADO > R-PIA supports A2 AR stimulation governing activation of halide permeability (29).

A2 AR stimulation functionally couples to Gs, activating adenyl cyclase and elevating cellular cAMP (29). We therefore compared cellular cAMP levels in COS-7 cells following incubation with forskolin (20 µM) or ADO nucleotides (ADO, AMP, ADP, and ATP; each at concentrations of 200 µM). The results in Fig. 5 demonstrate that ADO and three of its nucleotides elevate cellular cAMP levels above control values. Interestingly, ADO and related nucleotides elevated cellular cAMP levels far less than direct adenyl cyclase stimulation, despite strong activation of halide permeability by each of these agonists (Fig. 1, A and B). These findings suggest that activation of CFTR by this receptor-coupled mechanism may be greater than what would be expected based strictly on elevation of cellular cAMP. To further investigate the relationship between receptor-coupled cAMP elevation and CFTR activation, we performed dose-response studies of the effect of forskolin and ADO on cAMP levels and anion transport (Fig. 6, A–C). Figure 6A compares forskolin- and ADO-stimulated cAMP dose response curves and demonstrates that forskolin can elevate cAMP levels nearly 10-fold higher than ADO, with a half-maximal level achieved at ~5 µM. In contrast, cAMP levels after ADO, although significantly less than those achieved following forskolin stimulation, demonstrate dose dependency, with half-maximal levels achieved at ~0.1 µM (Fig. 6B). In functional SPQ experiments (Fig. 6C), ADO is substantially more potent (activation of I– efflux begins at ~0.1 µM) than direct adenyl cyclase stimulation with forskolin (activation of I– efflux begins at ~10 µM) following the acute addition of agonists. The cumulative halide permeability measurements by SPQ such as those shown in Fig. 6 do not account for desensitization of cell surface receptors or other aspects of cellular tachyphylaxis. However, in all of our experiments (Figs. 1, 3, 6C, and 9), ADO elicited anion transport effects roughly equivalent to or above those of forskolin, even though cAMP levels in these cells are much less elevated following ADO stimulation compared with forskolin (Figs. 5 and 6, A and B). These results therefore raise the possibility that total cellular cAMP concentration may not directly predict CFTR activation and that mechanisms in addition to cAMP may contribute to ADO-dependent activation of wtCFTR (see Discussion).

The preceding experiments suggest that A2 AR activation mediates the ADO nucleotide-stimulated halide permeability in COS-7 cells. A2 ARs have been subclassified into A2A and A2B subtypes (29), both of which can signal through adenyl cyclase. COS-7 cells are not known to endogenously express A2 ARs, but at least one report suggests that ADO can elevate cellular cAMP in this cell line (35). To clarify the A2 AR subtype responsible for our findings, we performed immunoprecipitation studies in COS-7 and normal human bronchial epithelial cells using isoform-specific anti-A2A and anti-A2B antibodies. In both cell types (Fig. 7A), an ~31-kDa protein was recognized by the anti-A2B antibody, indicating the presence of the A2B AR in COS-7 cells and normal human bronchial epithelia. In contrast, the A2A AR polyclonal antibody failed to identify the A2A AR in either cell type (data not shown).

These studies indicate that ADO and ADO phosphates activate wtCFTR by utilizing an A2B AR in COS-7 cells. However, whether the stimulatory effects of ADO nucleotides are produced by these nucleotides or by their metabolites is unknown. Previous studies of A2 AR-activated Cl− secretion in colonic and airway epithelia suggest that surface ecto-5′-nucleotidase activity...
ity influences ADO nucleotide-activated Cl⁻ secretion by metabolizing ADO phosphates to ADO (37, 38, 40, 41). We therefore investigated the effects on ADO nucleotide-stimulated halide efflux of 1) ecto-5'-nucleotidase inhibition and 2) ADO depletion by conversion to inosine. In the first experiment, COS-7 cells were stimulated with ATP in the presence of the ecto-5'-nucleotidase inhibitor α,β-methyleneadenosine 5'-diphosphate. The ATP formulation used in these experiments does not contain measurable levels of ADO as judged by HPLC (Sigma technical specifications). Figure 8A indicates that inhibition of ecto-5'-nucleotidase activity attenuated the ATP-activated halide permeability, suggesting that ADO and/or adenosine mono- or diphosphates generated by surface nucleotidase contribute to CFTR activation. In the second experiment, COS-7 cells were stimulated with freshly prepared AMP (200 µM) pretreated with ADA (1 IU/ml). ADA catalyzes the conversion of ADO to the inactive nucleotide inosine. Figure 8B indicates that treatment of an AMP solution with ADA attenuates the AMP-activated halide permeability. Together, these two experiments suggest that metabolic conversion of ADO nucleotides to more active (less phosphorylated) forms contributes to the A₂B AR activation of halide permeability in our experiments. This correlates well with studies of other model epithelia, in which ADO phosphate stimulation of A₂ ARs in T84 cells has been shown to depend in part on conversion to ADO by surface ecto-5'-nucleotidases (25, 37, 41).

The results presented here demonstrate that A₂B AR stimulation elevates cAMP levels and strongly activates halide permeability in COS-7 cells expressing wtCFTR. We next asked whether a clinically relevant CFTR mutant protein could be activated following A₂B AR stimulation. We chose to study the R117H CFTR, which is known to localize to the cell surface and maintain normal PKA-dependent activation but which has reduced single-channel Cl⁻ conductance (34, 43). Figure 9 compares 10 µM ADO- and 10 µM forskolin-stimulated halide permeability in COS-7 cells expressing R117H CFTR, indicating similar strong responses. These results show that a surface-localized conduction mutant can be activated in vitro through an A₂ AR mechanism that should also be available in vivo. Furthermore, activation of R117H CFTR by ADO is qualitatively similar to that obtained by pharmacologic stimulation of adenyl cyclase.

**DISCUSSION**

It has been shown that ADO and its nucleotides regulate Cl⁻ channel activity in human airway and colonic epithelia, and this model provides a mechanism by which the local cellular environment may control luminal Cl⁻ secretion. The present studies, using a simplified system, demonstrate coupling of the A₂B AR to CFTR activation and indicate that ADO and its nucleotides can be potent agonists for CFTR-dependent halide efflux.
ATP alone, studies were not specified (23). The A1 AR antagonist activated whole cell Cl−-cyclopentyl-1,3-dipropylxanthine promotes cAMP-epithelioid cells (7). Mucosal AR activation stimulates independent purinergic receptor pathway in C127i capable of activating wtCFTR utilizing a cAMP-mediated process. ATP and UTP have been shown to activate a Ca2+-dependent Cl−-adenosine receptor pathway independent of CFTR through stimulation of P2u receptors (12, 27, 40). ATP is also capable of activating wtCFTR utilizing a cAMP-independent purinergic receptor pathway in C127i epithelioid cells (7). Mucosal AR activation stimulates Cl−-adenosine receptor-coupled elevation of cellular cAMP activation noted in our experiments. Additional pathways regulating Cl−-secretion have been described in CF and non-CF cells in the past, including mediators that potentially have synergistic or permissive effects with cAMP-activated Cl−-secretion (2–4, 7, 12, 14, 27, 28, 40). The evidence presented in our experiments, however, suggests that in COS-7 cells a primary mechanism is through the A2B AR and cAMP (Figs. 3–5).

Four human AR subtypes have been identified, cloned, and characterized in terms of pharmacology, distribution, and cellular function (29). The two A2 AR subtypes, A2A AR and A2B AR, differ in size, agonist potency series, and tissue distribution, but both classically coupled to Gs and adenyl cyclase (29). A2 AR activation has been found to regulate diverse tissue processes, including respiratory and colonic ion transport (2–4, 23, 25, 26). A2A AR transcripts have been identified in human lung, brain, heart, and kidney (29), whereas transcripts from the smaller A2B AR (330 amino acids; protein size ~31–33 kDa) have been identified in many tissues, including human brain, ileum, colon, and the human colonic T84 cell line (29, 30, 32, 36, 38). Neutrophil-derived AMP has been shown to mediate A2B AR activation, cAMP accumulation, and Cl−-secretion following removal of ADA at 600 s. Dequench following removal of ADA supports notion that some of AMP-stimulated ion currents in CF cells by block of ecto-5'-nucleotidase activity (A) and catalysis of ADO to inosine (B). A: cells were incubated with ecto-5'-nucleotidase inhibitor a,b-methyleneadenosine 5'-diphosphate (a,b-MeADP; 600 µM) from 200 s, stimulated with ATP (200 µM) in presence of inhibitor at 400 s, and then switched to ATP solution free of inhibitor at 600 s. An increased rate of dequench was observed following removal of ecto-5'-nucleotidase inhibition. This result is consistent with conversion of ATP to less-phosphorylated (more active) ADO nucleotides. Of 38 control ATP-stimulated cells (absence of inhibitor), 16 had a positive response, defined by a 100% increase in slope following stimulation. In contrast, only 5 of 36 cells treated with a,b-MeADP responded to ATP stimulation (P < 0.025 compared with ATP alone, χ² test). After removal of inhibitor, 15 of 36 cells revealed a positive response compared with 0 of 31 NO3− (no agonist) controls (P < 0.001, χ² test). B: adenosine deaminase (ADA; 1 U/ml) was added to AMP (200 µM) in NO3− buffer for 20 min before experiment. Cells were perfused in ADA-containing buffer from 200 s, stimulated with AMP (200 µM) in presence of ADA at 400 s, and then switched to an AMP solution free of ADA at 600 s. Dequench following removal of ADA supports notion that some of AMP-stimulated ion efflux due to ADA conversion by ecto-5'-nucleotidase. Of 31 AMP-stimulated cells, 10 responded to AMP in presence of ADA, whereas 9 of 31 cells responded following removal of ADA at 600 s (P < 0.01 for each time point compared with NO3− control at 400 and 600 s, χ² test). Numbers in parentheses are total numbers of cells included in curves (means ± SE).

ADO nucleotides elicit Cl−-secretion in CF cells and tissues by many pathways, although the underlying mechanisms are not fully understood. ATP and UTP have been shown to activate a Ca2+-dependent Cl−-conductive pathway independent of CFTR through stimulation of P2u receptors (12, 27, 40). ATP is also capable of activating wtCFTR utilizing a cAMP-independent purinergic receptor pathway in C127i epithelioid cells (7). Mucosal AR activation stimulates Cl−-secretion in normal, but not CF, primary airway cell monolayers, although the CFTR genotypes in these studies were not specified (23). The A1 AR antagonist 8-cyclopentyl-1,3-dipropylxanthine promotes cAMP-activated whole cell Cl−-currents in normal and CF respiratory cells (28) and may be capable of directly activating surface-localized ΔF508 CFTR (14). Transcripts of both A1 and A2 receptors are expressed in airway epithelium, but the subtypes mediating receptor-coupled Cl−-secretion are not known (28, 29, 36). In summary, the positive regulation of CFTR and other Cl−-conductive pathways by ADO and related nucleotides appears important to the regulation of epithelial Cl−-transport. Clinical trials based on previous observations with ATP, UTP, and 8-cyclopentyl-1,3-dipropylxanthine illustrate the importance of a thorough understanding of the processes by which this class of compounds activate Cl−-secretion.

Although AR-coupled cAMP accumulation has been demonstrated in the COS-7 cell line previously (35), no studies have shown that COS-7 cells endogenously express either A1 or A2 ARs. The results in Fig. 6 suggest that 1) ADO is a potent agonist for wtCFTR activation, 2) CFTR activity may be stimulated with relatively lower cellular cAMP levels in the setting of A2B AR activation, and 3) receptor-coupled elevation of cellular cAMP may activate CFTR more efficiently than non-receptor-mediated ( forskolin-induced) elevation of cellular cAMP. These results also raise the possibility that non-cAMP-dependent signaling pathways, such as PKC, might contribute to some of the AR-coupled CFTR activation noted in our experiments. Additional pathways regulating Cl−-secretion have been described in CF and non-CF cells in the past, including mediators that potentially have synergistic or permissive effects with cAMP-activated Cl−-secretion (2–4, 7, 12, 14, 27, 28, 40). The evidence presented in our experiments, however, suggests that in COS-7 cells a primary mechanism is through the A2B AR and cAMP (Figs. 3–5).

Fig. 8. Inhibition of halide permeability in CFTR-expressing COS-7 cells by block of ecto-5'-nucleotidase activity (A) and catalysis of ADO to inosine (B). A: cells were incubated with ecto-5'-nucleotidase inhibitor a,b-methyleneadenosine 5'-diphosphate (a,b-MeADP; 600 µM) from 200 s, stimulated with ATP (200 µM) in presence of inhibitor at 400 s, and then switched to ATP solution free of inhibitor at 600 s. An increased rate of dequench was observed following removal of ecto-5'-nucleotidase inhibition. This result is consistent with conversion of ATP to less-phosphorylated (more active) ADO nucleotides. Of 38 control ATP-stimulated cells (absence of inhibitor), 16 had a positive response, defined by a 100% increase in slope following stimulation. In contrast, only 5 of 36 cells treated with a,b-MeADP responded to ATP stimulation (P < 0.025 compared with ATP alone, χ² test). After removal of inhibitor, 15 of 36 cells revealed a positive response compared with 0 of 31 NO3− (no agonist) controls (P < 0.001, χ² test). B: adenosine deaminase (ADA; 1 U/ml) was added to AMP (200 µM) in NO3− buffer for 20 min before experiment. Cells were perfused in ADA-containing buffer from 200 s, stimulated with AMP (200 µM) in presence of ADA at 400 s, and then switched to an AMP solution free of ADA at 600 s. Dequench following removal of ADA supports notion that some of AMP-stimulated ion efflux due to ADA conversion by ecto-5'-nucleotidase. Of 31 AMP-stimulated cells, 10 responded to AMP in presence of ADA, whereas 9 of 31 cells responded following removal of ADA at 600 s (P < 0.01 for each time point compared with NO3− control at 400 and 600 s, χ² test). Numbers in parentheses are total numbers of cells included in curves (means ± SE).

ADOS are activated by ADO (10 µM) or forskolin (10 µM). Expression of R117H CFTR was performed as described (see METHODS). Cells were stimulated with ADO (PI = 0.93) or forskolin (PI = 0.72) at 500 s. Numbers in parentheses are total numbers of cells included in curves (means ± SE).
lowing conversion to ADO by surface-localized CD73 (ecto-5'-nucleotidase) in T84 cells (25, 37, 38). The A2 AR subtype(s) responsible for ADO-stimulated Cl− secretion in human airway cells, however, has not been defined. Our results demonstrate that A2B ARs mediate halide permeability effects in COS-7 cells following wtCFTR expression, and indicate the presence of A2B AR protein in primary human bronchial epithelial cells.

A2B receptor stimulation of COS-7 cells expressing either wtCFTR or R117H CFTR indicates that this G protein-coupled receptor can effectively activate CFTR-dependant halide transport (Fig. 9). The R117H CFTR represents a class IV CFTR mutation, characterized by intact protein production, maturation, surface localization, and regulation but defective single-channel Cl− conduction (34, 43). Therefore, this mutation represents an ideal candidate to investigate the effects of A2B AR stimulation. The phenotype of patients possessing the R117H mutation is unusual. These patients are predominantly pancreas sufficient but do suffer the typical respiratory sequelae of cystic fibrosis (16). It has therefore been suggested that the R117H mutation may rest at the boundary of required CFTR function in two organ systems, providing adequate function to protect the exocrine pancreas but failing to provide the necessary function in the lungs to protect the airways from the pulmonary manifestations of cystic fibrosis. Although Cl− transport in cells expressing the common ΔF508 CFTR trafficking mutant may not be expected to be stimulated by A2B AR activation, our studies raise the possibility that the function of R117H CFTR and possibly other class IV surface-localized CFTR mutations might be augmented through pharmacologic activation of A2B AR. Further studies will be required to test this possibility.

In summary, these experiments are the first to describe 1) AR-coupled CFTR activation by ADO and adenosine mono-, di-, and triphosphates in a cell line devoid of endogenous competing Cl− transport pathways, 2) A2B AR regulation of CFTR-dependent halide transport, 3) A2B AR protein in COS-7 and native human bronchial epithelia, and 4) A2B receptor activation of R117H CFTR. The findings help clarify the positive regulatory effects that ADO and its nucleotides can confer to wtCFTR and R117H CFTR through the A2B AR. COS-7 cells possess properties that may be similar to native secretory epithelia, including expression of G protein-coupled receptors. Previous studies indicate that several mutant CFTRs can be pharmacologically activated in cell-free systems with PKA or PKC plus ATP, by direct stimulation of adenyl cyclase, or by phosphatase inhibition. None of these methods, however, utilize endogenous receptor-coupled pathways that are present in vivo. The studies presented here provide a means to evaluate whether mutant CFTRs localized to the plasma membrane can be activated by A2B receptor coupling. For example, additional experiments in COS-7 cells have recently allowed us to identify two other surface-localized CFTR mutants (A455E and G1349D) that can functionally couple to A2B AR activation (11). We speculate that patients possessing these and other surface-localized mutant CFTRs might uniquely benefit from therapeutics that exploit AR-coupled pathways to activate CFTR.

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